Parvaiz Ahmad · Mohd Rafiq Wani
Mohamed Mahgoub Azooz
Lam-Son Phan Tran *Editors*

Improvement of Crops in the Era of Climatic Changes

Volume 1

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Preface

Increasing global population and climate change are the two robust forces that collectively challenge scientists to devise such result-oriented techniques which ensure better crop productivity to meet up the world's ever-increasing food demand. The plant development and productivity are detrimentally affected by diverse environmental stress factors such as heat, cold, drought and salinity which limit agricultural crop production worldwide. Stressed climatic conditions are the prime causes for diminishing the overall yield of major crops by more than 50 %, which causes huge financial losses worth hundreds of millions of dollars each year. Consequently, to nosh the global population under such adverse environment remains a major challenge for all nations. On such situation, molecular breeding and genetic engineering have significantly contributed to expand the fundamental knowledge of cellular mechanisms involved in stress response, thus suggesting novel strategies to augment stress tolerance. If productivity, all over the world, is not increased in the context of constantly varying climatic conditions, food insecurity may foster major economic and political uncertainty. A major constraint for recuperating yield under abiotic stress is our limited understanding of the diverse genes that underline stress tolerance, as well as the hitches faced by breeders and biotechnologists, who are in quest of combining favourable alleles to create desired stress-adapted high-yielding genotypes. Consequently, a better understanding of gene function in plant stress adaptation and means to exploit these genes to augment crop performance are essentially needed, if we have to comprehend the full potential of our efforts in crop improvement. In this context, the book *Improvement of Crops in the Era of Climatic Changes Volume 1* will serve as avant-garde resource for researchers and students who are engrossed in developing improved crop cultivars and management methods. Written by a varied group of internationally distinguished experts, *Improvement of Crops in the Era of Climatic Changes Volume 1* is a concise yet comprehensive resource for researchers, students and others seeking advancements in this burning area of research and will lead to new commands and pondering on the subject of climate change and crop improvement.

In this book, we present a collection of 13 chapters written by 48 reputed experts in the field of plant abiotic stress tolerance and crop improvement. It is a well-timed contribution to a topic that is of vast eminence. The chapters provide a state-of-the-art account of the information available on abiotic stress tolerance and crop improvement. Chapter 1 throws light on citrus rootstocks for improving the horticultural performance and physiological responses under constraining environments. The chapter reveals the horticultural benefits due to the use of citrus rootstocks for alleviating the deleterious consequences of abiotic stresses. Chapter 2 deals with role of silicon in enrichment of plant nutrients and protection from biotic and abiotic stresses. This chapter is aimed to cover all the aspects regarding the valuable performance of silicon for survival of plants. Chapter 3 addresses the transgenic approaches for phytoextraction of heavy metals. Here, the authors scrupulously reviewed various approaches used to develop transgenic plants having increased phytoextraction competence for effective remediation of heavy metal contaminated soils. Chapter 4 is about using an allometric model for the accumulation of mineral nutrients in crops under saline–water stress: a field experience in fertigation. In this chapter, the authors described the theoretical background and analysed the field experience on crops fertigation, following stages of model development and discussing the growth restrictions imposed by saline and water stress to the nonrestricted forecast.

Chapter 5 deals with control of biotic and abiotic stresses in cultivated plants by the use of biostimulant microorganisms. In this chapter, the authors gave an up-to-date overview on the recent breakthroughs in the use of biostimulant microorganisms on plants for improving crop vigour, yield and quality and for increasing plant tolerance against biotic and abiotic stresses. Chapter 6 describes cyclic nucleotides and nucleotide cyclases in plants under stress, wherein the authors have shown that cyclic nucleotides such as cAMP and cGMP are involved in signal transduction in response to various environmental stresses. Chapter 7 deals with breeding and transgenic approaches for development of abiotic stress tolerance in rice. This chapter summarizes the recent advancement in breeding and transgenic approaches for the improvement of abiotic stress tolerance in rice using paradigm from the research targeted at drought, salinity and temperature stresses. Chapter 8 describes mineral bioavailability through mutation breeding in pulse crops: a review. In this chapter, the authors have made an endeavour to divert the attention of think tanks of countries like India and their policy holders, agriculturists and decisive bodies to look upon the collaborative work with the associations actively working on mutation breeding. In a strategic development for curbing the malnutrition and food insecurity problems, the work on the neglected crops like pulses has to be straightaway enhanced, and the same has been critically highlighted in the chapter. Chapter 9 is about abiotic stress and control of yield in cereals. Here, the authors examined the physiological processes impacted by abiotic stresses leading to reduced grain yield. In addition, the chapter is themed around the challenge of finding ways to improve cereal grain yield under such stresses.

Chapter 10 is about improvement of crop production under saline stress by a biohydraulic approach. In this chapter, the authors reviewed the strategies by which plants can be enabled to grow on saline soils. Chapter 11 deals with induced

mutagenesis for the improvement of pulse crops with special reference to mung bean: a review update. In this review, the authors revealed various aspects of contemporary knowledge of pulse crop improvement programmes through induced mutations, biotechnological approaches, molecular advances and new parameters of selection. They concluded that cultivars with improved efficiency vis-à-vis yield, early maturity, uptake of micronutrients, tolerance to abiotic stresses like drought, cold and salinity and resistance to biotic stresses like disease and insect pests can be easily developed by using mutation breeding and marker-assisted selection. Chapter 12 describes crop improvement through tissue culture. This chapter presents an overview of in vitro propagation and regeneration via meristems, cell, tissue and organ cultures, organogenesis and somatic embryogenesis. Additionally, new methods and developments in protoplast isolation and culture, hairy root culture and transfer of genes in transgenic plants are covered. These technologies could significantly simplify breeding programmes and overcome some important agronomic and environmental traits that would not be achievable through conventional breeding and propagation. Chapter 13 deals with agricultural pollution: an emerging issue. In this chapter, the authors proposed that the current issues of agricultural pollution can be resolved by practices like agricultural waste management, pest management and manure recycling. In addition, the chapter recommended that proper planning and unprejudiced decisions at government level are essentially required to unravel this perilous issue.

We wish to express abstemious appreciation to our well-versed contributors, who readily accepted our invitation to write their chapters. Moreover, we would like to thank Springer Science+Business Media, LLC, New York, particularly Eric Stannard (Editor Botany, Springer), Kevin Wright (Developmental Editor, Springer), Andy Kwan (Assistant Editor, Springer), Flora Kim (Developmental Editor, Springer) and all the other staff members of Springer, New York, who were directly or indirectly associated with us in the current project for their steady support and efforts in bringing out the timely publication of this volume.

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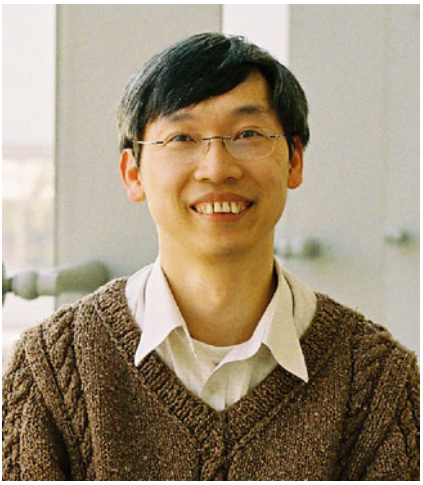
4. Dr. Lam-Son Phan Tran (Co-editor)



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Chapter 1
Citrus Rootstocks for Improving the Horticultural Performance and Physiological Responses Under Constraining Environments

Rafael V. Ribeiro, Erick Espinoza-Núñez, Jorgino Pompeu Junior, Francisco A.A. Mourão Filho, and Eduardo C. Machado

1 Introduction

Citrus trees are grown from latitude 40°N to 40°S, flowering and fruiting in climates ranging from tropical to temperate (Davies and Albrigo 1994). In those areas, fruit yield and tree development may be restricted due to environmental stresses, such as drought, salinity, flooding, and chilling/freezing and high temperature. To avoid the negative impact of environmental stresses, we may combine the best scion and rootstock to each condition, and this choice must consider the fruit yield and its sustainability in long term as citrus trees are perennial plants and production costs are not low.

We know that the sensitivity to environmental stresses is markedly affected by the rootstocks through modifications in the supply of water, carbon, and mineral nutrients to shoots (Syvertsen and Lloyd 1994). In fact, rootstocks are able to

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modify the citrus response to a range of environmental stresses, affecting citrus growth, flowering, and fruiting even under non-limiting conditions (Davies and Albrigo 1994; Murkute et al. 2005).

This chapter has an aim to present our current understanding about how citrus trees respond to environmental changes with especial emphasis to the role of rootstocks in alleviating the stresses caused by water deficit, flooding, salinity, chilling/freezing, and high temperature. The recent advances for explaining stress resistance of citrus plants are also considered. To accomplish this task, only literature directly related to citrus rootstock was considered herein. Before dealing with this, we present an overview about the rootstocks and their importance for fruit production and citrus growth.

2 Benefits to Crop Yield and Plant Growth Due to Citrus Rootstocks: An Overview

The first use of rootstocks in citriculture was in 1842 to control root rot caused by *Phytophthora* in Azores Islands through the use of resistant rootstocks (Chapot 1975). Since that time, most commercial orchards, except in some Asian countries, have been formed by combining citrus scion and rootstock as an attempt to join the best characteristics of each species. The use of rootstocks allows the reduction of juvenility, unproductive initial period, and the cultivation of citrus in areas initially considered unsuitable due to soil characteristics, diseases, pests, and environmental stresses (Castle et al. 1993; Castle 2010). The rootstock is responsible for physical support, absorption of water and nutrients, biosynthesis of plant growth regulators like abscisic acid and cytokinins, and physicochemical interaction with surrounding soil. The physiology of the whole tree is affected by rootstock, including traits of economic relevance such as fruit yield, fruit size, juice quality, tree vigor, and resistance against biotic and abiotic stresses (Castle et al. 1993; Medina et al. 2005). Fruit maturation, fruit holding on tree, and postharvest preservation are also affected by rootstock (Hodgson 1967). The scion influence on rootstock is less studied, but it affects root growth and the resistance to cold, drought, pests, and diseases (Castle 1987).

There is no ideal rootstock for all soil and climate conditions; thus, it is essential to identify the most suitable species for each growing area, considering climate, soil, crop management, scion, pests, diseases, and fruit destination. In general, citrus have to overcome constraints imposed by abiotic (drought, cold, salinity, alkalinity) and biotic factors. For instance, sour orange (*Citrus aurantium* L.) is resistant to root rot, citrus blight disease, water deficit, and cold, inducing high yield and high fruit quality. However, sour orange is susceptible to citrus tristeza virus (CTV), and it cannot be used as rootstock in countries where the virus is present (Castle et al. 1993).

Rangpur lime (*C. limonia* [L.] Osb.), volkamer lemon (*C. volkameriana* V. Ten. & Pasq.), and rough lemon (*C. jambhiri* Lush.) confer resistance to drought and induce high fruit yield. These rootstocks, however, induce low fruit quality as compared to fruits obtained on sour orange, Cleopatra mandarin (*C. reshni* hort. ex Tanaka), Sunki

mandarin (*C. sunki* [Hayata] hort. ex Tanaka), Trifoliate (*Poncirus trifoliata* Raf.), and their hybrids (Pompeu Junior 2005). They are susceptible to citrus blight, citrus sudden death (CSD) (Rodriguez et al. 1979; Bassanezi et al. 2003), and nematodes *Tylenchulus semipenetrans*, *Platylenchus jaheni*, and *Radopholus citrophilus* (Calzavara et al. 2007). Rangpur lime has replaced sour oranges due to the occurrence of CTV. However, Rangpur lime is susceptible to CSD, which increased the use of Swingle citrumelo as rootstock in Brazilian citriculture in the last years (Pompeu Junior and Blumer 2008a). In fact, Swingle citrumelo (*C. paradisi* Macf. Bhp. Duncan × *P. trifoliata* Raf.) has become the main citrus rootstock in Florida (USA) and the second most planted in Brazil (Castle 2010). Scions grafted on Swingle citrumelo have good fruit yield in sandy and clay soils, but it does not have good performance in alkaline and poorly drained soils. In addition, Swingle citrumelo is moderately sensitive to water deficit and cold conditions (Wutscher 1979), requiring irrigation for high fruit yield. The Swingle citrumelo is incompatible with Pera sweet orange (*C. sinensis* [L.] Osb.) and Murcott tangor (*C. sinensis* [L.] Osb. × *C. reticulata* Blanco), requiring an interstock (Pompeu Junior 1991).

Due to the reduction of agricultural areas close to the major consuming centers and high production costs for orchard management, high-density plantations are an alternative for obtaining high yield and reduction of harvest costs. In this context, the selection of new rootstocks, dwarfing techniques, and the use of interstocks are necessary to obtain highly productive orchards (Castle 1978). Regardless of this trend, the use of a single rootstock for all kinds of scions, soils, and climates limits the potential yield of citrus trees and increases the vulnerability of citrus orchards. Scion and rootstock diversification is essential in a sustainable citriculture, less susceptible to unexpected biotic and abiotic stresses.

Although there are many reports dealing with the interaction of scion/rootstock, our current understanding about the physiological basis underlying the rootstock effect on entire tree is limited (Webster 2004; Jones 2012). Some studies have suggested the translocation of minerals, plant growth regulators, carbohydrates, and water (Castle 1995; Webster 1995). At the graft union, rootstock would modify the amount or proportion of growth regulators, sugars, amino acids, minerals, and water moving from roots to shoots and also from shoots to roots (Webster 1995). In general, rootstocks affect the citrus physiology and subsequently the plant growth and development. The physiological changes caused by rootstocks will be presented and discussed in the following sections of this chapter. There are several research works confirming the importance of the rootstock on tree growth and fruit production. For instance, Orlando tangelo (*C. tangerina* hort. ex Tanaka × *C. paradisi* Macf.) scions grafted on rough lemon, Palestine sweet lime (*C. limettioides* Tanaka), and Cleopatra mandarin exhibited higher vigor than on Rusk citrange (*P. trifoliata* [L.] Raf. × *C. sinensis* [L.] Osb. Bhp. Washington Navel) and trifoliate selections (Castle and Krezdorn 1973). Some field experiments in nonirrigated orchards with orange, mandarin, and lime trees revealed the significant effects of rootstocks on tree vigor and fruit yield and quality. Tangelos and lemons are invigorating rootstocks that induce higher tree height and fruit yield as compared to scions on trifoliate

(*P. trifoliata*) and its hybrids (Mourão Filho et al. 2007; Cantuarias-Avilés et al. 2011; Espinoza-Núñez et al. 2011).

The rootstocks have significant effects on citrus juice quality, causing large variations in soluble solids concentration (Wutscher 1988; Castle 1995). Gardner (1969) investigated the rootstock effect on fruit quality using reciprocal fruit grafts and showed that fruit harvested from Valencia sweet orange/rough lemon and grafted on Valencia sweet orange/sour orange and vice versa had the size and juice quality characteristic of the "adoptive mother tree." It was estimated that almost 40 % of the soluble solid variation in citrus juice is attributed to the rootstock. In a similar experiment, Rangpur lime and Butwal lemon (*Citrus limon* Burm. f.) fruits, differing in acidity, were reciprocally grafted. Fruits kept their natural acidity, demonstrating that organic acids are fruit-synthesized unlike sugars that are translocated from other organs.

Considering plant structure, citrus dwarf trees have advantages over larger trees. They produce more fruit per canopy volume and allow high planting density and increasing fruit production per area (Castle 1978). Smaller trees facilitate inspection and control of diseases and pests and then improve the orchard health. The use of dwarfing rootstocks is a method for obtaining small trees. Flying Dragon trifoliate is considered a dwarfing rootstock, allowing the formation of small mature trees in several climates and crop conditions (Donadio and Stuchi 2001). The use of interstock is another way to produce small trees, as discussed in the next section.

2.1 Combining Species: Scions, Rootstocks, and Interstocks

The grafting technique aims to create an association between two genetically different individuals. Eventually, a third individual called interstock can be inserted in order to prevent the occurrence of incompatibility or to induce dwarfing (Castle 1978; Pompeu Junior 2005). Recently, the use of two or more rootstocks has increased further the complexity of citrus trees (Setin et al. 2009).

Grafting promotes the contact of two plants with distinct anatomical, physiological, and biochemical traits, which may cause low affinity and even incompatibility between scions and rootstocks. At the first view, plants grow normally and after two or more years of grafting, they begin to present nutritional deficiencies, leaf abscision, drying of apical shoots, excessive sprouting in rootstock, and reduced fruit yield and finally die. Incompatibility is a premature senescence phenomenon caused by anatomical and biochemical processes, which are intensified under stressful conditions (Feucht 1988). There is no evidence of being associated with any known virus or pathogen, and the incompatibility may be classified as localized or translocated (Mosse 1962). It is considered translocated, when an interstock between scion and rootstock does not solve the incompatibility. It is noteworthy that a larger rootstock trunk diameter than the scion diameter or vice versa is not always an indicative of incompatibility. Several citrus species grafted on trifoliate and its hybrids have reasonable to high fruit yield and show differences in rootstock and in scion diameters without the occurrence of gum ring (Castle et al. 1993).

Bitters et al. (1982) have shown that interstock length had small effect on size and fruit yield. However, the height of interstock insertion affects tree size and fruit production. Sampaio (1993) reported that trifoliate interstock between Valencia sweet orange (*C. sinensis*) and Rangpur lime caused reduction in tree size and low fruit yield. The Flying Dragon trifoliate (*P. trifoliata* cv. monstrosa) interstock reduced, by 30–50 %, the size of orange and grapefruit trees, depending on the combination of scion/rootstock. In addition, the interstock improved the fruit production per canopy volume (Ashkenazi et al. 1994). Espinoza-Núñez et al. (2011) have found that the effect of Flying Dragon trifoliate as interstock depends on its interaction with the rootstock. This interstock reduced the tree size of Tahiti lime (*C. latifolia* [Yu. Tanaka] Tanaka) grafted on Catania 2 Volkamer lemon and increased tree size in plants grafted on Davis A trifoliate, when compared with trees without interstock. Similarly, Flying Dragon interstock increased fruit yield of Tahiti lime grafted on Swingle citrumelo and Davis A trifoliate, but reduced fruit yield on Morton citrange (*P. trifoliata* × *C. sinensis*). The use of interstock as "filters" of pathogens in disease control did not show positive results as the insertion of Cleopatra mandarin or Swingle citrumelo interstock did not prevent the citrus blight or CSD in susceptible combinations (Carlos 1996; Pompeu Junior and Blumer 2008b).

Inarching, or replacement of the rootstock, is used to save plants in which rodents, diseases, and mechanical or chemical agents have damaged the root system or trunk. The inarching technique was used to save the first Washington navel orange tree planted in California in 1877, whose rootstock was damaged by gummosis (Pompeu Junior 2005). Inarching with tolerant rootstocks may be used to control CTV and exocortis and also to overcome the incompatibility between scion and rootstock. Marcottage is the suppression of the rootstock by canopy rooting, and it is used when a disease affects only the rootstock. Among the limitations of this technique, the scion sensitivity to water deficit and root rot limits its use in commercial orchards. The simultaneous use of two rootstocks of different species in order to prevent diseases and increase drought resistance is a new technique with only preliminary results.

3 Improving Physiological Responses Under Constraining Conditions with Rootstocks

About the terminology, we will use the general terms resistant and resistance, in a broad sense, for genotypes that are able to withstand or support the stressful conditions. Stress resistance has two components, i.e., tolerance and avoidance (Fageria et al. 2006), and the component of resistance involved in stress acclimation and adaptation of citrus trees will not be specified along this chapter. Another point is that some research works deal with relative tolerance/resistance comparing citrus species under limiting conditions. Actually, one must consider the sensitivity/resistance of a given genotype by comparing the plant performance between non-stressed (control) and stressed individuals.

3.1 Water Deficit

Water scarcity is the main factor limiting crop productivity (Boyer 1982), and the frequency and severity of drought events are expected to increase (IPCC 2007). As an evergreen species, citrus trees are subjected to the seasonal variation of water availability in several growing areas. Besides the use of irrigation, one of the most effective strategies to deal with water shortage is the choice of an appropriate scion/rootstock combination.

Reductions in stomatal and mesophyll conductance, transpiration, and net CO_2 assimilation are found in citrus trees under water deficit (Medina and Machado 1998; Arbona et al. 2005a; García-Sánchez et al. 2007; Magalhães Filho et al. 2008; Melgar et al. 2010), which affects growth, flowering, and fruit yield in citrus orchards (Shrestha et al. 1996; Botia 2008; Pérez-Pérez et al. 2010). Decreases in stomatal and mesophyll conductances are key regulatory mechanisms in citrus trees under moderate water deficit (Erismann et al. 2008). Stomatal closure due to water deficit is not always dependent on leaf turgor pressure, suggesting the participation of chemical signals from roots to shoots (Forner-Giner et al. 2011b).

In Valencia sweet orange trees grafted on Rangpur lime and Carrizo citrange, leaf gas exchange rates decreased progressively under water deficit. On the other hand, ABA concentrations in roots, leaves, and xylem sap increased between three and seven times (Gomes et al. 2003, 2004; Melgar et al. 2010; Forner-Giner et al. 2011b). These data suggest ABA may play a role as a chemical signal regulating stomatal function in citrus trees. In fact, Rangpur lime tetraploid trees with increased production of ABA by roots have enhanced resistance against water deficit (Allario et al. 2013). In Carrizo citrange, Forner-Giner et al. (2011b) observed increases in pH of xylem sap, with plants showing stomatal closure and decrease of transpiration. These authors suggested the modulation of ABA action through pH changes in xylem sap.

Besides the diffusional limitation due to mesophyll and stomatal resistances, photosynthetic activity may be affected by biochemical and photochemical limitations. However, the quantum efficiency of photosystem II of Rangpur lime, Swingle citrumelo, and Sunki mandarin trees was not affected by moderate water deficit, indicating that primary photochemistry is resistant to water shortage. Increases in the ratio between apparent electron transport rate and photosynthesis have suggested increases in electron flow to alternative electron sinks, such as photorespiration and Mehler reaction (Erismann et al. 2008). Water deficit causes significant reduction in the carboxylation rate and concentration of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in Valencia sweet orange trees, being a significant limitation to citrus photosynthesis under drought (Vu and Yelenosky 1988).

Under severe water deficit, the impairment of photosynthesis will cause excessive energy pressure on photochemistry, with probable generation of reactive oxygen species. In fact, some authors have investigated the influence of water deficit on antioxidative metabolism of citrus trees (Campos et al. 2011; Pérez-Clemente et al. 2012; Carvalho et al. 2013). In addition, the interaction between citrus trees and mycorrhizal fungus caused reductions in lipid peroxidation and in reactive oxygen

species production under water deficit, increasing the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate (APX), and guaiacol peroxidases (G-POD) (Wu et al. 2006, 2007). However, those research works did not involve any rootstock comparison. Although the enzymatic and nonenzymatic antioxidant systems are responsible for the neutralization of harmful effects of reactive oxygen species (Mittler 2002), there are few comparative studies about the influence of citrus rootstocks on antioxidant responses in both leaves and roots under water deficit.

As an overall consequence of water deficit on citrus physiology, plants have reduced growth and fruit yield (Hilgeman and Sharp 1970; Davies and Albrigo 1994; Davies and Bower 1994; Shrestha et al. 1996; Pérez-Pérez et al. 2010), with severity depending on water deficit intensity and also on the phenological phase affected by drought (Ginestar and Castle 1996; Camargo et al. 1999; Pérez-Pérez et al. 2010; Carr 2012). In general, citrus rootstocks have differences related to distribution and depth of root system, uptake of nutrients and water, anatomy of vascular system, production of phytohormones, and the regulation of water status and carbon assimilation, changing the sensitivity of the scion to water deficit (Castle and Krezdorn 1977; Vasconcellos and Castle 1994; Medina et al. 1998; Magalhães Filho et al. 2008; Rodríguez-Gamir et al. 2010; Jover et al. 2012). Root hydraulic conductivity, aquaporin expression, and osmotic adjustment determine the ability of rootstocks to provide water and nutrients to the scion and then maintain leaf gas exchange (Sinclair and Allen 1982; Syvertsen and Graham 1985; Rieger 1995; Medina et al. 1998; Molinari et al. 2004; Boscariol-Camargo et al. 2007; Rodríguez-Gamir et al. 2010).

Soil matrix is the main limiting factor to water uptake under low soil water availability, whereas the root hydraulic conductivity may represent up to two-third of the total limitation under well-watered conditions (Huang and Eissenstat 2000). Root hydraulic conductivity has been positively correlated to shoot growth rate. Citrus trees on invigorating rootstocks (rough lemon, Rangpur lime, and Carrizo citrange) have higher root hydraulic conductivity, stomatal conductance, and transpiration rates than trees on non-invigorating rootstocks such as Cleopatra mandarin and sour orange (Syvertsen 1981; Syvertsen and Graham 1985; Medina et al. 1998). Likewise, high root hydraulic conductivity is observed in rootstocks with high specific root length, with plants showing high photosynthesis, high water and mineral transport capacities, and fast root turnover (Huang and Eissenstat 2000). As invigorating rootstocks exhibit higher density and diameter of xylem vessels than non-invigorating rootstocks (Vasconcellos and Castle 1994), xylem characteristics appear to determine hydraulic conductivity and govern tree growth (Rodríguez-Gamir et al. 2010). Rootstocks with high hydraulic conductivity probably keep leaves more hydrated throughout the day with longer periods of stomatal opening and photosynthesis (Medina and Machado 1998; Medina et al. 1998).

Under water deficit, water tension inside xylem vessels increases and may reach a species-specific tension threshold, when little air bubbles may cause interruption of water supply to tree canopy. This phenomenon is called cavitation and the susceptibility to cavitation is a key characteristic to understand the resistance to water deficit (Tyree and Sperry 1989; Brodribb 2009; Nardini et al. 2011).

In Clementine mandarin (*C. clementina*) grafted on Carrizo citrange and trifoliate orange, cavitation occurs in leaf water potential below –1.0 MPa, triggering decreases in stomatal conductance and transpiration (Poggi et al. 2007).

3.1.1 Mechanisms of Resistance

Anatomically, each rootstock shows peculiar features (Castle and Youtsey 1977; Eissenstat and Achor 1999). Drought resistance could be attributed to fibrous root distribution and quantity, the horizontal and vertical extent of root system development, and water uptake and transport efficiency (Castle et al. 1993). For instance, rough lemon and Rangpur lime are rootstocks that confer high drought resistance and high fruit yield due to their spread, well-distributed, and deep root systems that occupy large soil volume and access more efficiently the soil nutrients and water (Syvertsen 1981). Citrus ability to transport water and nutrient is a physiological characteristic largely affected by rootstocks (Kriedemann and Barrs 1981; Syvertsen and Graham 1985; Medina et al. 1999). Scions on rough lemon and *P. trifoliata* have higher transpiration rates than on Cleopatra mandarin and sour orange. Those differences in transpiration were associated to the root hydraulic conductivity (Sinclair 1984).

Osmotic adjustment and water transport intrinsic proteins—aquaporins—may be additional mechanisms of drought resistance in citrus trees. In fact, in silico analysis of ESTs from roots of Rangpur lime under water stress revealed that several proteins often associated with water deficit were induced, such as proline-related synthase, aquaporins, and dehydrins (Boscariol-Camargo et al. 2007). The Cl^- inclusion mechanism in Rangpur lime rootstock may represent another physiological trait to avoid water deficit, enabling plants to accumulate Cl^- in leaves and then adjust osmotically (Brumós et al. 2010). As consequence, leaf turgor is maintained and citrus growth is less impaired by water deficit. Thus, the good performance of Rangpur lime under drought is probably related to its root capacity to absorb water and maintain shoot water status.

Solute accumulation through osmotic adjustment decreases the leaf osmotic potential while maintains water potential gradient to support water uptake and stomatal aperture (Molinari et al. 2004; García-Sánchez et al. 2007; Campos et al. 2011). Osmotic adjustment was observed in several rootstocks such as Carrizo citrange and Cleopatra mandarin (García-Sánchez et al. 2007), Swingle citrumelo (Molinari et al. 2004; Campos et al. 2011), F-A 5 hybrid, and trifoliate trees (Rodríguez-Gamir et al. 2010). Even fruits are able to adjust osmotically under water deficit (Yakushiji et al. 1996).

Rodríguez-Gamir et al. (2010) reported that F-A 5 rootstock was more resistant to water deficit than its parentals Cleopatra mandarin and *P. trifoliata*, which was associated with osmotic adjustment, causing higher relative water content, higher leaf turgor, and improved gas exchange as compared with other rootstocks. These same physiological traits caused the higher resistance of Cleopatra mandarin, when compared with Carrizo citrange under water deficit (García-Sánchez et al. 2007). Interestingly, reduction in plant hydraulic conductivity may affect the effectiveness of osmotic adjustment in maintaining shoot water status (Rieger 1995).

3.1.2 Improving Plant Performance Under Water Deficit

Swingle citrumelo plants transformed with genes that encode key enzymes of proline synthesis (*P5CSF129A*) have increased the constitutive proline concentration. Under water deficit, those transformed plants had turgid leaves and higher stomatal conductance and photosynthesis as compared to non-transformed ones (Molinari et al. 2004; Campos et al. 2011). Proline is also involved in protective mechanisms against oxidative damage, and its accumulation in transformed Swingle citrumelo plants determined low lipid peroxidation and maintenance of photosynthesis under water deficit (Campos et al. 2011). In addition, Carvalho et al. (2013) found that proline acts as a regulatory/signaling molecule, altering gene transcription levels related to the antioxidant metabolism.

Under low soil water availability, sweet oranges and mandarins scions budded on Cleopatra mandarin have higher water uptake, shoot water status, and leaf gas exchange than scions on Carrizo citrange (Romero et al. 2006; Pérez-Pérez et al. 2008). Comparing the root mass densities, Cleopatra mandarin has higher values and also higher plasticity than rough lemon, throughout the year (Syvertsen and Lloyd 1994). These results lead to the following question (Jones 2012): What governs the tree water relations, the tree vigor, or a direct effect of rootstock? Apparently, invigorating rootstocks are better than non-invigorating rootstocks for crop production under seasonal water deficit conditions. Although invigorating rootstock uptakes more water and causes rapid soil drying, its larger root system is able to explore more efficiently the soil resources when there is available water (Espinoza-Núñez et al. 2011).

Another interesting aspect is the symbiotic relationship between vesicular–arbuscular mycorrhizal fungus and citrus roots. Volkamer lemon in symbiosis with mycorrhizal fungus (*Glomus*) shows improvements in root growth and transpiration under both well-watered and moderate water deficit conditions. Under severe water deficit, the symbiosis caused fast recovery of transpiration in Volkamer lemon seedlings after soil rehydration (Fidelibus et al. 2001). Satsuma mandarin (*C. unshiu* Marc.) grafted on trifoliate rootstocks with the inoculation of mycorrhizal fungus developed large root system, which improved plant performance under water deficit (Shrestha et al. 1996). The association between citrus and mycorrhizal fungus also improved the antioxidant metabolism of citrus trees (Wu et al. 2006, 2007).

3.2 Flooding

Among the environmental stresses, the effects of low oxygen availability and soil chemical disturbance caused by flooding are relatively less studied as compared to water deficit, salinity, and temperature. Flooding is an important agricultural factor in citrus-growing regions at low altitudes, where the water table is relatively high or the irrigation is not properly used. Excessive rainfall is another seasonal cause of soil flooding. In general, citrus species are sensitive to low oxygenation caused by flooding (Syvertsen and Lloyd 1994), with sensitivity depending on scion/rootstock combination (Syvertsen and Levy 2005).

Leaf wilting, senescence, and abscision are among the main plant responses to flooding. However, the intensity of those symptoms depends on the rootstock (Vu and Yelenosky 1991; Arbona et al. 2008). For instance, Hamlin sweet orange grafted on sour orange died after 30 days of flooding, whereas only 20 % of the plants of this same scion cultivar on rough lemon did not survive after 60 days under the same stress condition (Vu and Yelenosky 1991). The occurrence of flooding with saline water changes the volatile composition from lemon trees, which may affect pollination and crop production (Velikova et al. 2012).

Reductions in leaf water potential and leaf turgor due to increases in resistance to water flow were reported in lemon trees (*C. limon* [L.] Burm. f.) grafted on sour orange and on *C. macrophylla* subjected to flooding (Ruiz-Sánchez et al. 1996; Ortuño et al. 2007; Gimeno et al. 2012). Reduced vegetative growth is another consequence of flooding, with citrus trees showing reduced shoot length (Syvertsen et al. 1983; Ruiz-Sánchez et al. 1996). As consequence of growth impairment and reduced phloem transport, starch may accumulate in leaves of trees under flooding conditions (Vu and Yelenosky 1991), with increases in root starch concentration being also noticed (Gimeno et al. 2012).

As mentioned above, flooding causes impairment of citrus growth, and this is a consequence of physiological changes caused by low soil redox potential and low soil oxygen availability. The main plant responses include the reduction in root hydraulic conductivity and structural changes in fibrous roots (Syvertsen et al. 1983; Hartmond et al. 1987). The reduction in root hydraulic conductivity results from a downregulation of aquaporin (PIP1 and PIP2) expression, triggered by decreases in pH of root xylem sap (Rodríguez-Gamir et al. 2011).

Leaf CO_2 assimilation of citrus rootstocks is also reduced by flooding, which was associated with stomatal closure, low chlorophyll content, reduced activity of RuBisCO, low RuBP regeneration driven by electron transport rate, and photochemical damage at the PSII level (Hartmond et al. 1987; Schaffer 1991; Vu and Yelenosk 1991; García-Sánchez et al. 2007; Gimeno et al. 2012; Velikova et al. 2012). Although flooding has caused low stomatal conductance, the intercellular CO_2 concentration remained similar between control and stressed plants and was not a limiting factor to photosynthesis (Vu and Yelenosky 1991; Ruiz-Sánchez et al. 1996; García-Sánchez et al. 2007; Gimeno et al. 2012; Velikova et al. 2012). On the other hand, stomatal closure has an important role in preventing leaf dehydration and maintaining leaf water status once flooding consequences were similar to those of water deficit (García-Sánchez et al. 2007; Ortuño et al. 2007). Leaf water balance may be maintained by low stomatal conductance as it reduces leaf transpiration (demand) in plants that have reduced hydraulic conductivity (supplying capacity). As compared to water deficit, flooding is less restrictive to citrus plants in terms of photosynthesis and leaf water-use efficiency (García-Sánchez et al. 2007).

Although previously suggested by Arbona and Gómez-Cadenas (2008), the stomatal closure under flooding is not related to increases in leaf ABA concentration in Carrizo citrange during the first days of stress. Stomatal behavior and ABA were related to each other only at the latter stages of flooding, when an inverse relationship was found (Rodríguez-Gamir et al. 2011). In addition, the accumulated ABA was not driven from roots, and Rodríguez-Gamir et al. (2011) have suggested that

old leaves have increased the ABA synthesis and transported it to young tissues. The recovery of stomatal aperture after flooding events is an important physiological trait determining citrus performance, which is affected by rootstock (Ruiz-Sánchez et al. 1996). In fact, the recovery capacity is an essential trait for plant survival and development after stressful events.

Osmotic adjustment is another citrus response to flooding found in Cleopatra mandarin, being related to the accumulation of osmotically active amino acids. Although proline accumulation has been found under flooding (García-Sánchez et al. 2007; Arbona et al. 2008), this molecule is suggested to be effective in protecting cellular function with nonsignificant role in osmotic adjustment (García-Sánchez et al. 2007). However, proline role in the detoxification of reactive oxygen species and in the protection of cell membranes was not confirmed under flooding (Arbona et al. 2008).

While leaf respiration was not affected by flooding, the respiration and total concentration of nonstructural carbohydrates were reduced in roots of sour orange and rough lemon (Vu and Yelenosky 1991). Velikova et al. (2012) have found increased leaf respiration in lemon trees subjected to flooding. Such discrepancy in relation to the data reported by Vu and Yelenosky (1991) may be due to water quality and experimental design as Velikova et al. (2012) evaluated the response of lemon trees to recurrent flooding with saline water.

Regarding the nutritional status, flooding decreased the root concentration of nitrogen and potassium due to soil leaching and reducing uptake, respectively (Gimeno et al. 2012). Citrus sensitivity to flooding is influenced by growth substrate type, with plants being more affected when waterlogging occurs in organic soils (Schaffer 1991). In soils with high organic matter, Tahiti limes grafted on *C. macrophylla* were more resistant to flooding when compared to *C. aurantium* and *C. paradisi* (Schaffer 1991).

As photosynthesis is almost abolished in plants under flooding and they continue to intercept light energy, the generation of reactive oxygen species is a probable consequence. Besides the generation of H_2O_2 through the Mehler reaction and photorespiration, the fermentative metabolism may be indirectly another source of H_2O_2. In such situation, the enzymatic and nonenzymatic antioxidant systems have pivotal function in the detoxification of plant tissues (Blokhina et al. 2003). The onset of lipid peroxidation (membrane damage) may play an important role in flooding sensitivity, because as fast as this specific oxidation occurs, the more sensitive is the rootstock to flooding (Arbona et al. 2008). The delay in membrane damage is caused by an active antioxidant system, which has increased activities of superoxide dismutase, ascorbate peroxidase, catalase, and glutathione reductase, as well as recycling of antioxidant metabolites.

3.2.1 Mechanisms of Resistance

Rough lemon is considered a rootstock resistant to flooding. Some of the specific traits of this rootstock may be related to its higher flooding resistance such as high root growth and root hydraulic conductivity, and high photosynthetic capacity and low leaf abscision (Syvertsen 1981; Vu and Yelenosky 1991). The ability to replace

damaged roots under flooding is another factor causing better performance of rough lemon in these conditions (Syvertsen and Lloyd 1994; Hardy et al. 2012). The rootstocks F-A 5 and F-A 13 are also resistant to flooding. However, the morphophysiological basis of such resistance has not been identified (Forner et al. 2003).

Desired traits in flooding-resistant plants are rapid recovery of leaf water status and stomatal aperture and less sensitivity of carbon metabolism, with maintenance of photosynthesis and carbohydrate availability to resume growth after the stressful event. Apparently, those traits would be linked to rapid replacement of dying roots. In addition, an active antioxidant system is another physiological trait important under flooding. Although anatomical adaptations to flooding have not been observed in citrus rootstocks, studies comparing the root and leaf anatomy of citrus plants in such stressful condition were not found in literature. Also, another interesting characteristic would be the resistance against opportunistic diseases, such as those caused by *Phytophthora* (Hardy et al. 2012).

3.2.2 Improving Plant Performance Under Flooding

Some researches have been carried out with the aim of improving citrus performance under flooding, a difficult task due to our little understanding about the genotypic variation in relation to physiological and anatomical responses of citrus to this kind of environmental stress.

Inoculation of vesicular–arbuscular mycorrhizal fungus did not improve the performance of sweet orange, Carrizo citrange, and sour orange rootstocks under flooding (Hartmond et al. 1987). The use of interstock has not improved the performance of Verna lemon trees under flooding, with plants presenting higher physiological sensitivity as compared to plants without the interstock. The high sensitivity to flooding when using interstock was probably caused by high O_2 demand due to high root biomass (Gimeno et al. 2012). The interstock also affected the sugar translocation from shoot to root. Grafted plants without interstock showed an increase in root sugar concentration, whereas those with an interstock had reduced sugar concentration in the roots (Gimeno et al. 2012).

Transgenic Carrizo citrange expressing the Mybleu transcript factor has increased the resistance to flooding through enhancements in expression of genes of carbohydrate metabolism (alcohol dehydrogenase, pyruvate decarboxylase, and sucrose synthase) and antioxidant system (superoxide dismutase). Higher expression of class-1 hemoglobin proteins (*HB1*) was also noticed in transgenic plants as compared to the wild type (Caruso et al. 2012).

3.3 Salinity

Salinity is a well-known environmental stress, affecting plant growth and development, and it becomes an important constraint in semiarid and arid areas where irrigation is used as a common agricultural technique to improve crop production

(García et al. 2002; Ferguson and Grattan 2005). Among crops, citrus species are especially sensitive to salt stress (glycophyte), and this is a relevant aspect in many growing areas around the World, such as Israel, Australia, the USA, Pakistan, and Spain (Mass 1993; Ruiz et al. 1997; Storey and Walker 1999; García et al. 2002; Murkute et al. 2005). Besides salt ions naturally occurring in soils, salts are also dissolved in irrigation water, and their accumulation in plant tissues may become excessive and cause physiological disturbance (Mass 1993). As consequence, plant growth is impaired (Zekri and Parsons 1992) and fruit production may be affected (Storey and Walker 1999; García-Sánchez et al. 2003; Grieve et al. 2007). These plant responses are also caused by reduced osmotic potential in root medium, which affects water uptake and plant water relations. Therefore, reduced plant growth and crop yield may be related to the toxic effect of accumulated ions and/or to the osmotic stress caused by them (Zekri and Parsons 1990; García-Legaz et al. 1993; Ruiz et al. 1999; Storey and Walker 1999; Ferguson and Grattan 2005).

The uptake and transport of ions in salt-stressed plants are well discussed in the review paper by Storey and Walker (1999). In general, the uptake and transport of salt ions are affected by membrane structure. H^+-ATPase pumping and ion channels are regulated by sterol composition of membranes and cause less resistance to ion transport as compared to the diffusion through membrane lipids. While the influx seems to be passive, the efflux of Na^+ occurs by active transport. However, unequivocal evidence about the nature of Cl^- accumulation in citrus plants has not been found (Storey and Walker 1999). Once salt ions have overcome the physical barriers imposed by hypodermis and endodermis cells and reached the vascular tissue, there is an imminent chance of shoot tissues to have increased Na^+ and Cl^- concentrations.

As ions accumulate in plant shoots, leaf necrosis and abscision are observed (Mass 1993; Gómez-Cadenas et al. 2003; Arbona et al. 2005b). Then, salinity also affects the photosynthesizing leaf area by decreasing leaf size and canopy volume (Syvertsen et al. 1988; Ruiz et al. 1999). Leaf abscision due to salt stress is associated with increases of aminocyclopropane-1-carboxylic acid (ACC) due to abscisic acid (ABA) synthesis and Cl^- accumulation, with consequent ethylene production (Bar et al. 1998; Gómez-Cadenas et al. 1998, 2003; Arbona et al. 2003, 2005b). Regarding citrus growth, Ruiz et al. (1997) have proven through the classical growth analysis, the great impact of salinity on biomass accumulation. As leaf turgor may remain high in plants subjected to salt stress (Behboudian et al. 1986), the excessive accumulation of Na^+ and/or Cl^- seems to be the main factor causing leaf abscision (Storey and Walker 1999; Gómez-Cadenas et al. 1998). Besides the recognized effects on shoot development, salt stress also causes severe reduction in root growth even at low NaCl concentration in nutrient solution (Zekri and Parsons 1990; Ruiz et al. 1997). However, this root response to salt stress varies according to the rootstock. For instance, Cleopatra mandarin has shown an increase in the allocation of biomass to roots under these conditions (Cámara-Zapata et al. 2004).

Although salinity reduces the leaf water potential, turgor pressure is not necessarily affected as leaves accumulate ions and experience osmotic adjustment (Behboudian et al. 1986; Mass 1993; Bañuls and Primo-Millo 1995). In addition to ions, leaves also accumulate proline with consequent change in osmotic potential

(Bañuls and Primo-Millo 1992; Gómez-Cadenas et al. 1998; Arbona et al. 2003). At this time, two important points must be clarified about the osmotic adjustment: (1) it is not necessarily related to salt resistance as salt-stressed plants also present this physiological response, and (2) it does not happen in plants that have the ability to exclude Cl^- or Na^+ from leaves, if we consider these salt ions as the main osmolytes. The accumulation of osmolytes in roots may be involved in the osmotic adjustment of root tissues, a physiological response poorly studied in citrus trees. However, the role of carbohydrates in such osmotic adjustment in both root and leaves was discarded by Arbona et al. (2005b). These authors reported reductions in hexose, sucrose, and starch fractions in both plant organs of Clementina Nules clementine grafted on Carrizo citrange subjected to saline conditions.

Salt-resistant rootstocks have less sensitivity of root hydraulic conductivity to saline conditions, and the reduction in this root trait is associated with increase in root diameter, reduced proportion of fine roots, suberization of hypodermis, and altered permeability of root membranes (Storey and Walker 1999; García-Sánchez et al. 2000). By reducing the root hydraulic conductivity, the salinity also limits the plant transpiration (Syvertsen and Yelenosky 1988; Storey and Walker 1999; Rodríguez-Gamir et al. 2012). The salt-resistant rootstock Cleopatra mandarin has lower expression of aquaporins (PIP1) as compared to Carrizo citrange and *P. trifoliata* (Rodríguez-Gamir et al. 2012). In addition, the salt stress caused by NaCl (80 mM) did not change the aquaporin expression in Cleopatra mandarin. Therefore, the low root hydraulic conductivity under saline conditions is probably caused by changes in root structure. Under salt stress, the number of lateral roots is reduced (root order from eight to seven), and the impact of salt ions is dependent on root order (Rewald et al. 2012). Curiously, cortex and stele dimensions were affected by salinity only in roots of the third and fourth orders in *C. volkameriana*, more persistent roots as compared to the first and second orders. In general, changes in root hydraulic conductivity, stomatal conductance, and photosynthesis due to saline conditions vary among citrus species (Zekri and Parsons 1990; Mass 1993; Storey and Walker 1999).

Some studies have pointed out the Na^+ or Cl^- accumulations as the causes of reduced photosynthesis in plants under salt stress (Behboudian et al. 1986; Lloyd et al. 1987, 1990; García-Legaz et al. 1993; Storey and Walker 1999; García-Sánchez et al. 2002a). Chloride is the main salt ion accumulated in leaves, whereas Na^+ tends to be more retained in roots and stem tissues as compared to Cl^- (Ferguson and Grattan 2005). As salinity causes stomatal closure (García-Sánchez and Syvertsen 2006), the mesophyll photosynthetic capacity may be reduced due to low CO_2 partial pressure during the initial stages of salt stress. An indirect relationship may exist between increased Na^+ and stomatal closure as Na^+ may replace K^+ in the vacuoles and then affect the turgor of guard cells (Behboudian et al. 1986; Zekri and Parsons 1990). However, such hypothesis remains to be tested. Increase in root-synthetized abscisic acid (ABA) and its transport to leaves may cause sustained stomatal closure due to salt stress (Gómez-Cadenas et al. 1998; Arbona et al. 2003). Decrease in stomatal density, due to saline conditions, can also reduce stomatal conductance and afterwards affect leaf gas exchange (Hepaksoy et al. 2002).

Salt stress may affect directly the photosynthetic capacity (García-Sánchez et al. 2002a) and also the stomatal apparatus by feedback regulation (Mass 1993; Storey and Walker 1999). In fact, stomatal closure may occur without any change in leaf turgor (Behboudian et al. 1986). As compared to leaf Cl^- concentration, a stronger negative relationship between leaf Na^+ concentration and photosynthesis was found ($r=0.92$ vs. $r=0.79$) in citrus species under salt stress, suggesting an important regulatory role of Na^+ on photosynthesis (Behboudian et al. 1986). Accordingly, the photosynthetic impairment under salt stress was caused by Na^+ accumulation (García-Legaz et al. 1993), with plants grafted on *P. trifoliata* exhibiting better photosynthetic performance than ones grafted on Cleopatra mandarin (Lloyd et al. 1987). In fact, trifoliate rootstock has the ability to exclude Na^+ of plant shoots by sequestering it in the roots and in the basal stem regions (Lloyd et al. 1987). However, some researchers have found that Cl^- accumulation was the cause of low photosynthesis and stomatal conductance (Bañuls et al. 1997; López-Climent et al. 2008), and others have proven that both or neither Na^+ nor Cl^- were responsible for low photosynthetic performance (Walker et al. 1982; Syvertsen et al. 1988; García-Sánchez et al. 2002a). According to Storey and Walker (1999), such discrepancy of results may be caused by different rates of Na^+ and Cl^- influx in leaves and the subsequent charge stabilization and ion compartmentation.

The citrus photochemistry is resistant to salt damage even with chlorophyll content being reduced (Behboudian et al. 1986; Lloyd et al. 1987; Zekri and Parsons 1992; Mass 1993; Walker et al. 1993; Almansa et al. 2002; García-Sánchez et al. 2002a; Hussain et al. 2012). Therefore, the photosynthetic impairments due to salt conditions are most caused by stomatal and biochemical limitations. The biochemical limitation was suggested because plants exhibited low photosynthetic rates even under high intercellular CO_2 concentration (García-Legaz et al. 1993; García-Sánchez and Syvertsen 2006, 2009). Leaf respiration rates, both in light (photorespiration) and in the dark, are also reduced by saline conditions (Behboudian et al. 1986; Anjum 2008). Root respiration is also suppressed by salt stress in sensitive rootstock (Karstens et al. 1993).

Regarding the oxidative damage caused by salt stress, Arbona et al. (2003) have reported that the sensitive Carrizo citrange rootstock has enhanced antioxidant enzymatic and nonenzymatic responses and maintained the lipid peroxidation under moderate levels in leaves. In conclusion, the deleterious effects of salt stress are related to the high Cl^- levels rather than to salt-induced oxidative damage. Leaf accumulation of flavonoids, phenols, and nonpolar molecules was also related to the Carrizo antioxidant protection (Arbona et al. 2003). The antioxidant response to salt stress is regulated by rootstock, with salt-resistant genotypes showing increases in the activities of SOD, CAT, and peroxidase (Balal et al. 2012). For instance, Verna lemon had salt-induced decrease in the activities of Fe-SOD and Mn-SOD isoforms when grafted on *C. macrophylla* or *C. reticulata* and this response was not found on *C. aurantium* rootstock (Almansa et al. 2002). An important aspect is that the significant increases in the activities of antioxidant enzymes such as peroxidases and SOD are also found in salt-sensitive rootstocks (Patel et al. 2011), revealing that the relationship between antioxidant response and salt resistance must be evaluated

carefully in order to avoid misleading conclusions. As salinity affects primarily the root system, investigation on antioxidant responses of citrus roots may reveal new information about the rootstocks. However, such data are not yet available.

An important aspect is that the response of leaf gas exchange is affected by the scion-rootstock combination and this sensitivity depends on the ability to tolerate the ions Na^+ and Cl^- in leaf tissues (Bañuls and Primo-Millo 1995). Although both Na^+ and Cl^- ions have been considered in salt-stressed citrus plants, some research works have reported that Na^+ accumulation in leaf tissues does not cause any injury and most of sensitivity to saline conditions is due to Cl^- accumulation in leaves (Zekri and Parsons 1992; Mass 1993; Bañuls et al. 1997; Moya et al. 2002, 2003; Brumós et al. 2009). There is a large range of leaf Cl^- concentrations that are considered toxic to citrus trees, varying from 2 to 20 g $(kg\ DM)^{-1}$ (Cole 1985; Levy and Shalhevet 1990; Levy et al. 1999; Raveh and Levy 2005). However, it is important to keep in mind that the toxic threshold varies according to the scion-rootstock combination.

Salt treatment also promotes nutrient imbalance, changing distribution and disturbing uptake and utilization rates of some important elements such as K, Ca, Mg, N, and P (Zekri and Parsons 1992; Ruiz et al. 1997, 1999; García-Sánchez et al. 2002a; Gimeno et al. 2010). For instance, salt-induced low Mg concentration may be related to the chlorophyll degradation previously commented, while low Ca concentration is a probable factor leading to root growth inhibition. Lea-Cox and Syvertsen (1993), García-Sánchez et al. (2002a), and Forner-Giner et al. (2011a) also reported decreases in N uptake in citrus rootstocks under salt stress. However, any impact of reduced N uptake on photosynthesis is unlikely, as the amount of stored N is commonly in excess in woody trees (Syvertsen 1987; Walker et al. 1993).

Salt stress also affects the citrus flowering and fruit set, which has a significant impact in fruit yield due to decreases in the number of fruits per tree (Mass 1993; Storey and Walker 1999; García-Sánchez et al. 2003). The threshold values of electrical conductivity of the saturated-soil extract in root zone for citrus species is between 1.2 and 1.5 dS m^{-1}, with fruit yield decreasing around 13 % for each 1.0 dS m^{-1} increase in salinity (Mass 1993; Ferguson and Grattan 2005). The effects of salinity on fruit quality are dependent on citrus species (Mass 1993; Grieve et al. 2007). In general, the external attributes are less affected as compared to the juice quantity and quality. Smaller fruit size and significant changes in soluble solids and titratable acidity have been reported in citrus trees subjected to salt stress (Mass 1993; García et al. 2002; García-Sánchez et al. 2003; Grieve et al. 2007).

3.3.1 Mechanisms of Resistance

The conclusions about salt resistance are, in some cases, built by comparing the performance of some rootstocks under saline conditions. However, the actual resistance should consider the dry matter production of a given scion-rootstock combination, comparing non-stressed and stressed plants. In this way, most studies have

identified rootstocks with relative salt resistance, which is dependent on exposure time, salt concentration and type, plant age, and scion species (Grieve et al. 2007). Another important question is that most of scientific research carried out about this topic deals with plant growth, giving less emphasis on fruit production under salinity (Mass 1993).

One important argument to explain the main mechanism of salinity resistance is the plant capacity to restrict the transport of Na^+ and Cl^- from roots to leaves (Storey and Walker 1999). Therefore, the primary challenge to explain this mechanism is to understand the physiological bases of ions uptake and transport. Ion transport depends on the activity of membrane channels that in turn depend on rootstock species (Storey and Walker 1999), with the capacity for ion accumulation being affected by both scion and rootstock (Fernandez-Ballester et al. 2003).

Sequestration of salt ions in cell vacuoles is a suggested mechanism (Lloyd et al. 1989). Salt-resistant cells have bigger vacuoles than salt-sensitive ones, indicating the compartmentation as an important strategy to avoid the deleterious effects of salt ions (Ben-Hayyim and Kochba 1983).

Accumulation of Na^+ in woody tissues restricts the transport to leaves, an exclusion mechanism (Mass 1993). The exclusion of Na^+ is related to the capacity of ion compartmentation in the vacuoles of parenchymal cells in roots and stems (Walker 1986; Bañuls et al. 1997; Gonzalez et al. 2012). Storey and Walker (1999) have suggested that part of the Na^+ reabsorbed from the xylem may be translocated to the phloem and directed to roots of trifoliate orange. However, such recycling is not yet proven in citrus plants. In fact, García-Sánchez et al. (2002a) have suggested that root ion accumulation and low ion transport ability are important traits for salt resistance.

The exclusion of Cl^- is controlled by root membranes, which limit the ion intake in both roots and vascular tissues (Storey and Walker 1999), being the membrane permeability affected by sterols. Protection of scion against Cl^- accumulation is mediated by limited ion uptake, accumulation of Cl^- in root and basal stem regions, osmotic adjustment, and decreased water uptake (Moya et al. 2002; Ferguson and Grattan 2005). Such ability to exclude Cl^- from leaves has a threshold related to the ion concentration in root medium (Mass 1993). For instance, Cleopatra mandarin has lost the ability to exclude Cl^- under high salt concentrations, where the osmotic potential was −0.35 MPa (Zekri and Parsons 1992). Murkute et al. (2005) have reported that exclusion capacity is lost at osmotic potentials lower than −0.2 MPa.

The rootstocks Cleopatra mandarin (Bañuls et al. 1997) and Rangpur lime (Grieve and Walker 1983) are among the Cl^- excluders, whereas Troyer citrange (Bañuls and Primo-Millo 1995) and *P. trifoliata* (Bañuls et al. 1997) are considered Na^+ excluders. Although *P. trifoliata* has the ability to exclude Na^+, on the other hand, it is a Cl^- accumulator (Zekri and Parsons 1992; Mass 1993). Ferguson and Grattan (2005) have suggested that Cleopatra mandarin is an excluder of both Na^+ and Cl^-, whereas Sykes (1992) and Zekri and Parsons (1992) have considered Cleopatra mandarin a good Cl^- excluder and a poor Na^+ excluder. In general, Cl^- exclusion is an effective trait to maintain the physiological activity of citrus leaves, whereas Na^+ exclusion is not effective to avoid the growth impairment due to saline conditions.

Considering the previous information, the most resistant plants must have the ability to exclude salt ions from scion—a rootstock function—and also the ability to maintain the physiological processes even with tissues presenting an increased concentration of salt ions—a scion function. Besides the resistance to high ion concentration, scions' ability to transport ions is related to the plant vigor and internal water flow driven by canopy transpiration (Mass 1993), which affects the ion accumulation in leaves. On the other hand, citrus rootstocks have an important role in preventing Na^+ and Cl^- accumulation in leaf tissues by restricting the axial transport (Mass 1993).

Although rootstocks have the ability to prevent the accumulation of Na^+ and Cl^- in leaf tissues, the direct comparison of several studies dealing with rootstock capabilities is complex. First, the plant age, the substrate type, and also how the salinity was applied must be considered. Second, the salt treatment must simulate what happens under field conditions (Mass 1993; Zekri 1993a; Storey and Walker 1999). Several attempts to establish rankings of salt resistance have been done (see the reviews of Mass 1993 and Storey and Walker 1999). However, comparison across experiments is difficult as previously mentioned. High plant growth, low water consumption and consequent high water-use efficiency, and low leaf Cl^- accumulation under saline conditions have been suggested as adequate salt-resistance indicators (Syvertsen et al. 2010).

Cleopatra mandarin has been considered a salt-resistant rootstock (Mass 1993; Storey and Walker 1999), inducing low water consumption. As consequence, less Cl^- and Na^+ are transported to leaves. This rootstock has also the ability to exclude Cl^- in roots (Storey and Walker 1999; García et al. 2002; Moya et al. 2003). In this way, any morphophysiological response limiting transpiration will reduce ion accumulation in leaves and improve salt resistance (Brumós et al. 2009). Therefore, an important trait related to salt resistance is water saving by citrus plant (García et al. 2002). The salt resistance of Cleopatra mandarin is related to the maintenance of root hydraulic conductivity, nutrient uptake, and root structure and also to the compartmentation of salt ions (García-Sánchez et al. 2000). In addition, Cleopatra mandarin has the ability to reduce rapidly the leaf Cl^- concentration during desalinization (Conesa et al. 2011).

Regarding gene expression under saline conditions, salt-resistant Cleopatra mandarin exhibits downregulation of genes related to photochemical reactions, photorespiration, and Calvin cycle through a rapid root signaling (Brumós et al. 2009). The strategy of Cl^- exclusion in Cleopatra mandarin was related to the ability of restricting net Cl^- loading in xylem roots, which was associated to the repression of *ICln* gene (Brumós et al. 2010).

Fine-root turnover allows the continuous root formation and may be suggested as a mechanism to avoid or delay salt accumulation in *P. trifoliata* leaves (Tozlu et al. 2000). However, young tissues tend to be saturated with saline ions more rapidly as compared to mature root tissues (Fernandez-Ballester et al. 2003). Although rootstocks have the ability to exclude salt ions from leaves and improve the citrus tree performance under field conditions, salt resistance is also related to scion sensitivity to salt damage (García-Legaz et al. 1993; Storey and Walker 1999).

In such scenario, the best option is to combine scion and rootstocks with resistance to saline conditions, keeping in mind that citrus trees should grow and have reasonable fruit yield.

3.3.2 Improving Plant Performance Under Saline Conditions

Some mechanisms of salt resistance have been recognized in citrus plants, and this section will focus on alternative ways to enhance tree performance under saline conditions. Although the plant growth and development are not similar among seedlings and field-grown mature trees, research carried out with seedlings is very helpful to uncover the physiological basis of salt resistance.

Salt resistance depends on growth media, with citrus growth being less impaired by saline conditions when plants grow in perlite and clay-loam soils (Gimeno et al. 2010). In fact, the sensitivity of Carrizo citrange to salt stress varies according to the growth media, and the characterization of salt resistance must take this factor into account (García-Sánchez and Syvertsen 2009).

In plants grafted on Cleopatra mandarin, the supplementation of calcium causes increases in Na^+ concentration and reduction in K^+ concentration in roots, indicating that the accumulation of Na^+ inhibits the uptake of K^+ (Bañuls et al. 1991). Experiments carried out with nutrient solution containing NaCl have high Na/Ca ratio, which represents a sodic condition rather than saline and causes ionic imbalance (Bañuls et al. 1991; Mass 1993). In fact, the adverse effects of salt stress on plant growth and leaf injury are mitigated by exogenous application of calcium, with Ca^{+2} limiting the transport of Na^+ and Cl^- to leaves (Bañuls et al. 1991; Boman et al. 2005; Chatzissavvidis et al. 2008; García-Sánchez and Syvertsen 2009). Cramer et al. (1985) and Bañuls et al. (1991, 1997) have suggested that the beneficial effect of Ca^{+2} under saline conditions remains in the maintenance of membrane selectivity and integrity.

Supplementation with nitrate has also affected citrus performance under saline conditions, improving total biomass production and reducing leaf abscision (Iglesias et al. 2004). Such responses were likely to be the consequences of increases in leaf N, chlorophyll content, and photosynthetic rates, with N-supplemented citrus showing reduced leaf Cl^- concentration due to a dilution effect induced by increasing leaf dry matter (Iglesias et al. 2004). The application of potassium nitrate in salinized citrus avoided the negative impact of excessive Cl^- accumulation in citrus leaves, and the underlying mechanism is probably related to the antagonistic relationship between Cl^- and NO_3^- uptake by roots (Gimeno et al. 2009a). Application of ABA on plants has reduced the Cl^- accumulation in leaves and then decreased the leaf abscision. ABA-induced stomatal closure, leading to reduction of leaf transpiration, was associated to decreases in leaf abscision under salt stress (Gómez-Cadenas et al. 2003).

Besides being a potential cause of soil salinization when fertigation is applied, irrigation is a management technique to remove the excess of salt ions from root zone (Mass 1993; Boman et al. 2005). Such leaching effect is also promoted by

rainfall in well-drained soils. Ferguson and Grattan (2005) and Boman et al. (2005) have suggested that irrigation management (timing and quantity) is one of the few practical ways to alleviate the effects of long-term salt stress, which is in agreement with the results obtained by García-Sánchez et al. (2003) in lemon trees and Grieve et al. (2007) in sweet orange trees. In fact, reasonable fruit yield can be reached with appropriate water management even using saline water to irrigate citrus trees (Grieve et al. 2007). Some practices are related to the spatial irrigation uniformity, to avoidance of the contact of saline water with leaf blade, and also to a fine irrigation schedule (Boman et al. 2005).

According to the review by Mass (1993), mycorrhizal fungi have no effect on salt resistance of citrus species, causing in some cases increased Cl^- uptake, an undesirable response. However, a recent study has shown that the inoculation of arbuscular mycorrhizal fungi has improved the plant growth of Volkamer lemon and sour orange seedlings under saline conditions (Khalil et al. 2011). In addition, Murkute et al. (2009) have also found positive effects of mycorrhizal fungi on plant growth and survival of *C. jambhiri* and trifoliate plants under salt stress.

The interstock has proven to be an interesting alternative to mitigate the saline effects on citrus growth. The performance of Verna lemon grafted on sour orange under salt stress was improved by using Valencia sweet orange as interstock (Gimeno et al. 2009b). The use of interstock under salt stress caused accumulation of Cl^- ions in roots and dilution of ion concentration due to increased leaf biomass, avoiding the deleterious effects of Cl^- accumulation in citrus scions (Mass 1993; Cámara-Zapata et al. 2003, 2004; Gimeno et al. 2009b). Such effect of interstock is determined by xylem diameter, with differences among rootstock, interstock, and scion causing restriction in water flow and then in ion accumulation (Cámara-Zapata et al. 2004; Gimeno et al. 2009b). According to Cámara-Zapata et al. (2003), ion accumulation can be reduced by a half when using interstock, which enables citrus plants to maintain the physiological activity under saline conditions. As mechanisms to protect citrus metabolism under salt stress, we may cite the low flux of ions to leaves due to interstock, the ion sequestration in leaf vacuoles, and the scion metabolic resistance to increased cytoplasmatic ion concentration (Cámara-Zapata et al. 2004). Although the interstock has the ability to change the salt ion accumulation in leaves (Storey and Walker 1999), more research is needed to prove the importance of such technique for salt resistance and crop yield in field-grown citrus trees.

Considering the transmissibility of resistance traits between scions and rootstocks, Moya et al. (2002) found that reduction in leaf Cl^- accumulation and plant vigor and small size of xylem vessels were the most transmissible traits in reciprocal grafts between Cleopatra mandarin (resistant) and Carrizo citrange (sensitive) rootstocks. Screening for salt resistance based on seedling emergence is not a suitable index, as there is no correlation between the sensitivity of seedling emergence and growth under saline conditions (Zekri 1993a, b). Leaf Cl^- concentration has been considered a good index to evaluate the salt resistance (Zekri and Parsons 1992; Zekri 1993a; Hussain et al. 2012), being leaf Cl^- accumulation dependent on rootstock (Behboudian et al. 1986; Moya et al. 2002). Accordingly, García et al. (2002) have suggested the use of Cl^- concentration in leaves and also the ratio between Cl^- concentration in shoot and root, an indirect measurement of the ability to restrict Cl^- transport.

A period of 56 days growing under saline conditions is considered long enough to select resistant rootstocks with salt-exclusion ability (Sykes 1992). However, the salt accumulation in mature trees is a continuous process during several years, and the results of short-term studies comparing rootstocks are probably different from those long-term studies developed under field conditions (Mass 1993; Grieve et al. 2007). In fact, salt resistance must consider the fruit yield under saline stress (García et al. 2002), a difficult task for breeding programs due to juvenile period of citrus trees.

Citrus ability to exclude Cl^- is not related to the ability to exclude Na^+, and such physiological capacities are inherited (polygenic traits), with progenies having better performance under saline conditions than their parental species (Sykes 1992). Using this assumption, citrus breeding to obtain salt-resistant hybrids is an alternative to overcome such stressful condition. Ferguson and Grattan (2005) also believe that the plant breeding will provide salt-resistant citrus species. In fact, Forner and colleagues (Forner et al. 2003) have bred the salt-resistant rootstocks Forner-Alcaide 5 (F-A 5) and Forner-Alcaide 13 (F-A 13) by crossing Cleopatra mandarin and *P. trifoliata*. As mechanisms against salt damage, F-A 5 and F-A 13 are able to exclude Cl^- from leaves, with lower Na^+ uptake as compared to Cleopatra mandarin and trifoliate orange (Forner-Giner et al. 2009, 2011a). Under salt stress, F-A 5 has the ability to maintain the photosynthetic activity (Forner-Giner et al. 2009), which is the opposite response as compared to Cleopatra mandarin (López-Climent et al. 2008). This latter salt-resistant rootstock presents lower Cl^- accumulation in leaves and reduced stomatal conductance, CO_2 assimilation, and photochemistry under salt stress.

As the resistance against Cl^- accumulation is an inheritable trait (Mass 1993; Saleh et al. 2008), the crossing between Cleopatra mandarin and Rangpur lime has been suggested. Besides mandarins (*C. reticulata*) as source of resistance, Hussain et al. (2012) have suggested the use of pummelo (*C. maxima*) for breeding new rootstocks with resistance to abiotic stresses. Good performance of the hybrid RT803 (Rangpur lime × Troyer citrange) to salt stress was reported by Levy et al. (1999), with plants being able to exclude both Cl^- and Na^+ and maintain low concentration of salt ions in leaves. However, its feasibility as citrus rootstock must be tested in field trails. Some *P. trifoliata* selections were able to exclude both Cl^- and Na^+ from leaves under salt stress, revealing an interesting option for citrus breeding (Sykes 2011). Ariel-Meloni et al. (2008) have found a rootstock that is more resistant than Cleopatra mandarin to salt stress, the hybrid Citrumelo 75AB (*C. paradisi* × *P. trifoliata*), which exhibited less impairment of plant growth and less accumulation of salt ions in leaves as compared to Cleopatra mandarin.

The salt resistance of tetraploid (4×) citrus genotypes has been evaluated in some recent studies (Saleh et al. 2008; Mouhaya et al. 2010). Leaf abscision due to salt stress was lower in 4× trifoliate (salt sensitive) as compared to its 2× genotype, whereas the saline conditions improved the plant growth of 4× Cleopatra mandarin (Saleh et al. 2008). However, responses to salt stress are affected by specific changes in anatomy (root anatomy, cell size, leaf thickness, number and size of stomata) and may cause lower performance of 4× genotypes as compared to 2× equivalents (García-Sánchez et al. 2002b; Mouhaya et al. 2010).

Regarding the biotechnological resources, Cervera et al. (2000) have successfully transformed Carrizo citrange plants with the halotolerance *HAL2* gene isolated from yeast. However, the salt resistance of those plants should be proven in controlled or field experiments. Another important finding was reported by Fu et al. (2011), who transformed *P. trifoliata* to overexpress a betaine aldehyde dehydrogenase gene (*AhBADH*) and increase accumulation of glycine betaine. As consequence, plants had improved performance under salt stress, showing less membrane damage and higher accumulation of K^+ in leaves as compared to the wild type.

When considering the interaction between environmental stresses, salinity may improve cold resistance due to low transpiration and reduced physiological activity of citrus species (Syvertsen and Yelenosky 1987, 1988). Under salinity, the continuous and previous uptake of salt ions may promote osmotic adjustment and then improve the plant performance under water deficit. Comparing drought and salinity, Pérez-Pérez et al. (2009) have found that lemon trees are less affected by salinity, being this response caused by efficient mechanisms of acclimation such as osmotic and elastic adjustments. Osmotic adjustment (by Cl^- accumulation) associated with increased rigidity of cell wall can maintain cell integrity and allow water uptake through the maintenance of water potential gradient between soil and leaves (Pérez-Pérez et al. 2009). Poor soil aeration caused by flooding limits the regulatory function of roots in excluding salt ions and then plant response to salt stress (Mass 1993). Even though, it is important to keep in mind that the simultaneous occurrence of environmental stresses has a greater impact on plant performance as compared to the each stress factor alone, as also suggested by Mass (1993).

3.4 Temperature

Temperature outside the optimum range for citrus trees is another limiting factor to plant growth and development. About this topic, not only chilling/freezing temperatures should be considered but also high temperatures that are known to impair physiological processes in citrus plants. Although there are many reports about the citrus response to temperature changes, varying from plant to molecular analyses, few researches have addressed the effects of rootstock in plant performance under unfavorable temperatures. After flooding, temperature stress is the second environmental factor less studied when considering the use of rootstocks. In addition, the effects of high temperatures in citrus physiology are still less understood as compared to low temperatures.

3.4.1 Low Temperature

The physiological responses to low temperatures are caused by changes in cellular level, mainly in cell membranes. As low temperatures affect membrane structure, changes in the activity of membrane-bound proteins as well as channels and

transporters are expected. This effect is intensified by freezing temperatures, causing extracellular ice formation and symplast dehydration due to reduced apoplastic water potential (Guy 1990). Leaf abscision and stem dieback are results of freeze damage, with stems being more resistant than leaves (Wilcox et al. 1983; Yelenosky and Vu 1992; Vu and Yelenosky 1993). Threshold temperatures to kill stem tissues are around −12 °C (Nesbitt et al. 2002), and in general citrus species tolerate up to −10 °C (Yelenosky 1985).

Low temperatures have significant effect on photosynthesis of citrus even under subtropical conditions, where citrus trees show reductions in stomatal conductance, low leaf CO_2 assimilation, and decrease in the activity of RuBisCO and regeneration of RuBP during the winter season (Ribeiro et al. 2009). Such photosynthetic responses are dependent on rootstock, with citrus grafted on Swingle citrumelo being less affected by night chilling as compared to those ones grafted on Rangpur lime (Machado et al. 2010). Such photosynthetic resistance was based on maintenance of primary photochemistry and less sensitivity of leaf gas exchange and carboxylation efficiency.

Exposure to chilling temperatures not only causes reduction in leaf CO_2 assimilation but also increases the activity of phosphoenolpyruvate carboxylase (PepCase) and the concentration of leaf soluble sugars. These plant responses were not changed by the use of Cleopatra mandarin, Carrizo citrange, and sour orange as rootstocks (Vu and Yelenosky 1993). As a natural consequence of environmental stress, citrus plants are also subjected to oxidative damage under chilling/freezing temperatures, and the rootstocks are able to alter the sensitivity to this kind of stress by modulating the antioxidant protection. Lipid peroxidation was lower, and the activity of ascorbate peroxidase was higher in oranges grafted on *P. trifoliata* rootstock as compared to plants on Carrizo citrange or sour orange under low temperature (Tajvar et al. 2011).

Besides air temperature, soil temperature also significantly affects citrus trees. Soil temperature of 5 °C was sufficient to cause reduction in leaf water potential of citrus trees, without changing the leaf relative water content (Wilcox et al. 1983). Leaf water potential of rough lemon and Carrizo citrange seedlings was not affected by soil temperatures between 10 and 15 °C. However, stomatal opening, transpiration, and root hydraulic conductivity were reduced due to low temperature (Wilcox and Davies 1981; Wilcox et al. 1983). In fact, low substrate temperature is more deleterious to photosynthesis of citrus plants than low air temperature, causing both diffusive and biochemical limitations in orange plants grafted on Rangpur lime (Santos et al. 2011).

Citrus trees have the potential to cold acclimation, and this trait can be improved by rootstocks (Yelenosky 1985; Durham et al. 1991; Zhang et al. 2005b). Freezing resistance can be induced through cold acclimation, which is an alternative for improving citrus performance by exposing plants to low soil and air temperatures (Wilcox et al. 1983; Nesbitt et al. 2002). Carrizo citrange and trifoliate orange are cold-hardy species that may improve the survival of plant shoot to freeze (Wilcox et al. 1983; Yelenosky and Vu 1992). Although rough lemon and citron (*C. medica* L.) have been considered sensitive to cold, some studies have not found this pattern (Wilcox et al. 1983; Yelenosky and Vu 1992). In fact, the freezing temperature and

also the plant age are important factors in determining the influence of rootstocks on cold acclimation of citrus trees.

As mechanism of cold resistance, *P. trifoliata* seedlings present significant cold acclimation, changing the LT_{50} from −9 °C to −18 °C without losing leaves (Durham et al. 1991). Among physiological traits associated with chilling/freezing resistance, increases in leaf carbohydrate and proline concentrations as well as decreases in leaf water content in citrus trees may be cited (Yelenosky and Vu 1992). Less sensitivity of photosynthesis (Machado et al. 2010) and an active antioxidant metabolism (Tajvar et al. 2011) are also important traits to cold resistance. The signaling between rootstock and scions must be more explored once rootstocks are sources of nutrients and hormones to the scions (Yelenosky 1985; Huang et al. 2011).

Among the upregulated genes of *P. trifoliata* trees under cold acclimation, Zhang et al. (2005a) reported increases in expression of a betaine/proline transporter, aquaporin, an aldo-keto-reductase protein, an early-light inducible protein, and a nitrate transporter, with potential role in osmotic balance and antioxidant response. Recently, the upregulation of the *Ptcorp*, a gene associated with cold acclimation, was reported in *P. trifoliata* seedlings under freezing conditions (Long et al. 2012). Some genes related to photosynthesis, cell wall, and biotic defense are also downregulated during cold acclimation (Zhang et al. 2005b). Cold-regulated genes in trifoliate trees were well studied by Sahin-Çevik and Moore (2006a, b), Meng et al. (2008), and Huang et al. (2011), revealing several modifications in genes encoding both regulatory and functional proteins. From the molecular point of view, trifoliate plants showed a rapid expression of *CORc115*, a cold-induced LEA gene, in response to cold that was not verified in *C. paradisi* (Champ et al. 2007). Such changes in gene expression (up- and downregulation) seem to be part of a coordinate strategy to avoid or reduce freezing-induced damage in trifoliate trees. As consequence, this species is able to tolerate temperatures below −20 °C (Yelenosky 1985). However, the impact of changes in gene expression on physiological traits has not been reported, which limits our understanding about how rootstocks improve citrus performance under chilling and freezing conditions.

3.4.2 High Temperature

As compared to winter freezing, water deficit, salinity, and flooding, the occurrence of high temperatures is not a major limitation to distribution of citrus orchards. However, decreases in fruit yield due to high temperature are frequent under subtropical climates, mainly when heat waves occur during the flowering stage. Leaf temperatures may reach 8 °C or even 11 °C above air temperature in exposed portions of citrus canopy (Veste et al. 2000; Ribeiro et al. 2005) causing decreases in stomatal conductance and photosynthesis even in well-hydrated plants (Ribeiro 2006). Besides diffusive limitations, photosynthesis is also reduced due to low carboxylation activity of RuBisCO, degradation of chlorophyll, and impairments in primary photochemistry under high temperatures (Guo et al. 2006; Hu et al. 2007). Oxidative damage is also reported under heat stress, with production of reactive

oxygen species and increases in the activities of antioxidant enzymes, which depends on citrus scion species (Guo et al. 2006).

As previously commented for cold acclimation, citrus trees have the potential to acclimate to high temperature when growing in warmer temperature regimes, showing enhancement in photosynthesis, growth, and thermal resistance (Ribeiro et al. 2004, 2006, 2012). Photochemistry is able to acclimate through changes in chloroplast membrane properties, increasing the thermal stability in citrus plants grown in arid regions (Veste et al. 2000). In fact, heat damage is not common in nature as the threshold temperature for damage of citrus leaves is around 55 °C (Ahrens and Ingram 1988) and management techniques using reflective nets and shade screens are able to avoid excessive warming of leaves and improve the photosynthetic performance of young trees (Medina et al. 2002; Jifon and Syvertsen 2003).

Considering the interaction between high temperature and other environmental stresses, one important aspect is that heat stress is commonly associated with drought (Machado et al. 2007). Therefore, the discrimination between the effects of high temperature and drought on field-grown trees is not an easy task. As citrus species have C_3 metabolism, the interaction between increasing CO_2 concentration (360–720 μmol mol^{-1}) and increasing ambient temperature (up to 4.5 °C) improved citrus biomass production (Allen and Vu 2009).

Although the effects of instantaneous and growth temperature on citrus growth and development have been studied, including physiological and morphological changes (Khairi and Hall 1976; Hall et al. 1977; Ribeiro et al. 2004, 2012; Guo et al. 2006), few reports were found on the effects of high temperature on rootstocks and on citrus response to increasing temperature as affected by rootstock. Heat damage was studied in roots of sour orange, Carrizo citrange, and Swingle citrumelo rootstocks, with the lethal temperature varying between 51.6 °C for Carrizo citrange and 53.5 °C for Swingle citrumelo. However, a short exposure of 25 min to 50 °C was sufficient to cause leaf wilting and change the shoot structure in all three species (Ingram and Buchanan 1984). In another study about citrus sensitivity to daily temperature variation, Bueno et al. (2012) found that rootstocks affect the response of Valencia sweet orange scions. Under high daily temperature variation, Rangpur lime caused higher shoot growth due to high root carbohydrate availability as compared to Swingle citrumelo.

4 Conclusions and Future Perspective

The use of rootstocks in citriculture is an essential issue for increasing crop yield and improves orchard health in the main growing regions around the World. Therefore, the evaluation of canopy response to a specific environmental condition cannot be done without considering the rootstock, which is known to significantly affect the physiology and morphology of citrus shoots (Davies and Albrigo 1994; Syvertsen and Lloyd 1994; Spiegel-Roy and Goldschmidt 1996; Agustí 2000; Pompeu Junior 2005).

Although we have recognized the role of rootstocks for crop production and many countries have invested in research and development, our knowledge about the interaction between scions and rootstocks is still limited under environmental stresses. Considering the published data on the interaction between rootstock and the environmental stresses discussed herein, most of available scientific papers are on salinity (almost 60 %), followed by drought, temperature, and flooding. In addition, the mechanisms of citrus resistance against environmental stresses have been studied and revealed in few cases. In these cases, the physiological and molecular bases of resistance to salinity in Cleopatra mandarin (Brumós et al. 2009) and to drought in Rangpur lime (Boscariol-Camargo et al. 2007) have been reported.

Another important issue is the citrus responses to multiple and simultaneous stresses, which commonly happen in nature and may cause cross protection. Few studies about the interaction between stresses and the role of citrus rootstock have been found, and none of them reported the hardening imposed by recurrent stresses, considered a common situation as citrus trees are perennial species. In fact, the importance of rootstocks in alleviating the impact of stressful conditions on citrus physiology and fruit yield must also be considered in long term by evaluating bearing citrus trees under field conditions.

Under the concern of global warming, increases in air temperature will probably interact with salinity, increasing the vulnerability of citrus trees. If water demand by crops is increased in parallel to the air evaporative demand, less water is available for irrigation. As consequence, poor water quality is a possible alternative for crop production in arid and semiarid regions, causing problems associated with salinity (Paranychianakis and Chartzoulakis 2005). This is only an example of an interaction that should be addressed in citriculture. These interactions might be negative or beneficial, as reported by García-Sánchez and Syvertsen (2006). Under increased air CO_2 concentration (700 vs. 360 μmol mol^{-1}), salt-sensitive Carrizo citrange had increased resistance against salt stress. Such improvement was caused by reduction in stomatal conductance and transpiration rates, which in turn lead to lower accumulation of salt ions in leaves (Syvertsen and Levy 2005).

The biotechnology may help in citrus breeding for overcoming natural barriers related to the reproductive biology, being a complementary tool to reduce the citrus vulnerability to environmental constraints. Research has been done in somatic hybridization and genetic transformation (Grosser and Chandler 2003; Dambier et al. 2011). Additional and potentially useful work may be developed, once the molecular basis to stress resistance has been revealed. This development may produce transgenic citrus rootstocks more resistant to drought and saline stresses.

As there is no perfect rootstock that has all desired traits related to resistance against both environmental and biotic stresses (Davies and Albrigo 1994), the choice of the rootstock must consider the edaphoclimatic characteristics of a given growing region as well as the biotic pressure. According to their response to environmental constraints, we can highlight the importance of *P. trifoliata* as a rootstock for regions with occurrence of chilling/freezing, Cleopatra mandarin for saline conditions, rough lemon for areas affected by flooding, and Rangpur lime and rough lemon for improving plant performance in regions with seasonal water deficiency.

Regarding high temperature, more research should be carried out about how rootstocks modulate the response of citrus to heat stress. Besides considering the biotic pressure, rootstock selection must take into account the fruit quality and destination, i.e., industry or fresh market. About this topic, Davies and Albrigo (1994) have presented a meticulous analysis of the tradeoffs between the resistance against environmental limitations, biotic factors, and fruit quality in several rootstock species.

Concluding, our research effort must be directed to improve our understanding about the physiological and molecular basis of the citrus rootstock resistance against environmental stresses and also to the breeding of new rootstock species with enhanced performance under multiple stress conditions. The susceptibility to biotic stress should also be considered, mainly when dealing with flooding. For this task, citrus physiologists, ecologists, molecular biologists, and phytopathologists have to interact with citrus breeders for the citriculture sustainability in a changing environment.

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Chapter 2
Role of Silicon in Enrichment of Plant Nutrients and Protection from Biotic and Abiotic Stresses

Durgesh Kumar Tripathi, Vijay Pratap Singh, Savita Gangwar, Sheo Mohan Prasad, Jagat Narayan Maurya, and Devendra Kumar Chauhan

1 Introduction

The Earth consists of several elements which are generally characterized as macro- (N, P, K, S, Mg and Ca) and micronutrients (Fe, Mn, B, Zn, Cu, Mo, Ni, Na, Si, Cl and Co). These elements play an immense role in enhancing the quality, quantity and protection of several plants (Fig. 2.1). Among nutrient elements, silicon (Si) is regarded as one of the most beneficial elements for the plant life (Epstein 1999, 2009) (Fig. 2.1). It is the sister element of carbon and occupies the same group in the periodic table. After oxygen, it is the second most abundant element on the Earth's crust (Epstein 1999). Silicon has multiple advantageous roles in the plant biology that is why in the past few decades, extensive studies have been carried out to know the nature, structure and benefits of silicon in plants. In the nineteenth century, scientists demonstrated the significant availability of silicon in different parts of plants, and therefore, its vital role in the agriculture and plants is considered (Guntzer et al. 2012).

In the present era, increased industrialization and urbanization has resulted into undesirable physiological, chemical and biological changes in the environment which has harmful effects on the crop quality and productivity. Biotic and abiotic stresses

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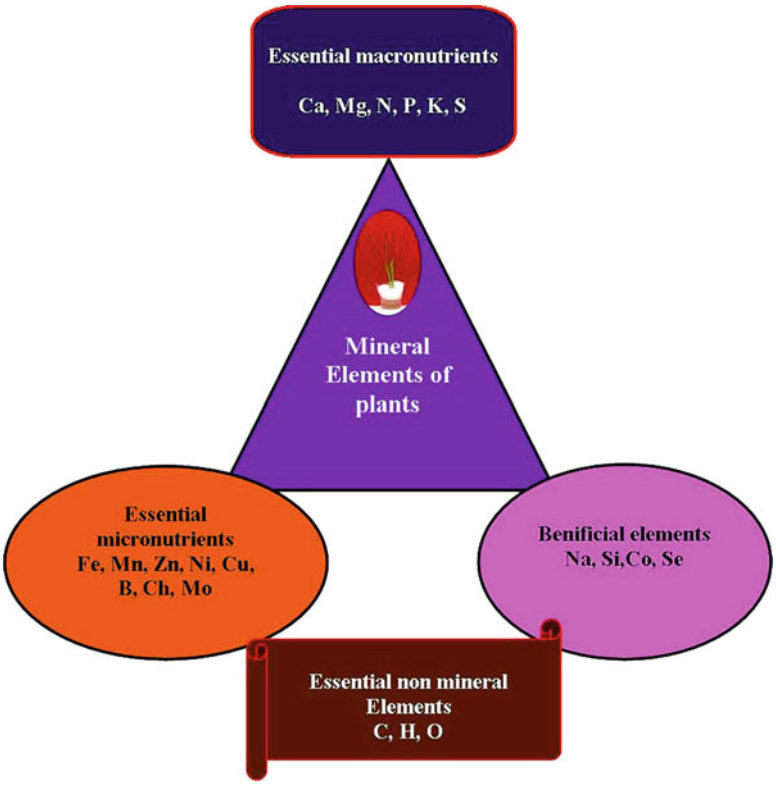

Fig. 2.1 Elements required for the growth and development of plants

are both major areas and challenges to the scientists working on the agricultural and agronomical aspects because both types of stress factors considerably reduce agriculture production per year (Broadhurst et al. 2004, 2013; Corpas et al. 2011; Kolbert et al. 2012; Bockhaven et al. 2012). Therefore, research on these problems is in progress to innovate the methods so that the negative impact of these stresses could be reduced. In the recent years, integrated nutrient management has been used as an effective method to protect plants from various abiotic stresses (Cakmak 2000, 2002; Shin and Schachtman 2004). A number of studies showed that Si has the capability to protect plants not only from biotic and abiotic stresses, but it also plays a role in enhancing the availability and also regulating the nutrient balance in plants during stress and non-stress periods (Marschner 1995; Waraich et al. 2011).

Generally silicon is not known as an essential element, but it is regarded as one of the most advantageous elements for the numerous plant species. It has been shown that under the abiotic and biotic stress environment, silicon plays an imperative role and protects plants of various species such as halophytic grass (*Spartina densiflora* Brogn.), corn salad (*Valerianella locusta* L.), makoi (*Solanum nigrum* L.), tomato (*Solanum lycopersicum* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), sugarcane (*Saccharum officinarum* L.) and several dicotyledons from

the hazardous effects of various stresses (Jones and Handreck 1967; Yoshida 1975; Elawad and Green 1979; Takahashi et al. 1990; Epstein 1994; Belanger et al. 1995; Savant et al. 1997, 1999a, b; Gong et al. 2005; Gottardi et al. 2012; Liu et al. 2013; Naranjo et al. 2013). Silica is absorbed by plants in the form of monosilicic acid and deposited in and between the plant cells which are commonly regarded as phytoliths. The name of phytolith is originated from the Greek word which means stone of plants; it is also known by various other names such as plant opal, biogenic silica and siliceous plant remains (Piperno 2006). Silicon has now become the element of interest for several disciplines of science like archaeobotany, palaeobotany, palaeoecology, plant taxonomy, plant physiology, nanotechnology and agriculture (Epstein 1999; Piperno 2006; Tripathi et al. 2011, 2012a, b; Chauhan et al. 2011). In this chapter, we summarized the beneficial role of Si in protecting plants against various biotic and abiotic stresses and also its probable role in management of nutrient uptake in plants.

2 Silicon and Plant Nutrients

Macro- and micronutrients are known to play an essential role in the entire plant life such as growth, productivity and metabolism (Marschner et al. 1996; Waraich et al. 2011). Mineral nutrients also protect plants by enhancing the resistance power against biotic and abiotic stresses (Marschner 1995; Waraich et al. 2011). It is also reported that imbalanced, deprived delivery of nutrients and poor soil fertility are meticulous troubles which may lead to reduced global food production (Peng and Zhou 2010; Moharana et al. 2012; Waraich et al. 2012). Plant physiologists and agriculturists are working in the area of nutrition research to generate the appropriate methods for protecting plants from the various hurdles. Some recent studies suggested that sufficient and impartial supply of mineral nutrients at the proper time is important for the growth and development of plants and is also required for intensive cropping scheme (Sarwar et al. 2010; Moharana et al. 2012). Exogenous supplies of mineral nutrients also play a key role against the heavy metal toxicity in plants (Pankovic et al. 2000; Hassan et al. 2005; Sarwar et al. 2010). It is also important to mention that Si plays a key role against the various stresses in plants by improving the mineral status. Till now very few studies have been carried out to find out the probable role of Si in nutrient management, and thus, detailed studies on the beneficial role of Si in nutrient management may contribute largely in stress physiology. Kaya et al. (2006) observed that water stress decreased C and K contents in maize plants. However, addition of Si improved the status of these nutrients in plants. Tripathi et al. (2012a, b) noticed that macro- (Mg, Ca and K) and micronutrient (Zn and Fe) contents in plant were decreased under Cr stress; however, Si addition improved the status of these nutrients. It is an established fact that various elements are involved in enhancing the internal integrity of plants. For example, Ca is essential for the development of the cell wall and maintenance of membrane structure, and under Cr toxicity, Ca level in plants gets reduced; however, addition of Si enhances the accumulation of Ca in plants (Marschner 1999; Waraich et al. 2011).

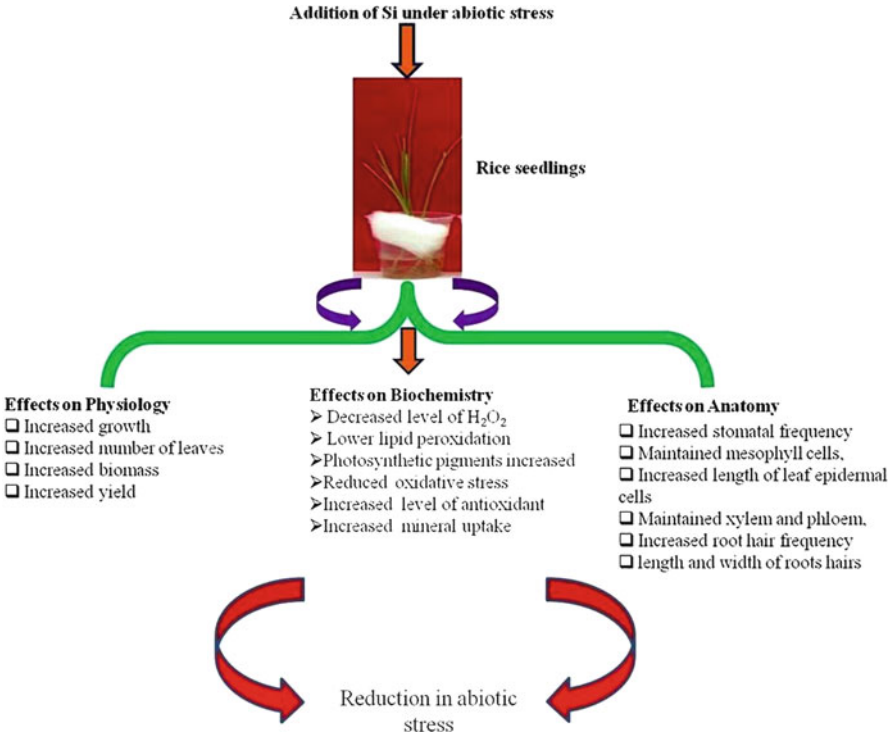

Fig. 2.2 Effect of silicon addition in plants against the biotic and abiotic stresses

Studies also confirmed that due to the metal toxicity, activity of indole acetic acid (IAA) oxidase is increased, which causes a deficiency of IAA (Morgan et al. 1976). Furthermore, the studies reveal that Zn^+ is required for the synthesis of IAA, and upon addition of Si in the growth medium, Zn content in plant increased. Hence, it is suggested that addition of Si might have increased IAA synthesis in plants by enhancing the Zn content, thus maintaining growth of plants under heavy metal (Al, Cd, Cr) stress (Singh et al. 2011a; Tripathi et al. 2012a, b). Further, in another study (Tuna et al. 2008), it was recorded that concentrations of Ca and K were significantly lowered in plants grown under high NaCl treatment but their concentrations were brought to the required level by the Si addition in both shoots and roots (Fig. 2.2). Ashraf et al. (2001) suggested that K plays a significant role in adjusting the osmotic pressure in plants, and appropriate K level in plants is the most advantageous condition to get rid of from water stress. Furthermore, under stress, decreased concentration of K in plants is reported; however, the addition of Si has been shown to increase its level in plants (Umar 2002; Sangakkara et al. 2001). Silicon seems to activate H-ATPase in the membranes, and therefore, K uptake in plants is significantly stimulated in the presence of Si (Liang 1999; Kaya et al. 2006; Miao et al. 2010). Thus, it appears that the application of Si may protect plants against various abiotic stresses by regulating the status of mineral nutrients (Fig. 2.2).

3 Silicon and Abiotic Stress

3.1 Silicon and Heavy Metal Stress

Pollution from heavy metals in soil, air and water is a global hitch that is causing a great loss to crop yield and exerts hazardous effects on human health when these metals enter into the food chain (Vernay et al. 2007). Chromium (Cr), aluminium (Al), cadmium (Cd) and arsenic (As) are the most toxic heavy metals which cause considerable hazardous effects on soil microflora, plant and other living organisms (Cervantes et al. 2001; Singh et al. 2011a, b). These heavy metals are widely used as industrial chemicals and their discharge effluents contaminate soil water and plants by enhancing their availability to plants (Pandey et al. 2005). By counting the loss and risk, heavy metal pollution is a serious problem for the researcher as well as the farmers. Therefore, there is a need to design methods to alleviate the toxicity of these heavy metals for safer food production.

Several studies confirmed that application of Si increases the capability of plants to withstand against stress and reduce the adverse effects and accumulation of various heavy metals in plants (Neumann and zur Nieden 2001; Nwugo and Huerta 2008; Singh et al. 2011b; Tripathi et al. 2012a, b; Huang et al. 2012). A literature survey shows that Si played a huge and significant role in alleviating metal toxicity. Wang et al. (2004) reported that formation of hydroxyaluminum-silicates in the root is responsible for Si-mediated detoxification of Al toxicity. Further, Neumann and zur Nieden (2001) showed that by forming Zn silicate in the cell walls, Si was directly involved in the detoxification of Zn. Heavy metals such as Cr, Al and Cd declined uptake of mineral elements, biomass accumulation, pigments and protein content in different parts of the plant (Singh et al. 2011a, b; Tripathi et al. 2012a, b) (Fig. 2.2). Morphological and anatomical studies revealed that metals alter the external and internal structures of leaves such as mesophyll cells, stomatal frequency, length of leaf epidermal cells, xylem and phloem and that of roots also such as root hair frequency, length and width of roots, xylem and phloem (Shanker et al. 2005; Singh et al. 2011b; Tripathi et al. 2012a, b; Soares et al. 2012; Vaculık et al. 2012). However, Si addition protects plants against metal toxicity by decreasing heavy metal uptake, root-to-shoot transport and MDA level and also by increasing mineral element uptake, TPCs and antioxidant capacity (Song et al. 2009; Singh et al. 2011b; Tripathi et al. 2012a, b). Application of Si also maintained the morphological and anatomical features such as increasing the number and length of root hairs and length of leaf epidermal cells, increasing the number of stomata and also maintaining leaf chlorophyll contents which are reduced by the metal toxicity (Fleck et al. 2011; Singh et al. 2011a, b; Tripathi et al. 2012a, b; Vaculık et al. 2012; Lukacova et al. 2013). These observations pointed out that Si addition under heavy metal contaminated soils may help in safer food production by reducing heavy metal load in grains and other edible parts of plants (Fig. 2.2).

Further, Iwasaki et al. (2002) showed that Si improved capacity of resistance against manganese by lowering Mn^{2+} concentration in the leaf apoplastic washing

fluid in *Vigna unguiculata*. Aluminium is a recognized major hazardous heavy metal and leads to reduced food production every year. Studies suggested that Si moderates positively Al toxicity in various plant species like conifers, rice, barley, soybean, sorghum and maize (Galvez et al. 1987; Barcelo et al. 1993; Baylis et al. 1994; Hammond et al. 1995; Ryder et al. 2003; Singh et al. 2011a, b). Like Al, As contamination is also an alarming environmental problem that is posing continuous threat to living beings (Acharyya et al. 2000; Shah 2010). In the environment, As exists in two main forms, i.e. arsenate (As^{V}) and arsenite (As^{III}), depending on the redox potential of the environment (Cullen and Reimer 1989). As^{V} is a phosphate analogue and is predominantly found in aerated soils and is easily transported into plants through the high-affinity phosphate transport system (Verbruggen et al. 2009; Indriolo et al. 2010). However, in reducing environment (partially aerobic) such as paddy field, As^{III} form is predominant and it enters in plants through nodulin 26-like protein (NIP) of the aquaporin subfamily (Verbruggen et al. 2009). After entry of As^{V} in plant roots via a phosphate transporter, it is rapidly reduced to As^{III} by arsenate reductases (Zhao et al. 2009). Further, it has been shown that As mobility from root to shoot varies among different plant species, indicating that it is under genetic control (Mirza et al. 2010). Loading of As^{III} into the xylem is important phenomena in arsenic translocation from root to shoot but it is not yet well understood. A study by Ma et al. (2006) verified the existence of the gene encoding silicon/arsenite efflux protein *Lsi2*, which is accountable for the loading of As^{III} into the xylem of rice. It is known that As accumulation caused oxidative stress, reduced the growth of plants and adversely affected the metabolisms, morphology and biochemistry of plants (Milton et al. 1989; Meharg and Hartley-Whitaker 2002; Raab et al. 2004; Zhao et al. 2009; Hoffmann and Schenk 2011). Recently, Tripathi et al. (2013) observed that application of Si significantly alleviated oxidative stress caused by arsenic (As) in Triguna (rice cultivar) by reducing the arsenic (As) accumulation and enhancing the antioxidant system. These results could contribute to an understanding of the mechanisms of Si-induced increase in metal tolerance of plants as well as in increasing productivity of crops under stress conditions.

3.2 Silicon and Radiation Damage

The problem of enhanced UV-B radiation in the environment is of scientific interest globally as it causes severe damages to plant tissues and ultimately leads to reduction in crop yield and quality (Correia et al. 1999; Costa et al. 2002; Kakani et al. 2003; Riquelme et al. 2007). Depletion of the stratospheric ozone layer is a cause of enhanced level of solar UV-B radiation at the Earth's surface. Enhanced UV-B radiations affect many physiological processes such as seed germination, growth, photosynthesis, status of mineral elements, water balance and various metabolic processes and also cause considerable negative impacts on crop production (Alexieva et al. 2001; Brown et al. 2005; Riquelme et al. 2007; Shen et al. 2010).

Thus, protection of plants against UV-B radiation for safer food production becomes a major scope of investigation for scientists and researchers. Shen et al. (2010) showed that drought stress and ultraviolet-B radiation adversely affected the soybean plants and caused membrane damage, as evaluated by lipid peroxidation and osmolyte leakage. However, Si plays a protective role against the combined stress of drought and UV-B radiation by enhancing the growth, photosynthesis and antioxidant parameters. Further, Yao et al. (2011) reported that Si improved the tolerance of wheat seedlings against UV-B stress by increasing antioxidant compound. Shen et al. (2009) suggested that allocation of mineral elements is reduced under UV-B stress; however, addition of Si improved the translocation of K and Ca and enhanced dry mass production. Gotoa et al. (2003) demonstrated that exogenous application of Si reduced UV absorbance (280–320 nm) in the leaf blades of rice crop. Further, it has been suggested that the accumulation of Si in the plant leaves can be associated with decreased level of phenolic biosynthesis (Gotoa et al. 2003), thus confirming that exogenous application of Si increases silica deposition in rice plants, reduced the activity of CAD and ferulic and *p*-coumaric acids which might be closely connected to alteration in the UV defence system (Gotoa et al. 2003). Further, Fang et al. (2011) concluded that UV-B tolerance in rice plants might be regulated by *Lsi1* gene, because exogenous application of Si increased the accumulation rate of Si in rice plants and activated photolyase and associated antioxidant enzymes in plants which increased repair ability of DNA and helped to reduce the injuries caused by UV-B radiation. Further, the detoxification and photosynthesis-related pathways in *Lsi1*-overexpressed lines were strengthened under UV-B treatment and thus contributed to enhanced rice defence mechanisms (Fang et al. 2011). It is important to note here that further investigation related to Si-induced UV-B tolerance is required at the molecular level to understand the appropriate pathways involved in enhancing the resistance power of plants.

3.3 Silicon and Drought Stress

Among the various stresses, drought is also one of the most serious worldwide problems for agriculture production, which showed a harmful effect and decreased the crop production. It usually reduces the photosynthetic pigments and photosynthesis of plants (Ormaetxe et al. 1998; Gong et al. 2005). Monakhova and Chernyadev (2002) reported that drought severely decreases the photochemical activities and inhibits the activities of enzymes of the Calvin cycle. High activities of antioxidant enzymes and high contents of non-enzymatic constituents are important for plants to tolerate environmental stress conditions such as drought (Gong et al. 2005). To cope with this, it has been shown that application of Si is supportive for drought tolerance of plants (Shen et al. 2010; Ahmed et al. 2011a, b). Gong et al. (2003) revealed that addition of Si could sustain better water status and improve dry matter in wheat (*Triticum aestivum* L.) plants. Pei et al. (2010) also reported the negative

impact of drought on wheat seedlings; however, addition of Si improved the tolerance in wheat seedlings to water-deficit stress induced by polyethylene glycol. Further, Gao et al. (2004, 2006) proposed that application of Si in maize plants minimizes the transpiration of plant leaf and water flow rate in the xylem vessel and improved water use capability. At the same time, Hattori et al. (2005, 2007) reported that Si assists transport and water uptake in sorghum plants (*Sorghum bicolor* L.) under drought conditions. In rice plants Si could reduce the transpiration rate and membrane permeability under water shortage tempted by polyethylene glycerol (Agarie et al. 1998). Other studies have also demonstrated that silicon induced supportive effects under drought; however, the mechanisms involved remain unclear and need further investigations.

3.4 Silicon and Salinity

Every year an ample amount of crop production is affected by salinity stress because nearly about one-third of the world's irrigated lands is suffering from excess salinity (Szabolcs 1994; Lopez et al. 2002). Salinity is one of the major factors limiting plant growth and crop productivity by unbalancing cellular ions which results in ion toxicity and osmotic stress (Tester and Davenport 2003). The reactive oxygen species (ROS) contents like singlet oxygen, superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) were increased due to the salinity stress which imposed the oxidative stress in plants (Alscher et al. 1997; Mittler 2002; Neill et al. 2002). Activity of ROS-scavenging enzymes like peroxidase in *Chloris gayana* was increased; however, at the same time greater tissue lignifications in *Atriplex prostrata* take place due to the salinity stress which leads to restricted plant growth (Wang et al. 1997; Sanchez et al. 2004; Ortega et al. 2006). Numerous studies have been performed to inspect the character of Si against the adverse effect of salinity on a variety of crop plants such as wheat, rice, barley, tomato, cucumber and mesquite and concluded that Si plays a protective role against the salinity stress (Matoh et al. 1986; Bradbury and Ahmad 1990; Ahmad et al. 1992; Liang 1998, 1999; Yeo et al. 1999; Liang et al. 1996, 2003, 2005; Liang and Ding 2002; Zhu et al. 2004; Al-Aghabary et al. 2004).

Liang et al. (2005, 2006) suggested that during NaCl stress, Si enhances the GSH content and maintains the optimal membrane fluidity and also plasma membrane H^+-ATPase, thus reducing oxidative stress. Further, Tuna et al. (2008) reported that addition of Si reduced the rate of sodium transportation into roots and shoots under salt stress; however, at the same time shoot K and Ca concentrations were appreciably improved. Romero-Aranda et al. (2005) accounted briefly that Si is capable to enhance water storage capacity within plant tissues by which salt dilution rate is increased, which resulted into higher growth rate of plants. From the above studies, it can be concluded that Si can be a better tool and play a significant role to reduce the severe toxicity symptoms of salinity.

4 Silicon and Biotic Stress

From many decades, biotic stress including insects, pests and diseases is a major threat to agriculture which causes considerable reduction in crop yield all over the world, and therefore, it put forth major global concern for the sustainable agriculture production. Surprising global climatic changes open the possibilities for pathogenic contaminations and infections in plants. Various evidences show a big loss of crops due to insects and pest attack, and thus, biotic stress is a focused area of agriculture research. Various studies have shown that availability of Si by the routine path in plant cells protects plants from insects, pests and diseases (Chérif et al. 1994; Belanger et al. 1995; Epstein 1999; Anderson and Sosa 2001; Massey et al. 2006; Reynolds et al. 2009, Bockhaven et al. 2012). Therefore, studies have been conducted to find out the potential of this unique element having manifold roles against the various biotic stresses (Epstein 1994; Ma 2004), and thus, it is assumed that Si acts as a physical barrier to infection by inducing dynamic resistance mechanisms (Ma 2004; Fauteux et al. 2005).

4.1 Silicon and Pathogen Resistance

Generally Si is abundantly deposited in the monocots especially in the members of family Poaceae and shows a dynamic potential against pathogen resistance (Datnoff et al. 1997; Carver et al. 1998; Shettya et al. 2012). Plants accumulate silica beneath the cuticle, which makes a double layer of cuticle with Si, restricting the entry of fungal mycelium and preventing infection in plant tissues (Bowen et al. 1992; Yoshida et al. 1962). It has also been reported that plants which were treated with Si can produce the phytoalexins and phenolics when stressed by fungal infections (Fawe et al. 1998; Rémus-Borel et al. 2005; Kiirika et al. 2013). Furthermore, Cherif et al. (1994) reported that Si was able to generate a defence mechanism against pathogen infection. Additionally, it has also been reported that Si improved peroxidase, polyphenol oxydase and chitinase activities in cucumber plants under the *Pythium* infection (Cherif et al. 1994). It is noticed that addition of Si protects wheat and barley from *Blumeria graminis* and rice from *Pyricularia oryzae* (Fawe et al. 1998; Fauteux et al. 2005). In this context, application of Si has also been found suitable to protect the cucumber from various insect pests like *Pythium ultimum* and *Podosphaera xanthii* (Menzies et al. 1991; Cherif et al. 1992, 1994; Belanger et al. 1995; Ghanmi et al. 2004). Moreover, Si has been shown to reduce the harmful effects of powdery mildew in cucumber, barley and wheat, leaf spot in Bermuda grass (*Cynodon dactylon*), rust in cowpea and ring spot in sugarcane (Fawe et al. 2001). Figure 2.3 shows the cross section of bamboo leaf infected by fungal mycelium which clearly indicates that Si, which is deposited as a flat sheet between cuticle and epidermal cells, acts as a barrier for fungal mycelium growth and penetration into the tissues of plants (Yoshida et al. 1962).

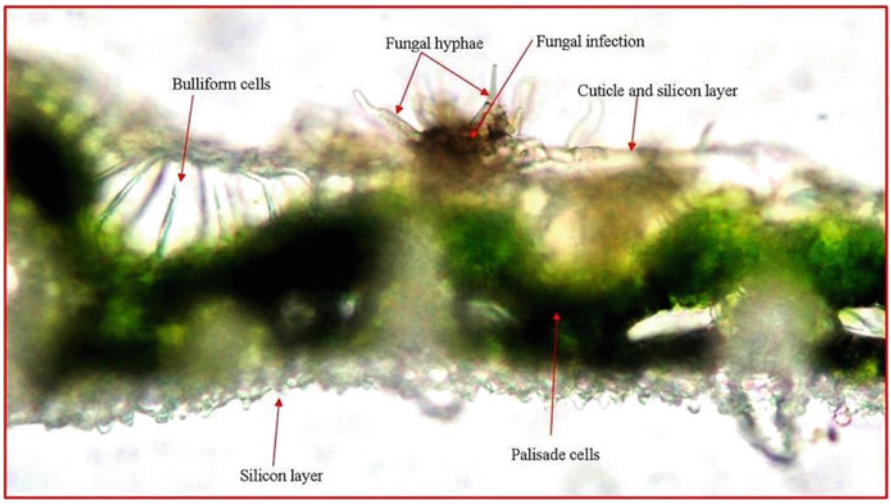

Fig. 2.3 Role of silica deposition in plants during the fungal attack

4.2 Insect, Pest Stress and Silicon

The problem of insects and pests is also of major concern in agriculture, basically for those farmers who have not been capable to purchase the costly chemicals for crop protection. Studies demonstrated that Si plays an important role in enhancing plant resistance against insect pest attacks (Chérif et al. 1994; Belanger et al. 1995; Epstein 1999; Anderson and Sosa 2001; Massey et al. 2006; Reynolds et al. 2009). Studies showed that Si deposition in various plant tissues provided a mechanical barrier against probing and chewing insects, and existence of silicified cells in plant tissues obstructs the feeding of insects (Savant et al. 1997; Massey et al. 2006). Some plants like rice, wheat and sugarcane accumulate high amounts of silica in their tissues that seem to interfere in the feeding of insect larvae (Epstein 1999). Savant et al. (1999a, b) reported that plants containing high Si content in their tissues showed better resistance against the infection of pests. Further, Sujatha et al. (1987) explain the positive association among the deposition of silica substance and insect pest resistance in rice plants. Several studies have also exemplified the positive effect of Si and proposed that Si plays a beneficial role in enhancing the resistance against various insects and pests such as brown plant hopper, stem borer, sugarcane stalk borer, leaf spider, green leaf hopper and non-insect pests such as leaf spider and mites (Ota et al. 1975; Tanaka and Park 1966; Maxwel et al. 1977; Yoshida 1975; Sujatha et al. 1987; Coulibaly 1990; Sawant et al. 1994; Savant et al. 1997).

5 Conclusions and Future Perspective

In the present instant, environmental pollution is a big problem before the humanities and scientific communities, limits agricultural production and also causes serious health problems to all living beings. Maintenance of better food availability for the increasing global population is a big challenge; hence, an appropriate scientific method is needed for the enhancement of productivity and protection of crops. From the last few decades, investigations have been carried out in order to minimize the impact of stress exerted by heavy metals, radiation, insects, pests, drought and mineral deficiency. Application of exogenous Si has appeared as an important implement which provides considerable protection to plants. Furthermore, studies illustrated substantial profits of this element against various biotic and abiotic stresses. However, our knowledge regarding the mechanism of Si accumulation and its deposition in plant tissues is slightly known. Additionally UV-B stress is one of the most important abiotic stresses that could influence every aspect of the physiology and biochemistry of plants. However, to our knowledge, limited efforts have been made to understand the physiological roles of Si in plants subjected to UV-B stress, and mechanisms of Si-mediated alleviation of damage caused by enhanced UV-B stress also remain unclear. Since enhanced UV-B radiation has been shown to affect growth and yields of crop plants severely, studies related to the effects of Si on UV-B-stressed plants will be interesting and helpful for protecting crop plants.

Further, studies related to the effects of biogenic silica and its nanoparticles on proteomics would be interesting, and results may further contribute to the understanding of mechanisms of Si-mediated impact on stressed as well as non-stressed crop plants. Since Si recycling and its effects on proteomics and genetic engineering are still lacking, it would also be interesting to investigate these aspects.

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Chapter 3
Transgenic Approaches for Phytoextraction of Heavy Metals

Atul Bhargava and Shilpi Srivastava

1 Introduction

A heavy metal is a member of an ill-defined subset of elements that include the transition metals, some metalloids, lanthanides, and actinides, all of which are known to exhibit metallic properties (Babula et al. 2008). The heavy metals have a specific gravity of more than 5 g/cm^3 in their standard state. Of the 90 naturally occurring elements, 53 can be considered as heavy metals, of which 17 are of importance for organisms and are bioavailable (Weast 1984). Iron (Fe), manganese (Mn), and molybdenum (Mo) are important as micronutrients; zinc (Zn), nickel (Ni), copper (Cu), vanadium (V), cobalt (Co), tungsten (W), and chromium (Cr) are toxic and required in traces; arsenic (As), mercury (Hg), silver (Ag), antimony (Sb), cadmium (Cd), lead (Pb), and uranium (U) are not nutritionally important and show toxicity towards various living forms (Godbold and Hüttermann 1985; Breckle 1991; Nies 1999; Schützendübel and Polle 2002). According to Wood (1974), most of the heavy metals can be categorized as toxic and their concentration in the soil varies between 1 and 100,000 mg/kg (Blaylock and Huang 2000). Arsenic (As), though a nonmetal, is often studied in heavy metal contamination since the reaction of most living forms to this element is similar to their reaction to the metal ions.

Large areas of developed as well as developing countries have been contaminated with high concentration of heavy metals that are the result of air emissions from combustion plants, oil, mining and other industrial processes, incinerators, and military and waste practices (Bhargava et al. 2012a, b). Heavy

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metal-contaminated soils pose a health hazard to all forms of life primarily humans, plants, and animals (Bhargava et al. 2012a) and cause serious danger to public health by entering the food chain or by leaching into drinking water. Besides this, they may have negative impact on ecosystems and other natural resources. The situation is compounded by the fact that heavy metals cannot be easily degraded and are difficult to remove from the environment (Sriprang and Murooka 2007). Since heavy metals can only be transformed from one oxidation state or organic complex to another, they need to be removed from the contaminated sites (Garbisu and Alkorta 2001).

2 Toxicity of Heavy Metals to Plants and Animals

Several metals are considered hazardous wastes that can accumulate in living forms and have a relatively large half-life. Heavy metals are detrimental to the growth of plants and survival of animal life (Sandalio et al. 2001; John et al. 2009; Bhargava et al. 2012b).

2.1 Toxicity of Heavy Metals to Plants

Plants are sensitive to heavy metals in a variety of ways that are enumerated below:

1. Uptake and accumulation of metals by binding to extracellular exudates and constituents of the cell wall
2. Extrusion of metals from cytoplasm to the extranuclear compartments
3. Complexation of the metal ions inside the cells by complex molecules
4. Concentration of osmolytes and osmoprotectants and induction of enzyme systems
5. Alteration of plant metabolism (Cho et al. 2003)

Heavy metals are known to induce changes at morphological, physiological, and molecular levels in the plants (Hall 2002; DalCorso et al. 2013). Heavy metal toxicity in plants varies according to the plant species, type of metal, their concentration, chemical structure, and edaphic factors (Schützendübel and Polle 2002; Nagajyoti et al. 2010). These are absorbed through the root systems, and once inside the plant, they induce destruction of chlorophyll, deficiency of essential minerals, and inhibition of root penetration (Kim et al. 2003; Manousaki et al. 2008; Shakya et al. 2008; Lamb et al. 2010; Singh et al. 2013). The uptake and accumulation of nutrients is influenced by alteration in the water absorption and solute permeability caused by the heavy metals (Hernández et al. 1997). The accumulation of heavy metals in plants and their subsequent release during decomposition facilitates their recycling in the ecosystem (Kim et al. 2003). This pathway regulates the level of toxic metals in the biosphere.

2.2 Toxicity of Heavy Metals to Humans

Some of the heavy metals are of importance to man, but their dietary intake has to be maintained at regulatory limits since excesses may lead to toxicity resulting in clinically diagnosable symptoms (Nolan 2003; Young 2005). However, metals like Cd, Pb, As, and methylated forms of Hg do not have much importance in human physiological processes and are toxic even at low concentrations (Holum 1983; McCluggage 1991; EU 2002; Young 2005). The effects of the heavy metals could range from toxic (acute, chronic, or subchronic), mutagenic, teratogenic to even carcinogenic (Duruibe et al. 2007). The toxicity, persistence, and nonbiodegradable nature of the heavy metals are potent threats to the living organisms. The presence of metals has been correlated with birth defects, liver damage, renal damage, cancer, and a range of disorders in various parts of the human body (ATSDR 2001). This toxicity is compounded by the fact that several heavy metals accumulating in the human body have a very large half-life (Salt et al. 1995).

Cadmium is one of the most important pollutants in terms of food chain contamination (Liu et al. 2005). Cd intake below the FAO/WHO tolerable weekly intake of 70 μg/day does not constitute a health risk. The metal poses considerable risk to human and animal health at concentrations that are not generally toxic for plants. Cadmium accumulates in the human kidney for a considerably long period (20–30 years) and produces deleterious effects on the respiratory and skeletal systems (Jin et al. 2004; Johri et al. 2010). Cd accumulates in the kidney cortex and causes renal tubular dysfunction (Strehlow and Barltrop 1988; McKenna and Chaney 1991). More severe exposure leads to bone defects like osteomalacia, osteoporosis, and spontaneous fractures (Alfvén et al. 2000). Subchronic airborne particle exposure to Cd leads to pulmonary effects like emphysema, bronchiolitis, and alveolitis, while high exposure leads to cadmium pneumonitis, an obstructive lung disease characterized by chest pain, bloody sputum, and death of lung tissues (McCluggage 1991; EU 2002; Young 2005).

One of the greatest concerns for human health is caused by lead contamination. Lead poisoning is an environmental and public health hazard that has acquired global proportions. Exposure to Pb occurs through multiple pathways such as air, food, water, dust, and soil (Lasat 2002; Gupta et al. 2013). The danger of Pb is compounded by low mobility even under high precipitation. The toxic effects of Pb include apoptosis, excitotoxicity, alteration in the lipid metabolism, inhibition of superoxide dismutase, suppression of the activity-associated Ca^{2+}-dependent release of acetylcholine, dopamine and amino acid neurotransmitters, disruptive effects on dopamine systems, and toxic effects on both astroglia and oligodendroglia (Lidsky and Schneider 2003). Lead poisoning initially causes damage to the central and peripheral nervous systems and inhibition of hemoglobin synthesis and finally leads to dysfunction of the kidneys and cardiovascular and reproductive systems (Ferner 2001; LWTAP 2004; Ogwuegbu and Muhanga 2005; Gupta et al. 2013).

Zinc is relatively harmless as compared to several other metal ions having similar chemical properties (Plum et al. 2010). Only exposure to high doses has toxic effects.

Zinc causes similar symptoms as lead poisoning that often leads to confusion (McCluggage 1991). Common signs of Zn toxicosis are diarrhea, vomiting, anemia, icterus, and liver and kidney failure (Fosmire 1990). Excess amount of Zn causes system dysfunctions resulting in growth impairment and reduced reproductive capacity (INECAR 2000; Nolan 2003).

The functional significance of Hg in human physiology is not known. Hg poisoning causes neurological disorders and extensive damage to the brain and central nervous system, abortion, corrosive esophagitis, hematochezia, congenital malformation, erethism, acrodynia, gingivitis, stomatitis, and kidney injury (Ferner 2001; LWTAP 2004; Charles et al. 2013; Miller et al. 2013).

Arsenic is known to coagulate proteins and forms complexes with coenzymes leading to inhibition of production of adenosine triphosphate, the main energy-yielding molecule in the body (INECAR 2000). Arsenic is carcinogenic with high exposure often causing death (Ogwuegbu and Ijioma 2003; USDOL 2004; Charles et al. 2013). Arsenic toxicity causes an immune disorder in which the body's immune system attacks its own peripheral nervous system which results in muscle weakness (Kantor 2006; Richards 2007).

Apart from plants, heavy metals have a detrimental effect on other forms of life. Livestock and wildlife have long been reported to be suffering from heavy metal poisoning (Rosenfeld and Beath 1964; Ohlendorf et al. 1986; Maracek et al. 1998).

3 Phytoremediation Technologies

The term phytoremediation refers to the remediation methods that efficiently utilize plant systems to remove pollutants (inorganic and organic) or render them harmless (Salt et al. 1998; Ali et al. 2013). This technique can be applied to reduce the pollutants present in soil, water, or air. The origin of the term phytoremediation can be traced to two words, the Greek word "phyto" meaning plant and the Latin suffix "remedium" meaning curing or restoring. This technique uses various approaches as depicted in Fig. 3.1.

Technologies for phytoremediation can be categorized into the types as shown below:

1. Phytoextraction. Phytoextraction refers to the removal of contaminants from soils by plants and their transportation and concentration in the harvestable parts. This technique utilizes plants that concentrate contaminants in their aerial parts so that the contaminant-enriched biomass can be properly disposed of (Kramer 2005). Several plant species are known that flourish in the presence of high concentration of contaminants in the soil and even hyperaccumulate them in their shoots (Baker and Brooks 1989).
2. Phytostabilization. Phytostabilization is the utilization of plants in mechanical stabilization of polluted soil for preventing bulk erosion, leaching, and reducing

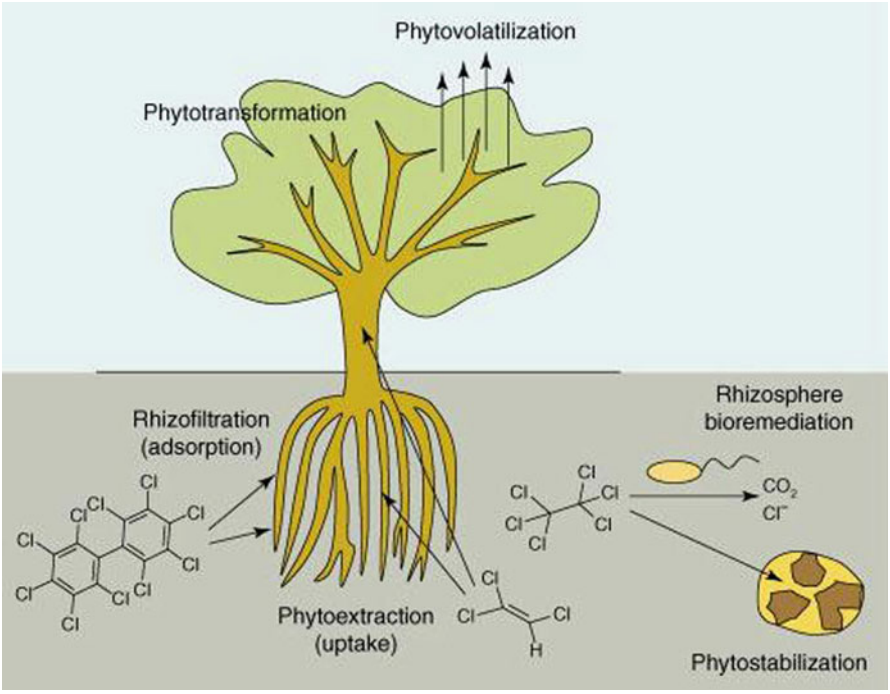

Fig. 3.1 Different phytoremediation strategies employed to clean up the environment (Reprinted from Aken (2008), with permission from Elsevier)

airborne transport of pollutants (Gonzaga et al. 2006). Such plants develop extensive root system, possess tolerance to the contaminant, and immobilize the contaminant in the rhizosphere. These plants provide a cover of vegetation for the contaminated site, thus preventing water and wind erosion (Kramer 2005).

3. Phytovolatilization. This involves biologically converting soil metals into volatile forms and releasing them into the environment (Chaney et al. 1997; Garbisu and Alkorta 2001; Lasat 2002; McGrath et al. 2002; Ernst 2005; Sakakibara et al. 2007). Contaminants like mercury or selenium, once taken up by the plant roots, can be converted into nontoxic forms and volatilized through various plant parts (Prasad 2003).
4. Phytoimmobilization. This involves decreasing the mobility and bioavailability of pollutants by the plants primarily through alteration of soil factors which lowers mobility of the pollutant and sorption on roots.
5. Phytotransformation. Phytotransformation, also referred to as phytodegradation, is the sequestering and breakdown of pollutant by plants into simple compounds that are integrated with plant tissue and foster plant growth (EPA 1998).
6. Rhizofiltration. Rhizofiltration is the form of bioremediation that makes use of plant roots to absorb, concentrate, and/or precipitate pollutants from contaminated

water bodies (Salt et al. 1998). This process removes contaminants by trapping them into harvestable plant parts, but is restricted for treatment of aquatic bodies.

Of the abovementioned techniques, phytoextraction seems to be quite attractive (Chaney 1983) and has been receiving increasing attention for decontaminating polluted soils. The advantages of this technique are low costs, generation of metal-rich plant residue, minimal environmental disturbance, and public acceptance (Kumar et al. 1995; Bhargava et al. 2008). The time required for remediation of the metal-contaminated site may range from 1 to 20 years depending on the type of contaminant, extent of contamination, growing season, and on how efficiently a metal is removed by the plant (Kumar et al. 1995; Blaylock and Huang 2000). Phytoextraction is most promising for the remediation of vast stretches of land contaminated with heavy metals at shallow depths (Kumar et al. 1995; Blaylock and Huang 2000).

4 Plants and Heavy Metals

Plants have been divided into three categories with respect to their response to excess amount of metals in their growing substrate (Baker 1981, 1987):

1. Excluders: These survive by avoidance mechanisms and are sensitive over a wide range of metal concentrations in the soil. Excluders prevent uptake of toxic metals into root cells (de Vos et al. 1991). These species avoid spread of contamination due to erosion and can be used for stabilization of soil (Lasat 2002).
2. Indicators: Indicator plants accumulate metals in their aboveground biomass which mirrors external concentration of metal in the soils (McGrath et al. 2002).
3. Accumulators: These can survive by physiological tolerance mechanisms and accumulate metals in the aboveground biomass at varied soil concentrations (Bhargava et al. 2012a). Accumulators do not prevent metals from entering the roots, thus allowing bioaccumulation of metals.

5 Hyperaccumulators

The term "hyperaccumulator," introduced by Brooks et al. (1977), refers to those plant species that can uptake metals, transport them to aerial parts of the plant, and accumulate up to 100-fold greater amount of the metal as compared to the non-accumulator plants (Baker and Brooks 1989; Baker et al. 2000; McGrath and Zhao 2003). Metal hyperaccumulation refers to the uptake and sequestration of extremely high concentrations of metal in the aboveground biomass of the plant growing in normal field conditions (Pollard 2000). Metal hyperaccumulators have the natural capacity to accumulate heavy metals in their aboveground tissues without exhibiting any toxicity symptoms (Bhargava et al. 2012a). A metal hyperaccumulator can

concentrate the metals to a level of 0.1 % (of the leaf dry weight) for Ni, Co, Cr, Cu, Al, and Pb; 1 % for Zn and Mn; and 0.01 % for Cd and Se (Baker and Brooks 1989; Baker et al. 2000). Metal hyperaccumulators have been reported to occur in over 450 species of vascular plants from 45 angiosperm families that comprise Brassicaceae, Asteraceae, Caryophyllaceae, Cyperaceae, Cunoniaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae, Violaceae, and Euphorbiaceae (Padmavathiamma and Li 2007; Bhargava et al. 2012a). The highest number taxa that are established for hyperaccumulation of metals are found in Brassicaceae (approximately 11 genera and 87 species), especially in the genera *Thlaspi* and *Alyssum*, wherein accumulation of more than one metal has been reported (Reeves and Baker 2000; Prasad and Freitas 2003; Verbruggen et al. 2009; Vamerali et al. 2010).

6 Transgenics for Improved Phytoextraction

The goal of remediating soils contaminated with heavy metals is extraction of the contaminant from the large soil volume and its concentration into various plant parts for harvest and easy disposal (Bhargava et al. 2012a). Biomass production and bioconcentration efficiency are the two factors that categorize a plant as an efficient phytoextractor (Cherian and Oliveira 2005). Bioconcentration is the ratio between the concentration of the pollutant in the aboveground parts of the plant and the concentration of the pollutant in the soil. Plants ideal for phytoextraction should have high biomass and deep roots, should be easily harvestable, and should accumulate considerable amount of the contaminant in their aboveground parts. However, no plant fulfils all these criteria. Most of the plants that accumulate metals exhibit metal selectivity, show sluggish growth, produce little biomass, and cannot be utilized for remediation other than in their natural habitats (Kamnev and van der Lelie 2000). Thus, in spite of the fact that the amount of metal concentration per unit of plant biomass is high, little amount of metal is removed from the contaminated site during a certain period of time (Bhargava et al. 2012a). Apart from this, the use of hyperaccumulator plants can be limited because of less information about their agronomic characters, breeding potential, pest management, and physiological processes (Cunningham et al. 1995).

An interesting alternative can be modification of a rapidly growing non-accumulator plant to modify it to achieve some of the features of the hyperaccumulators. Plant breeders and environmental researchers have long strived to develop such improved plant varieties which can be used for effective phytoextraction. Conventional breeding approaches coupled with suitable agronomic practices like soil fertilization and conditioning, proper plant density, crop rotation, weed control, and irrigation practices can go a long way in enhancing the phytoextraction capacity vis-á-vis metals (Lasat 2000). Traditional plant breeding uses the available genetic variation within taxa to bring together the traits that can aid in successful phytoextraction (Li et al. 1995; Liu et al. 2005; Bhargava et al. 2008). However, several anatomical constraints severely restrict sexual compatibility between taxa and pose

serious limitations in developing hybrids with increased phytoextraction capability. Biotechnology, especially recombinant DNA technology, has opened new gateways in phytoremediation technology by offering the opportunity for direct gene transfer that would overcome sexual incompatibility, if present (Bhargava et al. 2012a; Mani and Kumar 2013). This approach of the development of recombinant plants having increased uptake, accumulation, and tolerance can be considered as a good alternative. Recent progress in determining the molecular basis for metal accumulation and tolerance by hyperaccumulators has been significant and has provided us a path to tread for achieving this goal (Clements et al. 2002). Genetic engineering has enabled us to transfer specific genes conferring metal tolerance into plants having high biomass. Development of transgenic plants containing alien gene from various life forms and overexpressing these genes have been carried out for improving the phytoextraction potential, and considerable success has been achieved in this direction (Cherian and Oliveira 2005). The transgenic plants exhibiting greater phytoextraction capacity are generally produced by the manipulation of genes from microbes and mammals both of which have the metabolic machinery (degradative enzymes) required to achieve complete mineralization of organic molecules (by virtue of being heterotrophs) (Aken 2008). Apart from this, the introduction of trace-element detoxification systems from yeast and bacteria into plants also holds immense potential. A number of strategies have been followed to achieve this goal which are provided below (Pilon-Smits and Pilon 2002).

6.1 Metal-Binding Molecules

Plants respond to metal stress by mechanisms like chelation and sequestration of metals by use of particular ligands. Plant metal tolerance and accumulation are significantly influenced by overproduction of metal chelator molecules. The two metal-binding ligands best characterized and found across most taxonomic groups are the metallothioneins (MTs) and phytochelatins (PCs) (Cobbett 2000; Cobbett and Goldsbrough 2002; Kim et al. 2013). PCs, a family of thiol-rich peptides, were first reported in fission yeast (*Schizosaccharomyces pombe*) (1981) and later in plants (1985). PCs consist of three amino acids (glutamine, cysteine, and glycine) of which the Glu and Cys residues are linked through a γ-carboxylamide bond. PCs consist of repetitions of the γ-Glu-Cys dipeptide followed by a terminal Gly with the basic structure (γ-Glu-Cys)*n*-Gly [(PC)*n*] where n has been reported as high as 11, but usually ranges from 2 to 5 (Memon and Schroder 2009). MTs are ubiquitous, low molecular weight (500–14,000 Da), cysteine-rich proteins that give rise to metal-thiolate clusters and were originally isolated in 1957 as Cu-, Zn-, and Cd-binding proteins in equine kidney (Gratao et al. 2005; Yang et al. 2009). Both PCs and MTs have been widely reported in the plant kingdom ranging from algae, gymnosperms, monocots, and dicots and are known to play a crucial role in the uptake, transportation, tolerance, and accumulation of heavy metals in plants (Hartley-Whitaker et al. 2001; van Hoof et al. 2001; Cobbett and Goldsbrough 2002; Kim et al. 2013; Zagorchev et al. 2013).

Table 3.1 Expression of metallothionein genes in transgenic plants (Reprinted from Bhargava et al. 2012a, with permission from Elsevier)

MT gene	Source	Plant species genetically modified	Reference
mt-IA	Mouse (*Mus musculus)*	*Nicotiana tabacum*	Pan et al. (1994)
mt-ß-glucuro-nidase fusion	Chinese hamster (*Cricetulus griseus)*	*Nicotiana tabacum*	Hattori et al. (1994)
mt-II	Humans (*Homo sapiens*)	*Nicotiana tabacum*	de Borne et al. (1998)
cup1	Yeast (*Saccharomyces cerevisiae*)	*Nicotiana tabacum*	Thomas et al. (2003)
tymt	Cattail (*Typha latifolia*)	*Arabidopsis thaliana*	Zhang et al. (2004)
hmt	Humans (*Homo sapiens*)	*Medicago varia*	Watrud et al. (2006)
mt-1	Mouse (*Mus musculus)*	*Lycopersicon esculentum*	Sheng et al. (2007)
smtA	Cyanobacteria (*Synechococcus* sp.)	*Arabidopsis thaliana*	Xu et al. (2010)
femt3	Buckwheat (*Fagopyrum* sp.)	*Nicotiana debneyii*	Nikolic et al. (2010)
ccmt1	Pigeon pea (*Cajanus cajan*)	*Arabidopsis thaliana*	Sekhar et al. (2011)
psmtA	Pea (*Pisum sativum*)	*Populus alba*	Turchi et al. (2012)

Overexpression of MTs has opened up new avenues for enhancing heavy metal tolerance and accumulation, especially of Cd and Cu. A number of MT genes from various organisms like mouse, hamster, humans, yeast, cyanobacteria, and plants have been overexpressed in plants for enhancing heavy metal uptake and accumulation (Table 3.1) (Bhargava et al. 2012a). Overexpressed MT genes in *Brassica napus* and *Nicotiana tabacum* have led to increased Cd tolerance in the transgenic plants (Misra and Gedamu 1989). Overexpression of MT genes of plant origin has also resulted in increased metal accumulation in many plants. Enhanced Cu accumulation was reported in *Arabidopsis thaliana* when the pea MT genes were overexpressed in the plants (Evans et al. 1992). Overexpression of the MT yeast gene (*CUP1*) in cauliflower led to a 16-fold increase in tolerance towards Cd (Hesegawa et al. 1997). In tobacco, the overexpression of yeast metallothionein (*CUP1*) promoted 7-fold increase in Cu uptake during copper stress (Thomas et al. 2003). Compared to the control, the genetically engineered plants accumulated 2–3 times more copper when grown in copper-rich soils.

Modification or overexpression of the enzymes involved in the synthesis of PCs has been successfully employed to genetically transform plants having high biomass into efficient phytoremediators (Zhu et al. 1999; Bhargava et al. 2012a). Several reports are available wherein overexpression of the genes encoding enzymes that stimulate the synthesis of cysteine and glutathione has resulted in an increase in

the formation of PCs. The tolerance of transgenic plants to metals such as Pb and Cd was reportedly enhanced by the induction and overexpression of a wheat gene encoding phytochelatin synthase (TaPCS1) in shrub tobacco using *Agrobacterium-mediated* transformation (Gisbert et al. 2003). The recombinant plants had longer roots and greener leaves as compared to the normal plants. When grown in metal-contaminated soil, the transgenic plants showed significant increase in Pb concentration in the aboveground parts as well as in roots. An *Arabidopsis* PC synthase (AtPCS1) when overexpressed showed increased production of PCs (1.3- to 2.1-fold) in the transgenics as compared to the wild-type plants (Lee et al. 2003). The lines showed hypersensitivity to Cd and Zn stress but not for Cu. The enzyme cysteine synthase [O-acetyl-L-serine (thiol) lyase] belongs to the family of transferases and is responsible for catalyzing the last step for L-cysteine biosynthesis in plants producing L-cysteine and acetate (Cherian and Oliveira 2005). The tolerance of recombinant plants overexpressing the cysteine synthase cDNA towards a range of heavy metals (Cd, Ni, Pb, Cu, and Se) was analyzed by Kawashima et al. (2004). When grown on an agar medium containing Ni, Cd, and Se, the recombinant tobacco plants exhibited more tolerance than the wild types and showed comparatively higher fresh weight and root length. Two transgenic *Arabidopsis* lines were obtained by employing genetic transformation using the *Atcys-3A* cDNA construct expressing the cytosolic *O*-acetylserine-(thiol) lyase (Dominguez-Solis et al. 2004). There was enhancement in the cysteine availability in the recombinants which enabled the plants to survive under conditions of heavy Cd stress with most of the metal accumulating in the trichomes. Similar results were obtained when *Atcys-3A* was overexpressed in *A. thaliana* which resulted in increased Cd tolerance (Dominguez-Solis et al. 2001). Thus, modification of the cysteine biosynthesis pathway along with an alteration in the number of leaf trichomes may be highly beneficial in increasing heavy metal accumulation. The induction of PCs by Cd suggests that metal tolerance and accumulation can be improved by biosynthesis of phytochelatins, but supporting evidence that this approach would yield a Cd phytoextraction plant is lacking (Bhargava et al. 2012a). Although Cd tolerance has been increased 3–7-fold by overexpression of the enzyme PC synthase, this increase is quite small as compared to the 200-fold higher tolerance exhibited by *T. caerulescens* for Zn and Cd (Heiss et al. 2003; Chaney et al. 2005; Wang et al. 2006). The overexpression of PCs or MTs for Cd accumulation in tobacco plants has yielded similar results (Lugon-Moulin et al. 2004).

Ferritins are a broad superfamily of ubiquitous, intracellular iron storage proteins found in animals, fungi, bacteria, algae, and higher plants, but have not been reported in yeast (Briat et al. 2010a, b). These were first discovered and isolated from the spleen of horses. These proteins play a crucial role in the iron homeostasis and help alleviate oxidative stress through detoxification of excess iron (Briat et al. 2010b). The 450 kDa globular ferritin protein has the shape of an inorganic microcrystalline hollow sphere that surrounds about 24 subunits and can accommodate between 2,000 and 4,000 ferric iron atoms (Arosio and Levi 2002; Li et al. 2012). The overexpression of ferritin in transgenic tobacco and rice showed manifold increase in the iron content in different plant parts (Goto et al. 1998, 1999). Overexpression of ferritin in transgenic tobacco plants was achieved by Vansuyt et al. (2000) that led to

an increase in the iron content in leaves and seed, but the results were soil dependent. However, due to paucity of work and lack of conclusive results, more detailed studies are needed to recommend use of ferritins in phytoextraction.

Siderophores are low molecular mass, iron-chelating compounds produced by microorganisms and members of family Poaceae that increase the availability and uptake of iron into roots (Neubauer et al. 2000; Devez et al. 2009). Apart from sequestering iron, siderophores can chelate various other metals like Cr, Cd, Cu, Ni, Pb, and Zn (Nair et al. 2007). This specific chelation of metals can be of great relevance to decontamination of soil having high metal content.

6.2 Membrane Transporters

The acquisition, transport, distribution, and compartmentalization of metals within different tissues and cells are extremely essential for healthy plant growth and development and are also extremely important for remediation of toxic metals (Hall and Williams 2003; Cherian and Oliveira 2005). Transporters play a crucial role by shuttling potentially toxic cations across the membranes. The genetic manipulation of metal transporters can be of immense importance in enhancing metal accumulation and tolerance in plants. Although a number of membrane transporter gene families have been identified using powerful genetic and molecular techniques (Table 3.2) (Bhargava et al. 2012a), the molecular physiology of the plant transport systems is still in its infancy. The prominent cation transporters are mostly in the ZIP (ZRT, IRT-like protein), NAS (nicotianamine synthase), NRAMP (natural resistance-associated macrophage protein), YSL (yellow stripe-like transporter), CDF (cation diffusion facilitator), SAM (S-adenosylmethionine synthetase), FER (Ferritin Fe(III) binding), HMA (heavy metal ATPase), CTR (copper transporters), and IREG (iron-regulated transporter) family (Guerinot 2000; Williams et al. 2000; Talke et al. 2006; van de Mortel et al. 2006; Kramer et al. 2007; Memon and Schroder 2009; Maestri et al. 2010).

Two yeast genes FRE1 and FRE2 that encoded ferric reductase were introduced in tobacco (*Nicotiana tabacum*) under the cauliflower mosaic virus 35S promoter for enhancing uptake of iron (Samuelsen et al. 1998). The transgenic plants accumulated higher iron content in the shoots than the wild-type plants. Overexpression of NtCBP4, the metal transporter gene from tobacco encoding a calmodulin-binding protein, was carried out and the recombinants showed enhanced tolerance to Ni^{2+} and hypersensitivity to Pb^{2+} (Arazi et al. 1999). Thus, selective ion tolerance in crops can be introduced using NtCBP4 which was involved in metal uptake across the plasma membrane. Sunkar et al. (2000) prepared a truncated version of NtCBP4 designated as NtCBP4ΔC that was devoid of the C-terminal, the calmodulin-binding domain and part of the cyclic nucleotide-binding domain. Overexpression of this truncated protein resulted in the transgenic plants showing enhanced Pb tolerance and attenuated accumulation of Pb. The transgenic plants obtained by overexpression of antiporter calcium exchanger 2 (*CAX2*) from *A. thaliana* in *N. tabacum* accumulated more Cd^{2+} and Mn^{2+} and exhibited elevated tolerance to high Mn^{2+}

Table 3.2 Important metal transporter genes in different plant species involved in heavy metal tolerance and accumulation (Reprinted from Bhargava et al. 2012a, with permission from Elsevier)

Family	Gene	Plant	Metal transported	Reference
Zn-regulated transporter (ZRT)	*zip1-12*	*Arabidopsis thaliana*	Zn	Weber et al. (2004),
	zip4	*Oryza sativa*	Zn	Roosens et al. (2008)
	zip	*Medicago truncatula*	Zn	Ishimaru et al. (2005) Lopez-Millan et al. (2004)
	znt1-2	*T. caerulescens*	Zn	van de Mortel et al. (2006)
Fe-regulated transporter (IRT)	*irt1*	*Arabidopsis thaliana*	Fe	Kerkeb et al. (2008)
	irt1-2	*Lycopersicon esculentum*	Fe	Bereczky et al. (2003)
	irt1-2	*T. caerulescens*	Fe	Schikora et al. (2006), Plaza et al. (2007)
Natural resistance-associated macrophage proteins (NRAMP)	*nramp1-3*	*Lycopersicon esculentum*	Fe	Bereczky et al. (2003)
	nramp4	*Thlaspi japonicum*	Fe	Mizuno et al. (2005)
	nramp1	*Malus baccata*	Fe	Xiao et al. (2008)
Cation diffusion facilitator (CDF)	*mtp1*	*Arabidopsis thaliana*	Zn	Kawachi et al. (2008)
	mtp1	*Arabidopsis halleri*	Zn	Willems et al. (2007)
	mtp1	*Thlaspi goesingense*	Zn, Ni	Kim et al. (2004)
	mtp1	*Nicotiana tabacum*	Zn, Co	Shingu et al. (2005)
Al-activated malate transporter (ALMT)	*almt1*	*Triticum* spp.	Al	Sasaki et al. (2004)
	almt1	*Secale cereale*	Al	Collins et al. (2008)
P-type, ATPase (heavy metal associated)	*hma8*	*Glycine max*	Cu	Bernal et al. (2007)
	hma9	*Oryza sativa*	Cu, Zn, Cd	Lee et al. (2007)
	hma4	*Arabidopsis halleri*	Cd	Courbot et al. (2007)
	hma3	*Arabidopsis thaliana*	Co, Zn, Cd, Pb	Morel et al. (2008)
Nicotianamine synthase (NAS)	*nas2, nas3*	*Arabidopsis halleri*	Zn	Talke et al. (2006)
Copper transporter	*copt1*	*Arabidopsis thaliana*	Cu	Sancenon et al. (2004) Andres-Colas et al. (2010)
Yellow stripe-like (YSL)	*ysl2*	*Arabidopsis thaliana*	Fe, Cu	DiDonato et al. (2004)
	ysl3	*T. caerulescens*	Fe, Ni	Gendre et al. (2006)

levels mainly due to increased transport of metal ions in root tonoplast vesicles (Hirschi et al. 2000). It was suggested that modulation of CAX2 could be of immense importance in enhancing plant ion tolerance.

Another approach of developing efficient hyperaccumulator plant by using metal transporters is to alter their metal specificity. The *Arabidopsis* root membrane protein (IRT1) has an important role in the uptake of Fe, Cd, Zn, and Mn (Rogers et al. 2000). However, the substitution of glutamic acid residue with alanine at position 103 resulted in the loss of Zn transport capacity. Two other mutations, at position 100 or 136, eliminated the transport of both Mn and Fe. The experiments clearly demonstrate that the transport profile of a ZIP family member can be altered to raise plants with an altered specificity for selective metals.

6.3 Metal Metabolism

The introduction of an entirely new pathway from a new organism can be a good strategy to remove toxic metals from the environment. This approach was followed to convert toxic methylmercury to volatile elemental mercury by introduction of two bacterial genes (*merA* and *merB*). This strategy has been successfully applied in the engineering of plants capable of removing methyl-Hg from contaminated soils (Rugh et al. 1996; Pilon-Smits and Pilon 2002). *MerA* gene encodes mercuric reductase, an enzyme that reduces ionic mercury to elemental mercury, while *MerB* gene encodes organomercurial lyase, which converts methylmercury to ionic mercury (Summers 1986). *Arabidopsis* was the first to be engineered with the gene *merA* from an Hg-resistant bacterium (Rugh et al. 1996). Researchers later developed transgenic *Arabidopsis* plants wherein *merB* enzyme was targeted to the endoplasmic reticulum (Bizily et al. 2003). The recombinants showed 10 to 70 times higher specific activity to degrade organic Hg than the recombinants with cytoplasmic *merB*. Bizily et al. (2000) accomplished the complete pathway from methyl Hg to metallic Hg by developing double transgenics in *Arabidopsis* in which both *merA* and *merB* were expressed. These double transgenics were 50 times more tolerant to concentrations of methyl Hg in comparison to the wild-type plants and 10 times more tolerant than plants transformed with *merB* alone. In contrast to the *merA-merB* double transgenics, the wild-type plants and single transgenics did not volatilize elemental mercury on supply of organic mercury. The double transgenics efficiently converted organic mercury to elemental mercury and released it in volatile form (Pilon-Smits and Pilon 2002).

The same strategy discussed above has been followed to raise plants having the capacity to volatize mercury from other plant species. Transformation of yellow poplar (*Liriodendron tulipifera*) and eastern cottonwood (*Populus deltoides*) with *merA* led to increased tolerance of the transformants to ionic Hg (Rugh et al. 1998; Che et al. 2003). The recombinant plants cottonwood plants showed normal growth in 25 μM Hg(II), a concentration sufficient to kill the wild-type plants. The recombinants produced up to 4 times more elemental Hg as compared to the wild types, proving the ability of the transgenic plants for efficient uptake and transformation of

Hg to less toxic form. Heaton et al. (2003) successfully transformed rice (*Oryza sativa*) by incorporating the *merA* gene. The recombinants slowly converted Hg^{2+} to the less toxic volatile form and were tolerant to Hg^{2+} concentrations that were detrimental to the wild-type plants (Heaton et al. 2003). The transgenics obtained by double transformation of *Spartina alterniflora* (Smooth Cordgrass or Saltmarsh Cordgrass), the common wetland deciduous grass, with *merA* and *merB* showed increased tolerance to phenylmercuric acetate and mercuric chloride (Czako et al. 2006). Eastern cottonwood when transformed with both *merA* and *merB* exhibited high resistance to phenylmercuric acetate and had a greater rate of detoxification as compared to the control plants (Lyyra et al. 2007). In the transgenics, *merB* released Hg^{2+} by catalyzing the protonolysis of the C-Hg bond, which was subsequently converted to the less toxic, volatile elemental Hg^0 by *merA*. The transgenics released 10 times of elemental Hg as compared to the wild-type plants. However, public acceptance to this approach is low because Hg^0 volatilizes and can redeposit on soil or water bodies (Chaney et al. 2007). In this case, the contaminant instead of being destroyed simply transformed from soil-bound to the airborne form.

6.4 Oxidative Stress Resistance Mechanisms

Another interesting alternative to improve metal tolerance is the overexpression of enzymes involved in general stress resistance mechanisms (Pilon-Smits and Pilon 2002). Nine genes from *Arabidopsis, N. tabacum*, wheat, and yeast involved in oxidative stress response were expressed in an *Arabidopsis* ecotype by Ezaki et al. (2000). The transgenic plants were highly resistant to Al due to the resistance conferred by *Arabidopsis* blue-copper-binding protein gene (*AtBCB*) and three genes from tobacco, viz., glutathione S-transferase gene (*parB*), peroxidase gene (*NtPox*), and GDP-dissociation inhibitor gene (*NtGDI1*). Overexpression of an alfalfa gene encoding a NADPH-dependent aldose/aldehyde reductase in tobacco provided the transgenic plants greater tolerance against oxidative damage induced by heavy metals (Oberschall et al. 2000). Reduced accumulation of Cd and enhanced tolerance to the metal has been reported by the overexpression of glutathione reductase (Pilon-Smits et al. 2000). In another study, the overexpression of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase in *Lycopersicon esculentum* (family: Solanaceae) led to more accumulation and less deleterious effects of heavy metals in the transgenic plants in comparison to the non-transgenic ones (Grichko et al. 2000).

7 Transgenics in the Field

Although transgenics show promise for phytoextraction of heavy metals during laboratory studies using hydroponic systems or when grown on agar media, their field testing has been rarely carried out. This necessitates thorough testing of the transgenic plants for ascertaining the actual risks involved with the use of such

plants in the field for phytoextraction. Field trials with transgenic poplars carried out in former mining areas in Russia and Germany to assess the risk of transgenic poplars developed for the remediation of contaminated soils have shown that the transgenic plants were genotypically stable with no adverse impact on the environment (Peuke and Rennenberg 2005). Theoretical risk assessment on the use of metal-volatilizating plants has shown that the use of transgenic plants having phytoextraction capacity is relatively safe (Lin et al. 2000; Meagher et al. 2000; Rugh et al. 2000). Still, safety issues on the use of phytoextraction technology remain that range from entry of metals in the food chain, accumulation of metals in the topsoil, and spread of the contaminant in the plant material to new environments (Perronnet et al. 2000; Linacre et al. 2003; Mertens et al. 2005, 2007). Although gene escape by the use of transgenics is not a significant problem, there should be proper analysis of the gene frequency for numerous generations over contaminated and non-contaminated soils by greenhouse or pilot experiments performed in the fields (Pilon-Smits and Pilon 2002). Also, the use of transgenics should be undertaken in consonance with the classical breeding approaches which would lower the risk of outcrossing to wild taxa (Bhargava et al. 2012a). The transgenic plant species should be carefully chosen in such a way that they do not have any compatible wild relatives. This may also require use of male-sterile transgenics and harvesting of the plants before blooming (Pilon-Smits and Pilon 2002).

8 Conclusions and Future Perspective

The role of transgenic plants as metal hyperaccumulators is emerging as a cutting-edge area of research and is likely to gain commercial significance in the broader realm of bioremediation. However, most of the studies have been carried in controlled conditions for short durations (Sheoran et al. 2011). Intense research efforts are needed to explore and optimize this underutilized technique for greater field use. Phytoextraction is an environmental friendly, cost-effective, aesthetically pleasing approach which is most suitable for not only the developing countries but also the developed world. An amalgamation of modern biotechnology with conventional breeding is likely to be fruitful in the cleaning of heavy metal-contaminated sites in the coming years.

Developing transgenic plants using biotechnological approaches for efficient phytoextraction of heavy metals requires comprehensive knowledge of the genetic and biological processes in metal hyperaccumulators (Sheoran et al. 2011). This knowledge can help us to develop an ideal hyperaccumulator plant having greater capacity for metal uptake, tolerance, and accumulation (Sheoran et al. 2011). The sequencing of complete genome of hyperaccumulators could go a long way in identifying promising functional noncoding regions which would reduce the burden of comprehensive laboratory and field testing (Wray and Babbitt 2008). The identification of genes associated with metal tolerance using genome sequencing can open up new avenues for the creation of transgenics having desired properties that would help in establishing phytoextraction as a potent technology for environmental

cleanup. The understanding of genome evolution in the hyperaccumulator species should be improved by amalgamation of ecological and molecular genomics (Verbruggen et al. 2009). Detailed study of the adaptive evolution across candidate genes associated with metal tolerance and accumulation could yield promising results (Bhargava et al. 2012a). Another possible approach could be the simultaneous expression of several genes in specific cellular components instead of a single gene.

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Chapter 4
Using an Allometric Model for the Accumulation of Mineral Nutrients in Crops Under Saline and Water Stress: A Field Experience in Fertigation

Enrique Misle, Besma Kahlaoui, Estrella Garrido, and Mohamed Hachicha

1 Introduction

Equilibrated crop nutrition is a main goal in an increasingly intensified agriculture, challenging farmers and scientists, since mineral nutrients are within the main factors determining yield. Food self-sufficiency has been achieved in many countries despite having been an apparently unattainable goal. However, hunger and malnutrition still exist in many countries, and limited availability of additional arable land and water resources, added to the declining trend in crop yields, globally makes food security a major challenge today (Cakmak 2002; Roy et al. 2006). Currently, low levels of available mineral nutrients in soils are within the major constraints contributing to food insecurity, malnutrition and ecosystem degradation (Cakmak 2002). Recently, it has been increasingly recognized that plant nutrition can best be achieved through an integrated use of diverse plant nutrient resources (Roy et al. 2006; Nayak et al. 2012; Zhang et al. 2012; Islam et al. 2013; Srinivasarao et al. 2013). The actual yield of current cultivars does not reflect at all their potential yield, and crop management, especially nutrients, still offers great possibilities to increase yields (Cassman 1999; Dobermann and Cassman 2002; Xie et al. 2007). Lal (2013) indicates that drought stress and nutrient deficiency/imbalance are among the causes of yield gap and encourages finding solutions through innovative research.

Soil salinization has long been identified as a major problem for agriculture; indeed, salinity in soils contributed greatly to the decline of several ancient

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civilizations (Alam 1999). It has been estimated that about 10 % of the total surface area of the world is salt affected; about 15 % of the arid and semiarid lands and one third of all agricultural lands are also becoming saline (Sen et al. 2002). Globally, more than 770,000 km^2 of the lands, 20 % of the irrigated areas and about 2 % of the agricultural lands are affected by secondary salinization (FAO 2000).

During the last decades, a growing number of technological advances have aimed to improve crop nutrition and irrigation (Rouphael et al. 2012). However, much of the published work is found as extension bulletins, manuals or website information, and the connecting link between plant physiology and the scheduling of field labour is not frequently found. A search for the word 'fertigation' in scientific papers in the search engine of one of the biggest editorial groups resulted in just 1,021 articles. In addition, if the search includes the word 'model', then articles are far fewer. However, this search reveals that Gärdenäs et al. (2005) offered a model of nitrate leaching for fertigation under micro-irrigation and Ajdary et al. (2007) developed a model for nitrogen leaching in onion under drip irrigation. In addition, Moreira Barradas et al. (2012) created a decision support system-fertigation simulator for the design and optimization of sprinkler and drip irrigation systems. Though these three recent research works are good examples of science being applied to field problems, the scarce number of results of a search of the word 'fertigation' means that further work is needed for defining current and daily nutrient requirements for specific crops.

Moreover, the application of a systems approach to the fertigation problem is not frequent, making it necessary to link the fertigation scheduling and crop requirements at the local and specific growth rhythm of crops. Models can contribute at different application levels in adapting agriculture to climate change (Anwar et al. 2012; Connor et al. 2012). There is much to do if physiological knowledge could be used to design specific but simple tools for crop nutrition. Dobermann and Cassman (2002) advanced that to meet the expected food demand in the next 30 years, research must seek to develop nutrient management approaches that optimize profit, preserve soil quality and protect natural resources. There are mechanistic models available, but they can prove difficult to use or validate over field conditions, especially when parameters need to be adapted locally and scaled (Boote et al. 2013). Alternatively, Misle (2013) has claimed that the allometric approach is a synthetic but powerful tool, which can determine the global behaviour of a system. At the same time, Günther and Morgado (1996) emphasized that even if we used simple allometric models, there remains open windows for interpretation and new research, which makes them attractive to researchers. While allometric theory is debated among scientists (West et al. 1997; Enquist 2002), it has a reasonable chance to be considered in a systemic approach to fertigation (Misle 2013).

In this chapter we describe the theoretical background and the development of an allometric model for the accumulation of mineral nutrients by crops, and we analyse field experience from 1998 on crops fertigation, following the integration of the model with available equations for saline and water stress. Finally, we discuss growth restrictions imposed by saline and water stress to the nonrestricted forecast.

2 Water and Fertilizer Use Improvement: The Challenge

Efforts to improve water usage in irrigation are old, as evidenced in ancient civilizations in the construction of canal networks in the Near East and North Africa, of dams such as the great dam of Marib or the oldest drip-like irrigation method known with clay pots employed throughout Atacama, North Africa and the Near East (Hillel 1997; Sethuraman and Naidu 2008). Possibly, the first experiments carried out with what is known as the modern drip irrigation lines seem to have occurred in Germany in 1860, when researchers began experimenting with subirrigation, using clay pipes to create a combination of irrigation and drainage systems. In 1913, E.B. House at Colorado State University improved methods of applying water to the root zone of plants. Later, in the 1920s, perforated pipe was introduced in Germany. Notably, in 1934, O.E. Robey experimented with irrigation through porous canvas hose at Michigan State University (Sethuraman and Naidu 2008). Currently, this method exists commercially as geotextile exuding hoses.

Water application through drip irrigation is a highly efficient method and is ideal for controlling the location of soluble fertilizers in soil, which is known as fertigation (Badr and Abou El-Yazied 2007). The application of fertilizers through drip irrigation systems can reduce fertilizer application rates and can result in less contamination of groundwater tables (Badr and Abou El-Yazied 2007) at times when farmers face increasing restrictions on production practices and a general demand for agro-ecosystems sustainability (Misle 2013). Thus, Hebbar et al. (2004) demonstrated that fertigation resulted in lesser leaching of NO_3–N and K to deeper layers of soil. Whatever the dominating mechanism driving the movement of mineral nutrients in soil, either convection in the water flow (mass flow) or diffusion along a concentration gradient (Badr and Abou El-Yazied 2007), when fertilizers are located close to the absorbing parts of roots, it is evident that availability of mineral nutrients can be maximized, leaving the fertilizer rating as the relevant factor on crop nutrition in practice. As traditional fertilization carried out as a separate management practice is being replaced by fertigation in vegetable production, this trend has eventually been taken to the extreme by continuous fertigation. In this regard, discrete practice of fertilization (typically at sowing or planting and mid-crop) leads to low fertilizer use efficiency by wasting salts applied at the beginning of the crop cycle. However, continuous fertigation does not make a difference, as it has been demonstrated that its application results in a far greater use of fertilizers than the nutrient uptake by plants (Badr and Abou El-Yazied 2007). Cook and Sanders (1991) demonstrated that daily or weekly fertigation of tomato significantly increased yield compared to monthly fertigation, but there was no advantage of daily over weekly fertigation. Similarly, Locascio and Smajstrla (1995) found that the yield of surface drip-irrigated tomato was not increased when fertilizer was applied as daily fertigation compared to weekly fertigation. Thus, while fertilizer timing plays an important role in crop nutrition, the fertilizer rate at each fertigation event and the way it matches crop requirements at each crop stage is a critical.

Unlike fertigation timings and rates, crop requirement is a topic much more attractive to scientists in the fields of ecophysiology and plant physiology. So it is not

surprising to find a vast body of research in this field. Bertsch (2003), for example, has published an exhaustive and precious compilation on absorption of mineral nutrients for a wide number of grains, processing, horticultural and ornamental crops from different research works.

3 Ratio and Proportion: A Fascinating History

The study of size correlated variations in organic form and process is traditionally called allometry in the biological sciences (Greek *allos*, 'other', and *metron*, 'measure', Huxley Huxley and Teissier 1936; Niklas 1994). In crops, the development comprises phenology and allometry, that is, time required to plant structures to complete their appearance and the proportions governing among them. Allometric relationships are characteristic of each crop and biological age. Indeed, Niklas (1994) has shown examples of scale relationships between morphological or physiological factors and the biomass or other growth measures of organisms, ranging from unicellular algae to terrestrial plants.

Allometry is a long-known concept, which has not always referred to by using this term. The roots of allometry are to be found in the use of ratio and proportion, a practical idea that was used even before the introduction of numeric systems; a system of proportion was first devised in the Mesopotamia, while geometry was also developed as an important tool in the Ancient Egypt (Idrisi 2005). Later, Greeks learned mathematics from these main sources and turned geometric knowledge into intellectual analysis. But formal abstraction was not used widely until the work of Al-Khawarizmi, more specifically until the work on proportion by Ahmad ibn Yusuf and the power studies of Al Karkhi (Rashed 1996). Later on, this knowledge flowed to the European renaissance, where proportion concepts were applied widely, especially in architecture, sculpture and painting. However, no clear traces can be found at that time about the modern use of proportions and power equations such as allometric relationships in living organisms. Notably the concept of proportion transmitted from the Greeks through Vitruvius 'resides in the correlations by measurement between various elements… and between each of these elements and the whole… and when all these parts have also their place in the total symmetry of the building, we obtain eurhythmy' (Ghyka 1977). This concept of symmetry is closely related to morphological allometry. With regard to rhythm, Ghyka (1977) quotes an old definition: 'rhythm is in time what symmetry is in space'. By following this terminology, allometry in plant nutrition should relate space and time in the search of crops growing at the optimum rhythm of mineral nutrient accumulation, that is to say, growing eurhythmically.

Allometry was later applied by Pearsall (1927) to plants. However, as reviewed by Gayon (2000), the modern terminology and its view as a power equation were invented by Huxley (1924); notably, before him, Dubois published an article in 1897 on the

relationship between the weight of the brain and the weight of the body in mammals, where he used the power equation. Nevertheless, for some years, the allometric approach seemed to lose strength among scientists, or at least it seemed to be an indifferent option among empirical formulas (France and Thornley 1984), unaware of the re-signification that it would gain soon. Finally, a complete revaluation of allometry has emerged from recent reinterpretations that revealed its integrative sense, raising it to the category of theory and generating controversy until now (West et al. 1997; Enquist 2002). Briefly, these authors explain the fundamentals of allometry: it constitutes the condition for *maximizing the scaling of surfaces where resources are exchanged with the environment (*e.g. *roots area, leaf area, lungs or gut surfaces),* while *physiological rates must match the ability of vascular networks to obtain and deliver resources*; as a consequence, *the energy needed to distribute resources is minimized*, which is *equivalent to minimizing total hydrodynamic resistance of the system*.

4 The Model: Theoretical Background

Crop growth has long been recognized to follow a well-known curve of accumulated growth characterized by a sigmoid shape in time (Leopold and Kriedemann 1975; France and Thornley 1984; Poorter 2002). This growth response is modulated by environmental conditions throughout crop development, mainly affected by temperature and photoperiod. Through analytical work it is feasible to eliminate the variability given by changes in temperature and then to obtain the typical shape of a growth curve. Different models can be used to achieve this by calculating a physiological time in poikilothermic organisms like plants (Sharpe and De Michele 1977; Van Straalen 1983; Ritchie and Nesmith 1991; Tijskens and Verdenius 2000). Misle (2013) employed the model proposed by Norero (1987a), which, like other models, acknowledge the nonlinear nature of the thermal response. It assumes dependence on the enzymatic dynamics, like the model of Sharpe and De Michele (1977), but it only requires two cardinal temperatures. Crop time period, expressed in effective growing days (as thermal time), is obtained through the integration in time t, from initial time, ti (e.g. from emergency), to final time, t_f (e.g. physiological maturity), of the temperature response function $f(T)$:

$$\Phi = \int_{t_i}^{t_f} f(T)dt, \tag{4.1}$$

where Φ is called *tautochrone* or total thermal time of a crop under optimum temperature and T is temperature (Norero 1987b). Thus, any value $\phi < \Phi$, over a time $t < t_f$, can be calculated as

$$\phi(t) = \int_{t_i}^{t} f(T)dt. \tag{4.2}$$

The biological age ratio λ (Norero 1987b), dependent only on temperature, and which does not take into account photoperiodical or vernalization responses, is defined as

$$\lambda = \frac{\phi(t)}{\Phi}. \tag{4.3}$$

The conversion of physical time to thermal time by (4.1) allows to set a constant value for different growth conditions, with independence of the thermal environment of crops. Following this procedure, Misle (2003) determined that $\Phi = 59.9$ days for a muskmelon crop, which is equivalent to a crop period (time span) of 60 days at optimum temperature (or thermally effective days) to complete the whole crop development. This thermal time implied 90 physical days (actual time).

To compare the growth period under specific crop growth conditions (by means of thermal time $f(T_t)$) with the minimum period at optimum temperature (Φ), it is practical to approximate the integral to a daily sum:

$$\phi(T_t) = \phi(T_{t-1}) + f(T_t)$$
$$\lambda(T_t) = \frac{\phi(T_t)}{\Phi}.$$

So that at any time, the biological age is:

Under isothermal conditions, when no water or nutrient stress is present, the growth rate $g = (dG/dt)$ can be expressed as a function of the biological age instead of the physical time ($dG/d\lambda$):

$$g = \frac{dG}{d\lambda} = k\left[(\lambda) - (\lambda)^2\right]. \tag{4.4}$$

If this condition can be maintained during the whole crop cycle, it can be integrated to obtain the following:

$$\int_{\lambda=0}^{1} g \cdot d\lambda = k'\left[\frac{\lambda^2}{2} - \frac{\lambda^3}{3}\right] \tag{4.5}$$

where k and k' are physiological constants which relate growth rate and biological age.

From this point, Misle (2013) links growth and nutrients proportions. Misle (2003) reported that results are more accurate when nutrient accumulation is expressed as a function of thermal time rather than as a function of physical time (chronological). Thus, crops from different environments can be comparable (Table 4.1). This has been employed by Fallas et al. (2010, 2011), but using the degree–day time method for thermal time.

The convenient biological timescale (0–1) based on a more realistic relationship of the thermal response leads us to the next step of the search: a function for nutrient accumulation that depends on thermal time. In this regard, Misle (2003) suggested

Table 4.1 Maximum nitrogen absorption rate for three different studies in muskmelon crop calculated on a thermal time basis compared to physical time basis

	Maximum nitrogen absorption rate ($mg.m^{-2}.d^{-1}$)	
Location	Thermal time basis	Physical time basis
Curacaví [Riveros (1988)]	2041.9	1115.6
Colina [Arqueros (1996)]	1886.6	1077.1
Curicó [Misle (2003)]	2013.0	1389.0
Mean rate	1980.5	1193.9
Coefficient of variation	0.0417	0.1424

Modified from Misle (2003)

that the quantity of a nutrient absorbed by a crop, M, can be expressed as a function of its biological age (λ). This idea was first proposed by Whitmore and Addiscott (1987) who used degree–days for the thermal time function, which was employed by Addiscott and Whitmore (1987) and Whitmore (1988). However, there was a need to derive a suitable function dependent on λ. Many structural and functional variables of organisms are, in general, accepted to exhibit a power relationship with the corporal mass or with measures as length or diameter (Niklas 1994; West et al. 1997; Enquist 2002). Hence, nutrient accumulation can be allometrically related to the total biomass in crops (Misle 2008, 2013):

$$\frac{M}{M_{\mathrm{T}}} = M_0 \left(\frac{B}{B_{\mathrm{T}}} \right)^b , \tag{4.6}$$

where M is any quantity of accumulated nutrient at any time, associated to a biomass quantity, B; M_{T} is the total nutrient quantity in the total biomass accumulated by the crop, B_{T}; and M_0 is a normalization constant, theoretically and for practical reasons, equal to unity. The equation is similar to that proposed by Hardwick (1987), except for the use of a nonlinear thermal time as follows: since B in (4.6) can be expressed as a function of thermal time, (4.6) can be coupled with (4.5) to obtain (Misle 2008, 2013)

$$\frac{M}{M_{\mathrm{T}}} = M_0 \left[k' \left(\frac{\lambda^2}{2} - \frac{\lambda^3}{3} \right) \right]^b . \tag{4.7}$$

For analytical convenience, the exponents, as well as the denominators of λ, must be regarded as undetermined constants at the data analysis stage (letters c, d, e and f in (4.11)), since, commonly, growth rate under actual growing conditions exhibits variations in the ideal symmetrical shape, accelerating at the beginning or at the end of the growth period, at either spring–summer or autumn–winter crops. Doing this, an important limitation was avoided when the isothermal condition was assumed first in (4.4). The specific equation obtained by following this procedure will determine the relationship between nutrient accumulation and biological age under local environmental conditions. Thus, requirements of mineral nutrients can be calculated at any

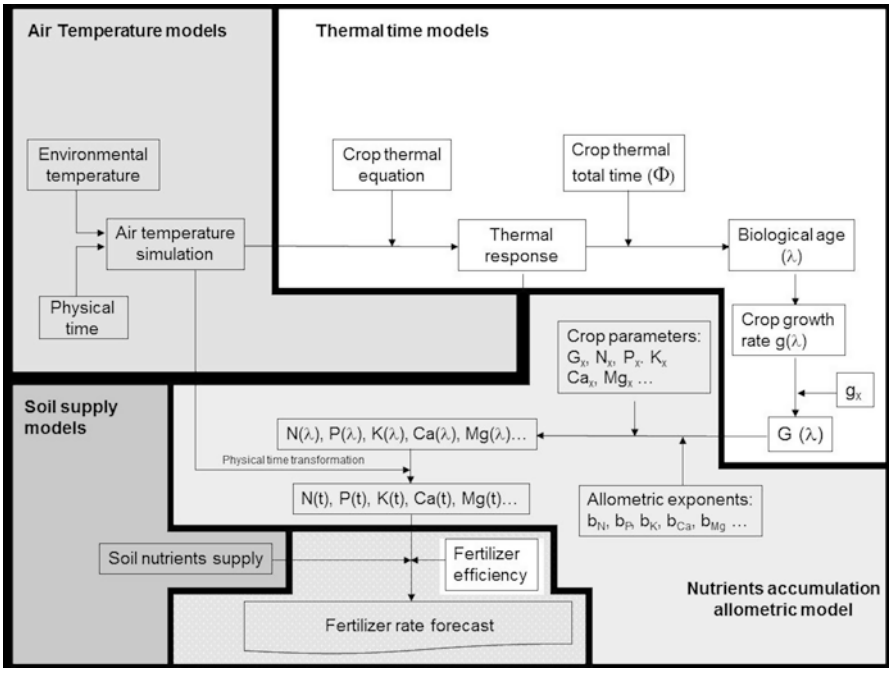

Fig. 4.1 Assembling of the allometric model for mineral nutrient accumulation with other sub-models for the calculation of fertilizer rate forecast on an actual time basis (modified from Misle 2013)

time of the crop cycle for a specific biomass target by means of (4.7), when growth rate is temperature dependent. Coupling this model with current models (as sub-models), including the estimation of air temperature, soil supply models and fertilizer efficiency, can give a fertilizer forecast on an actual time basis (Fig. 4.1).

The next step was to couple (4.9) with restrictions of water and salts in order to obtain reliable forecasts under actual conditions. These restrictions can be associated to stress, since, as pointed out by Alam (1999), *plants are considered to be under stress when they experience a relatively severe shortage of an essential constituent or an excess of a potentially toxic or damaging substance*. In this sense, we deal with external abiotic constrains. To determine the yield response to water, we used the well-known equation of Doorenbos and Kassam (1986), and once the daily reduction for the biomass was estimated, the allometric equation was applied to calculate each one of the main macronutrients. However, its direct use through the k_y (yield deficit to evapotranspiration deficit ratio) would mean to ignore the differing responses among nutrients reported in the literature. Thereby, Misle and Garrido (2008) introduced a hypothesis by which the allometric coefficient (b_M) between each nutrient and the biomass modulates the value of k_y, resulting in a specific ky_M for each mineral nutrient:

$$kyM = k_y \wedge b_M. \tag{4.8}$$

From the equation of yield response to the evapotranspiration deficit, a daily yield water restriction, Y_{WR} , and hence, by allometry, through each ky_M a nutrient water restriction, WR_M, is calculated, according to Doorenbos and Kassam (1986), so each day nutrient accumulation, $M(t)_{WR}$, becomes

$$M(t)_{WR} = M\left[\lambda(T_t)\right] * (1 - WR_M) * M_X,$$

where M_x is the total nutrient accumulation without restrictions.

In relation to the saline stress, the literature reports much evidence showing a trend towards the decrease of the nutrient concentration as the electrical conductivity is increased (Pessarakli 2002; Maggio et al. 2007). For this reason, the following hypothesis was proposed. Since any nutrient can be allometrically related with the biomass according to (4.6) and while the salinity increase causes a reduction in the biomass produced, then

$$1 - \frac{B_s}{B_x} = \left[\frac{S}{S_x}\right]^{b_S}, \tag{4.9}$$

where S ($dS.m^{-1}$) is the electrical conductivity of the soil in the actual condition, B_x is the maximum total biomass production without saline restriction and B_s is the estimate of biomass under saline conditions. S_x is the electrical conductivity in absence of saline stress. So, the new magnitude of produced biomass B_s with respect to the maximum value B_x can be associated to the corresponding relative quantities of accumulated nutrient:

$$\frac{M_{xs}}{M_x} = \left[1 - \left(\frac{S}{S_x}\right)^{b_S}\right]^{b_M}. \tag{4.10}$$

Then at any time t, daily nutrient accumulation will be found as

$$M(t) = M\left[\lambda(T_t)\right] * M_{XS}.$$

Finally, when water and saline restrictions are to be considered, daily maximum accumulation must be multiplied by water and saline restrictions simultaneously. In other words, the $M[\lambda(T_t)]$ entered in this case is the first calculated $M(t)_{WR}$.

Equation (4.9) is a simple and sound hypothesis, which is easy to couple with the other components of the allometric model, but it is not realistic enough. For this reason, a new proposal, coherent with the allometric approach, is under study (Misle et al. 2013).

5 Assembling of Sub-Models

The allometric model was coupled with the well-known equations for the effects of water and salinity restrictions on yield, making it necessary to associate them with the sub-models of environmental temperature, evapotranspiration and thermal time.

The methodology of Gutiérrez and Hajek (1979) was used for the forecast of maximum and minimum temperatures, but it was modified by a stochastic variability. For the rest of the environmental parameters, the protocol from Allen et al. (1998) was followed, except for the reference evapotranspiration, which was estimated through the equation of Priestley and Taylor (1972). For research purposes, the estimates were tabulated in a Quattro Pro X4 sheet, approximating all the time-dependent relationships to a daily sum. In this way, the series of calculations are abbreviated or prolonged in time, depending on the starting date of the crop (emergency), on the thermal characteristics of the agro-ecosystem and on their variability. A polynomial function ($n=3$) was used for the daily calculation of the crop coefficient, k_c and the k_y (maximum and minimum values, *kc*: 0.3 and 1.1; *ky*: 0.3 and 0.9).

6 Field Experience

During the first experiment, carried out between August 1998 and January 1999, 15 km from Curicó (SL: −34.9833; WL: −71.2333), we aimed to obtain the nutrient uptake of muskmelon cultivated in greenhouse, with fertigation at a plant density of 3.09 (pl.m^{-2}). The content of N, P, K, Ca, Mg and plant biomass was analysed periodically for 90 days after transplant. The total biomass production notably reached 1.95 (kg.m^{-2}), and the total macronutrients absorbed were (g.m^{-2}) 37.8 of N, 8.4 of P, 64.5 of K, 42.4 of Ca and 6.5 of Mg. The total thermal time required by the crop was determined, and the absorption rates were calculated in thermal timescale ($\Phi=59.9$ d) as well as in physical timescale (total physical time = 90 d in the field trial). Data from this trial were compared against two already published experiments available in the local literature. We concluded that the use of the biological scale (thermal) for the nutrients absorption rate as well as for the accumulated absorption in differing situations (soil, environment, cultivars) is a more exact procedure than the simple use of the physical time (Misle 2003). Two kinds of results were of relevance as the idea of modelling developed: (1) the quantification of the accumulation of mineral nutrients (N, P, K, Ca and Mg) in muskmelon and (2) a reliable thermal time associated to the accumulation of mineral nutrients (Table 4.1). Regarding the latter, Fig. 4.2 illustrates a sequential transformation of data from physical to thermal time. At first, the transformation of results to biological age does not appear to provide much benefit (column b), but it is necessary to consider the fact that different trials have different maximums for each nutrient, so that only when the relative accumulation data was considered, the full data set in each graph became close to a quasi-sigmoidal shape (Fig. 4.1c). In the sequence, the polynomial is closer to this shape as the data transformation proceeds. This polynomial fit was used just for illustration purposes. The results in Fig. 4.2 were our first suggestive step regarding the feasibility of the allometric model.

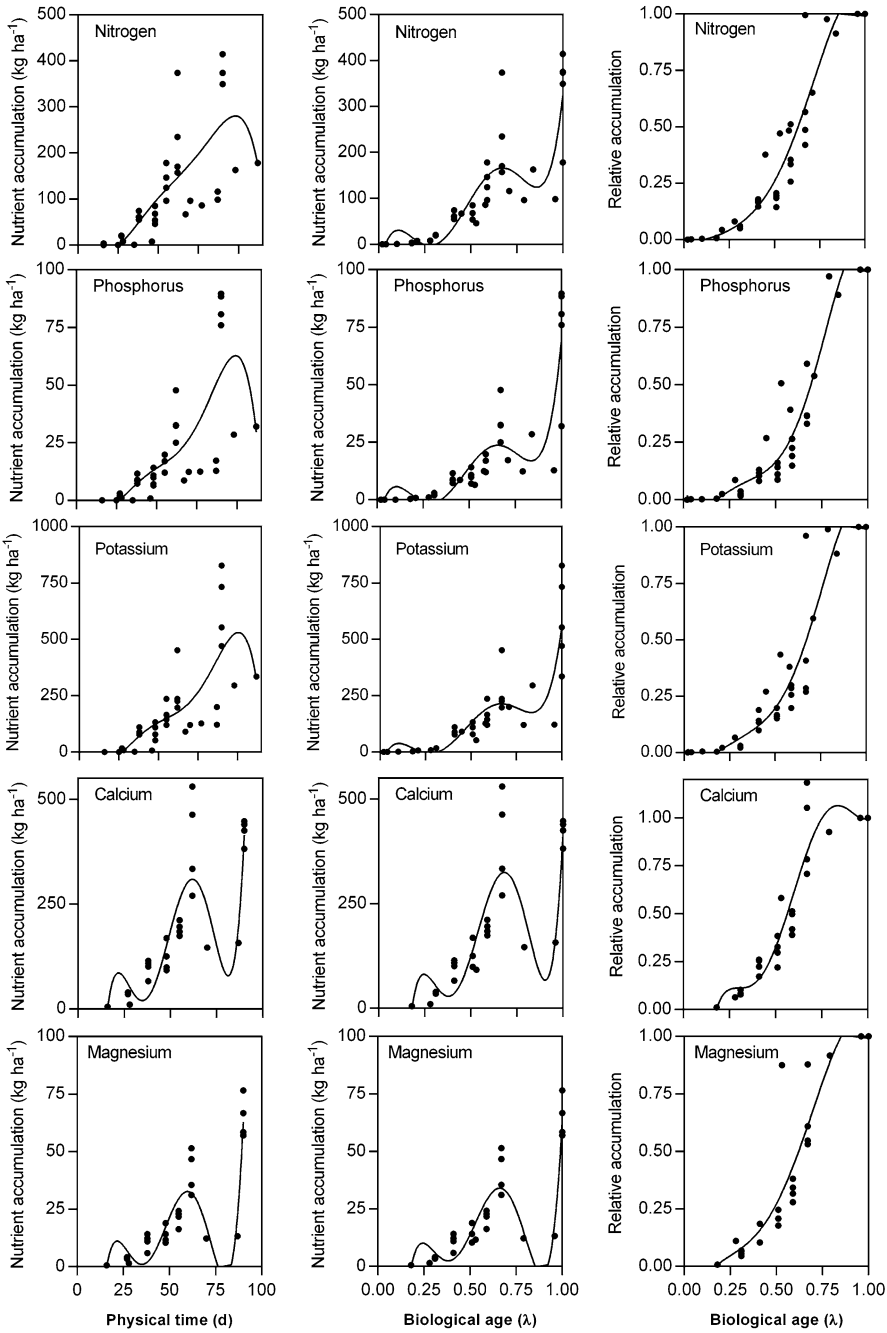

Fig. 4.2 Sequence of data transformation, showing results as (a) physical units of nutrient accumulation (kg.ha^{-1}) and physical time, (b) physical units of nutrient accumulation and biological age (**λ**) and (c) relative accumulation and biological age. Curves just illustrate the fit of a fifth order polynomial. The N, P and K graphs contain data from three different and independent trials; the Ca and Mg graphs contain data from two trials (modified from Misle 2003)

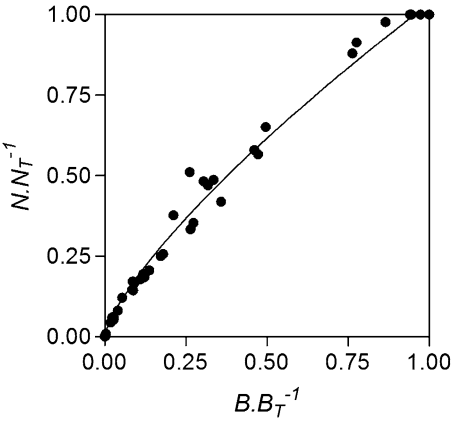

Fig. 4.3 Allometric relationship of N in muskmelon calculated with data reported in four published experiments (n = 46, $R^2 = 0.9911$) (Misle 2006)

In the following phase of research, the data of nutrients absorption recorded during the muskmelon development and, at the same time, the data from articles published by different authors were used to fit the model. Regression analysis was applied to the linear transformation of a power function. The data for the regression analysis were always relative values of absorbed nutrient and biomass obtained during the development of crops.

Analysing separately the case of nitrogen in muskmelon in four published field experiments (Riveros 1988; Arqueros 1996; Rincón et al. 1998; Misle 2003), the resulting power relationship was clearly revealed, with a notable proximity of points of the four different data sets to the curve. The magnitude of the allometric exponent b was 0.75 (Fig. 4.3). Similarly, it was possible to determine the coefficients of fit for the power equation in seven different vegetable and fruit crops (Table 4.2).

In the final step towards a model, we proceeded with (4.7) taking the expression (M/M_T)^(1/b) as a function of the biological age, for fit purposes, in the following way:

$$\left(\frac{M}{M_{\mathrm{T}}}\right)^{\frac{1}{b}} = k\left(\frac{\lambda^c}{d} - \frac{\lambda^e}{f}\right). \tag{4.11}$$

Parameters c, d, e and f were left as unknown constants for regression analysis (Table 4.3).

According to the parameters estimated, a simulation of nutrient accumulation was possible as a first verification of the allometric model (Fig. 4.4), but further experimental research is needed to validate this claim.

Table 4.2 Parameters of the allometric equation in different crop species for the relationship between relative nutrient absorption and biomass (Misle 2006)

Crop	Nutrient	$M_0{}^I$	b^I	Determination coefficient	Absolute sum of squares	Standard deviation of residues	References
Celery	N	0.98	0.88	0.9981	0.002077	0.01723	Rincón et al. (2002)
	P	1.01	0.96	0.9990	0.001199	0.01309	
	K	1.00	1.11	0.9996	0.000514	0.00857	
	Ca	0.99	1.15	0.9979	0.002349	0.01832	
	Mg	1.02	0.87	0.9991	0.001048	0.01224	
Broccoli	N	1.02	0.88	0.9957	0.003151	0.03241	Rincón et al. (1999)
	P	1.04	0.86	0.9829	0.013570	0.06726	
	K	1.02	0.93	0.9955	0.003333	0.03333	
	Ca	0.99	0.92	0.9981	0.001267	0.02055	
	Mg	1.03	0.70	0.9902	0.007487	0.04996	
Cauliflower	N	1.01	0.86	0.9992	0.000615	0.01109	Rincón et al. (2001)
	P	1.01	0.92	0.9985	0.001150	0.01516	
	K	1.02	0.96	0.9971	0.002318	0.02153	
	Ca	1.03	0.98	0.9917	0.007135	0.03778	
	Mg	1.06	0.89	0.9810	0.017600	0.05933	
Muskmelon	N	1.04	0.70	0.9934	0.003848	0.04386	Arqueros (1996)
	P	1.03	0.66	0.9929	0.004016	0.04481	
	K	1.05	0.77	0.9913	0.005423	0.05207	
	Ca	1.02	0.60	0.9770	0.012560	0.07925	
	Mg	1.04	0.40	0.8349	0.084470	0.20550	
Muskmelon	N	0.97	0.78	0.9624	0.033290	0.06896	Misle (2003)
	P	0.97	1.20	0.9788	0.017340	0.04978	
	K	0.96	1.02	0.9809	0.015640	0.04727	
	Ca	1.12	0.57	0.9385	0.080060	0.10690	
	Mg	1.02	0.74	0.9934	0.006459	0.03038	

(continued)

Table 4.2 (continued)

Crop	Nutrient	$M_0{}^l$	b^l	Determination coefficient	Absolute sum of squares	Standard deviation of residues	References
Muskmelon	N	1.04	0.67	0.9963	0.005049	0.02512	Riveros (1988)
	P	1.03	0.85	0.9954	0.005993	0.02737	
	K	1.04	0.83	0.9968	0.004260	0.02308	
	N	1.03	0.75	0.9953	0.003047	0.03187	Rincón et al. (1998)
	P	1.01	1.11	0.9988	0.000814	0.01647	
	K	0.99	0.97	0.9997	0.000176	0.00766	
	Ca	1.10	0.40	0.9072	0.057100	0.13800	
	Mg	1.04	0.61	0.9861	0.008833	0.05426	
Bell pepper	N	1.01	0.82	0.9996	0.000405	0.00761	Rincón et al. (1995)
	P	1.01	0.92	0.9986	0.001427	0.01428	
	K	1.03	0.70	0.9952	0.005328	0.02759	
	Ca	1.01	0.63	0.9897	0.010800	0.03928	
	Mg	1.00	0.75	0.9969	0.003162	0.02125	
Watermelon	N	1.01	0.65	0.9986	0.000893	0.01725	Arqueros (1996)
	P	1.06	0.61	0.9740	0.018630	0.07881	
	K	1.00	0.84	0.9992	0.000517	0.01313	
	Ca	1.00	0.65	0.9933	0.004260	0.03768	
	Mg	1.06	0.58	0.9626	0.027240	0.09530	
Grape	N	1.03	0.54	0.9734	0.023990	0.05163	Rodríguez et al. (1974)
	P	1.02	0.66	0.9935	0.005961	0.02574	
	K	1.03	0.97	0.9756	0.025420	0.05314	
	Ca	1.05	0.88	0.9914	0.009446	0.03240	
	Mg	0.99	1.23	0.9934	0.006322	0.02650	

[l] $M \cdot M_T^{-1} = M_O \bullet (B \cdot B_T^{-1})^b$ (M = nutrient quantity in crop biomass, B; M_T = total content of nutrient in total crop biomass, B_T; M_0 = normalization constant, b = allometric exponent)

Table 4.3 Fit parameters of (4.11) for N, P, K, Ca and Mg in muskmelon (modified from Misle 2013)

Parameters	N	P	K	Ca	Mg
K	7.210	3 .578	4.045	14.150	6.396
C	4.255	3.274	3.504	4.879	4.130
D	1.960	2.008	1.957	2.024	1.961
E	6.972	11.870	9.654	6.303	7.322
F	2.683	4.536	3.766	2.356	2.814
R^2	0.9212	0.9445	0.9124	0.9737	0.8692

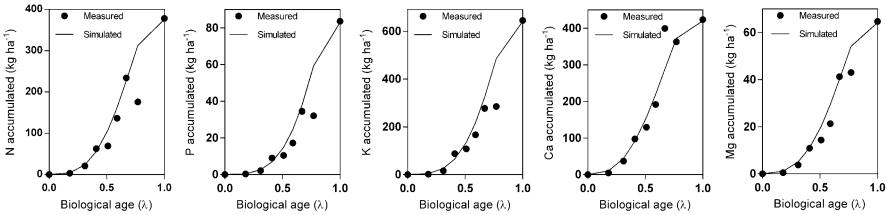

Fig. 4.4 Curves of nutrient accumulation generated by the allometric model for muskmelon, compared with experimental data obtained by Misle (2003) (modified from Misle 2013)

7 Using the Model for Organic Production

In other situations, during the season 2008/2009 a field experiment was carried out to compare a scheduling of conventional and of organic fertilization. The aim was to determine whether equivalent levels of accumulation of mineral nutrients, growth and yield can be obtained in organic as well as in conventional fertilization. Currently the nutritional management of crops acquires higher importance within production costs: the increasing price of oil causes permanent increase in the price of synthetic fertilizers, while its inappropriate use has led to the pollution of groundwater in many areas of agricultural lands throughout the world. The risks associated to this type of contamination reminds us of the need to optimize the use of fertilizers, while the steady state of the prices of agricultural products are a challenge to farmers for refining crops management in order to achieve acceptable economic productivity. Thus, it is of considerable relevance to accomplish fertilization scheduling according to the growth rate of crops. At the same time, the increasing demand for healthy (clean) products and particularly for organic production has opened market opportunities that are more profitable in agriculture. However, there is not enough research that can demonstrate that it is possible to maintain yield levels and to obtain crops nutritionally equilibrated under organic styles of agriculture.

After irrigation, fertilization is the second limiting factor of horticultural production, which aims to incorporate in soils the quantities of mineral nutrients required by crops. Therefore, it is very important to determine nutritional requirements in

Table 4.4 Nutrient requirement forecast to be applied as fertigation at each time interval by both organic and chemical fertilizers according to the biological age of a bell pepper crop in the irrigated valley of Central Chile

Physical time (crop age) (*d*)	Biological age (λ) (adim)	Estimated date	Estimated biomass kg ha^{-1}	N	P	K	Ca	Mg
0	0	15-11-2008	0	0	0	0	0	0
32	0.1	17-12-2008	3	1	0	0	1	0
53	0.2	07-01-2009	52	5	0	5	6	1
68	0.3	22-01-2009	231	15	1	15	14	3
80	0.4	03-02-2009	615	27	2	29	22	5
95	0.5	18-02-2009	1,338	45	4	54	32	8
111	0.6	06-03-2009	2,472	64	7	86	40	11
129	0.7	24-03-2009	3,959	76	10	116	43	13
164	0.8	28-04-2009	5,642	75	12	131	39	12

order to define the quantities that should be incorporated in soil for achieving optimal yields (Rincón et al. 1995).

Methodologically, the first problem of running such a simulation was to define the total nutrients requirements and the total biomass to be produced. Based on the bibliographical references (Rincón et al. 1995; Arqueros 1996), the following requirements were set for maximum accumulation (kg.ha^{-1}), 364 of N, 50 of P, 555 of K, 223 of Ca and 63 of Mg, and maximum biomass production 1.3 kg.m^{-2}. Then, a simulation of thermal time was required to be coupled with crop requirements. Our eurhythmical forecast became complete by back calculating the physical time through the thermal time and temperature sub-models in order to obtain a forecast of growth timings (Table 4.4).

This kind of scheduling, although it is a simple procedure compared to mechanistic models, is easy to set up and it is much more precise than fixed calendars with fixed doses. But frequently the conditions for growing crops are not the optimum, and then different stress limitations are to be considered. Results obtained in this research surprisingly revealed that the organic fertilized crop was able to follow the growth and production rhythm of the conventional fertilized peppers and both reached high biomass and yield (Misle et al. 2009). The data sets and further results analyses are being edited for future publication.

8 Using the Model Under Saline and Water Stress

At the current state of our research, we assembled different sub-models and obtained full simulations, but with the available data, it is not possible to carry out a wider verification due to the difficulty in having the necessary data sets. Other limitations should also be studied, such as those regarding to the biomass partition; so far the model has not considered changes that could occur in the harvest index.

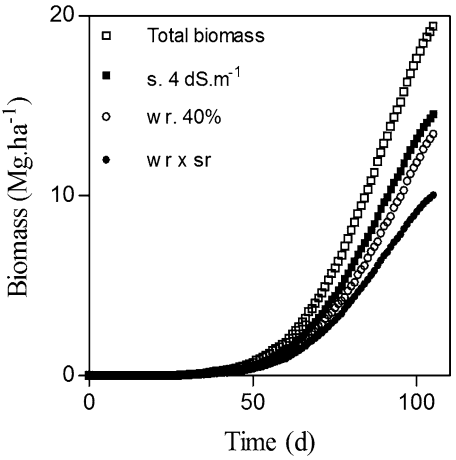

Fig. 4.5 Simulated biomass accumulation of muskmelon in the irrigated valley of Central Chile. In descending curves: normal condition, constant saline water of 4 $dS.m^{-1}$, water restriction of 40 % and both saline and water restrictions (Misle and Garrido 2008)

Other important assumptions for the simulations on muskmelon crop were total nutrient accumulation without limitations ($g.m^{-2}$): 36.8 N, 7.2 P, 68.9 K, 31.3 Ca and 10.6 Mg; the total biomass associated to this mineral uptake was 1.95 $kg.m^{-2}$ (Rincón et al. 1998; Misle 2003). This biomass requires defining a high plant density of 3.09 m^{-2}. The chosen location was the irrigated valley of Central Chile in Curicó, where previous research has been carried out on this crop. The accentuated effect of simultaneous stresses of water and saline can be noted in Fig. 4.5, while no simple additive effects are observed. Otherwise, the forecasted biomass production could fall even more. Such results are plausible as an increasing number of works quantify the accumulation of macro- and micronutrients in crops in response to salinity (Colla et al. 2013; Lao et al. 2013; Shiyab et al. 2013).

As can be observed, our work just constitutes an example of the possibilities of the assembling of the allometric model to other sub-models, which help to determine the nutritional requirements of crops through the case of the muskmelon under different stressing conditions (Fig. 4.5). However, the practical consequences of using this kind of simulation for scheduling fertigation are of considerable relevance. In some of our simulations, having saline water with 3 $dS.m^{-1}$ implies a difference compared to no restriction by more than 70 $kg.ha^{-1}$ of N. At the field level, this quantity is not negligible, but, at the regional level, it is of much importance. On the other hand, if 70 $kg.ha^{-1}$ is applied to the soil and not used by plants, an increase in salinity and groundwater contamination problems will be foreseeable. Figure 4.6 shows a sample of a simulation for N and K accumulation with separate water stress (40 % restriction), salinity stress (4 $dS.m^{-1}$) and the simultaneous simulation of both, water and saline stress.

However, taking into account the experimental annulation of photoperiodical or vernalizing responses, which are not considered here, additional evaluation of muskmelon is required, as much as in other cultivated species. The practical difficulty of using the model thoroughly is the scarcity of experiments with records of the absorption rhythm of crops and, at the same time, the records of meteorological data.

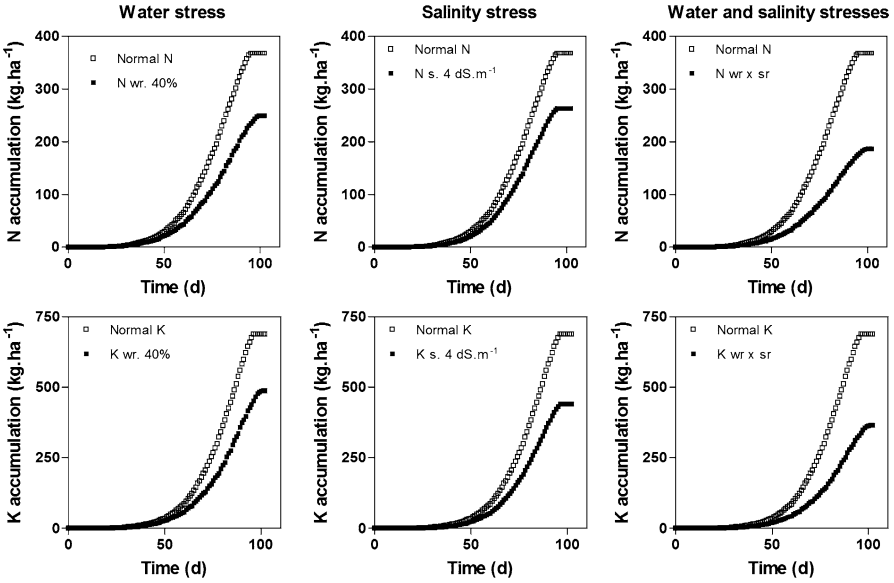

Fig. 4.6 Simulated N (up) and K (down) accumulation of muskmelon in the irrigated valley of Central Chile. Normal condition (upper) and stress condition (lower). Stress situations from left to right: constant saline water of 4 $dS.m^{-1}$, water restriction of 40 % and both saline with water restrictions (Misle and Garrido 2008)

Thus, it would be necessary to carry out specific experimentation aimed to obtain the parameters of (4.11) to have reliable simulations. Due to the variability of the temperature experienced annually by crops in any location, and even the variability occurred in any seasonal period, the possibilities of achieving a benefit or advantage when incorporating this model to crop nutrition management programmes through fertigation are of great practical interest. Moreover, still coarse approaches to the magnitude of the parameters of (4.11) should lead to an improved programming of the fertilization compared to fixed applications.

A question that arises at this point is: what is the advantage of using the power function over polynomials, linear or other equations? We contend that it is due to the allometry present in plants. Misle (2013) noticed that a good fit does not represent a reliable determination of the allometric exponent as a parameter, since when analysing data a high correlation coefficient can be obtained with a little curvature and scarce experimental points. A frequent criticism is also the regression method. Niklas (1994) warned that, in addition to the fit quality, attention should be paid to the scale change among variables. The biomass in the present work crosses two orders of magnitude in physical units ($kg{\cdot}ha^{-1}$) compared to the magnitude of the mineral nutrients, suggesting that, probably, the power function describes the relationship among nutrient quantity and biomass better (Misle 2013). In addition, Niklas (1994) highlighted that it is useful to know that the scaling exponent determined by reduced major axis regression equals that calculated by least-squares

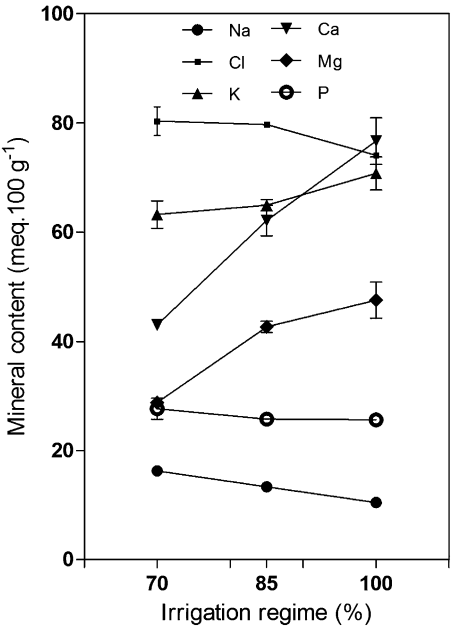

Fig. 4.7 Effect of saline water (6.57 $dS.m^{-1}$) on Na, Cl, K, Ca, Mg and P content (meq $100.g^{-1}$ DW) in Rio Grande cultivar under three irrigation regimes (100 %, 85 % and 70 % of water requirement) (elaborated with data from Kahlaoui et al. 2011a, b)

regression divided by the coefficient of correlation. In the results of Misle (2003) calculations are as follows: N, 0.7819/0.9810 = 0.7970; P, 1.198/0.9893 = 1.2109; K, 1.023/0.9904 = 1.0329; Ca, 0.5677/0.9688 = 0.5860 and Mg, 0.7373/0.9967 = 0.7397. This difference can be considered negligible.

At the current state the model does not consider the change in the electrical conductivity during the growing season, but it can be included easily. Even when the simulations were run assuming constant salinity media, the model flexibility allows its incorporation in future changing conditions of salinity during the growing season. Regarding changes on the nutrient concentrations in crops caused by different salinity conditions, the literature reports various effects according to the species and according to each nutrient. Hu and Schmidhalter (2005) discussed this point with detail, emphasizing the need for more research on this topic. Kahlaoui et al. (2011a, b) studied the response of tomato cultivars to water regime under saline water irrigation (6.57 $dS.m^{-1}$) and the results indicate that mineral nutrients decrease in general as the water levels also decrease, while Na^+ and Cl^- increase. The more sensitive were Ca^{+2} and Mg^{+2} (Fig. 4.7). Similarly, Rejili et al. (2007) studied the K^+/Na^+ balance, and their results confirm that as Na^+ increases, K^+ decreases when increasing the salinity. In our modelling work, a quantitative link with the saline restriction is presented; however, it is not possible at this stage to take in consideration the interactions that occur as a consequence of saline interference. Additional work with the model fully assembled with the other sub-models and new data sets are needed to further confirm whether our assumptions are sound to generate effects as in Fig. 4.7.

Since the harmful effects of a high Na^+ concentration has been pointed out as being due to (1) inhibition of water uptake due to osmotic effect, (2) disturbance of

normal metabolism caused by high Na^+ concentration in plant tissues and (3) inhibition of the absorption of other essential cations (Alam 1999), we ask about the sufficiency of the salinity response sub-model (Maas and Hoffman 1977). It seems that, for the first point, a direct way of calculation cannot be derived and that an additional interaction between the sub-models of salinity effect and water shortage effect could be needed. Clearly, relative mineral uptake is not equally affected for each nutrient when the crop is exposed to drought (Nawaz et al. 2012). Thus, the selective accumulation of ions (e.g. K^+ respect to Na^+) or a reduced ion accumulation may be reflected in numbers thanks to a proper relationship, but this issue needs additional verification. Interestingly, the imbalance in Na^+/K^+ under saline environment also occurs in a drought-resistant plant (Díaz-López et al. 2012) showing that ion interactions remain to be studied. For example, the importance of Ca^{2+} in saline soils is well-known, and it is accepted that as salinity increases, the requirement of plants for Ca^{2+} increases, while the uptake of Ca^{2+} from the soil solution may decrease due to ion interactions, precipitation and increases in ionic strength that reduce the Ca^{2+} activity (Alam 1999; Duman 2012). At the same time K^+ does not only have osmotic functions but also other relevant functions in alleviating detrimental effects of abiotic stresses in plants, even inhibiting Na^+ uptake (Fernandes Rodrigues et al. 2013); improvement of K nutritional status can greatly lower the production of reactive oxygen species by reducing activity of NADPH oxidases and maintaining photosynthetic electron transport (Cakmak 2005; Wang et al. 2013). Since this raises a complex issue, we introduced a conditional calculation for experimental simulations, in order to manually decide whether we applied our ky_M modified as in (4.8) for Ca^{2+}. This may be a weak assumption for which many data sets are needed to test simulations, currently not available to us. For example, the predicted concentration of nutrients can be obtained by further calculating our results. We compared N concentration estimated in this way with values measured in Misle (2003) and obtained a significant correlation with $r=0.8979$. But the confidence interval band ($\alpha=0.05$) revealed that forecasts are better in the middle part of the concentration values, that is to say, the middle part of the crop cycle. Predictions for all mineral nutrients studied decreased as time went on, and final magnitudes were close to the measured (N, P, K, Ca and Mg: 1.94 vs. 2.06, 0.43 vs. 0.47, 3.32 vs. 3.55, 2.18 vs. 2.31 and 0.33 vs. 0.35 %, measured vs. simulated, respectively). Thus, are we going to obtain reliable forecasts under stress conditions? This is the kind of question we suggest for future research. There are, in addition, physiological requirements that are ignored by allometry, as in cucurbits, in which Ca^{2+} plays an important role in the fraction of feminine/male flowers, observed in the field, especially after $\lambda=2/3$. At this stage, it is frequent to observe Ca^{2+} deficiency symptoms in fruits of noncontrolled crops. It is also well known that Ca^{2+} and K^+ are relevant to fruits quality. Probably Ca^{2+} helps to avoid leakage of K^+ and additional Na^+ to be taken up from cells in saline environments (Alam 1999). However, beneficial effects have been observed in tomato quality indicators, as soluble solids, pH and titratable acidity when irrigated by saline water (Kahlaoui et al. 2011a, b). In our experience, as biological age increases, intervals between fertigation events can be shortened for avoiding extreme salinity during the process, when

noncontinuous fertigation is used. Soil management also plays an important role in crop nutrition, as the key role of Ca^{2+} in the physical condition of soil (Alam 1999; Herrera et al. 2008) or the saturation level of soil C (Garrido and Matus 2012). The latter can be associated with inputs of organic materials (usually crop residues) exceeding the soil storage capacity. The application of organic manure decreased the adverse effect of salinity on the vegetative growth and green yield of sweet fennel plants (Abou El-Magd et al. 2008). However, the soil organic matter can affect the availability of some mineral nutrients (mainly micronutrients) due to chelation phenomena (Alam 1999; Hellal 2007; Wright et al. 2007). Our kind of modelling work focusing to the practical management of fertigation can be considered together with the C saturation degree to equilibrate the nutritional status of agricultural lands and to help to reduce the micronutrient malnutrition problem discussed by Welch and Graham (1999). The well-known effect of organic matter in damping the consequences of salinity for crops may be considered by us in future as an additional sub-model.

9 Conclusions and Future Perspective

Crop management is a complex decision-making process where multiple interactions are to be considered. Besides, the FAO has focused global attention on the need for large-scale adoption of integrated nutrient management (Roy et al. 2006). This practice should be considered into the context the increasing number of regulations on production practices focusing on human health and sustainability of agroecosystems. Balanced crop nutrition has also side benefits. Recently, very old perception of farmers has been made explicit: the equilibrated nutrition of plants leads to healthy crops, a hypothesis called trophobiosis (Chaboussou 1985; Paull 2008; García-Mina 2012). In support of this hypothesis, it has been observed that N deficiency sensitized cotton plants to water stress, causing the effects of stress to occur at higher water potential (Alam 1999). As this chapter ends, we have to note that trophobiosis is not so far from the idea of equilibrium contained in the concept of eurhythmical growth.

Although the allometric model was successfully used by us for fertigating crops as not controlled trials for some years, there is a need for additional research to validate this methodology. As Martín-García (2003) mentions, models are simply an ordered collection of suppositions about a complex system, where the validation and verification are, in rigour, impossible. We are convinced of the applicability of this approach, since its potential use is wide, and it spans from programming at the field level to regional planning and national support programmes for agriculture. Nevertheless, the model is concerned with fertigation planning, the target of high yield leads to ideal nonrestricted conditions of growth as a goal, but also realistic restrictions should be included when stress is not avoidable. Useless fertilizers and groundwater contamination can be avoided or at least reduced. The current methodological proposal is still under development and additional research is required to verify and to refine the integrated model.

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Chapter 5
Control of Biotic and Abiotic Stresses in Cultivated Plants by the Use of Biostimulant Microorganisms

Adriano Sofo, Maria Nuzzaci, Antonella Vitti, Giuseppe Tataranni, and Antonio Scopa

1 Introduction

Nowadays, agriculture is increasingly focused on the quality of products and on the environmental, hygienic and sanitary aspects. Therefore, agricultural practices are moving toward a sustainable management of the agricultural crops, in order to ensure quantitative and qualitative product properties. In this context, the main objective of agriculture is to suggest techniques and technologies able to guarantee environmental, human and animal protection. In the agri-food sector, the European Community approved numerous legislative dispositions (e.g. Reg. 1095/07 e 33/2008) and has recently indicated the new objectives of scientific and technological research in the agricultural sector (Horizon Program 2020). Crop management is actually carried out by chemical products that ensure an efficacious plant protection but often interfere with the other biological components of the environment, determining irreversible imbalances. In addition, these products can cause serious risks for consumer's health as a consequence of the residues in food products. From this scenario, it emerges the need of a gradual decline in the use of chemical tools in agriculture and specifically in the control of plant diseases. During the last decade, the studies on alternative environmental friendly technologies have received a strong impulse and have proposed a wide range of options, including agronomical, physical and biological control means (Verma et al. 2007; Shoresh et al. 2010; Bharti et al. 2013; Yeoh et al. 2013). Recently, it was growing the idea that the plants have enormous self-defence potentiality, and this would allow a natural disease control with positive effects on environmental and human health safeguard (Hogekamp and Küster 2013; Estrada et al. 2013; Wu et al. 2013).

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The studies on the biochemical mechanisms associated to systemic resistance in plants could allow individuating new control strategies against plant pathogens and parasites, based on the exploitation of the natural mechanisms of plant defence. This type of resistance mechanism, already documented by Ross (1961), is known as "systemic acquired resistance" (SAR); it is effective against a wide range of pathogens and its action differs in relation to the inducer agent.

2 Systemic Acquired Resistance and SAR Second Messengers

Systemic acquired resistance (SAR) against pathogens is associated with the expression of pathogenesis-related (PR) genes that are considered molecular markers of SAR (Van Loon and Van Strien 1999). The activation of PR genes is in turn mediated by endogenous salicylic acid (SA) as molecule involved in signal transduction. The first step is the activation of calcium channels in plasmalemma, mediated by G proteins (pathogen protein) (Legendré et al. 1992). The increase of cytoplasmatic calcium concentration stimulates the superoxide anion production (O_2^-), a reactive oxygen species (ROS) (Schwacke and Hager 1992). The O_2^- released into the apoplast is dismutated in H_2O_2 naturally or by the action of the enzyme family of superoxide dismutase (SODs) (Buonaurio et al. 1987; Scandalios 1993). Then, the H_2O_2 can be in turn reduced to water and molecular oxygen by enzymatic and nonenzymatic plant antioxidant defences, such as catalase (CATs) (Scandalios et al. 1997) and ascorbate peroxidase (APXs) (Asada 1992), ascorbic acid, tocopherols, flavonoids and anthocyanins (Dixon and Paiva 1995; Noctor and Foyer 1998). The radical (OH^-), a strong oxidant, is obtained from H_2O_2, by Hebert–Weiss and Fenton's reactions, completing the reaction chain that is known as oxidative "burst" (Bolwell et al. 1999). Among ROS, H_2O_2 plays a predominant and diversified role in the events which lead to induction of resistance and to the transduction of the molecular signal of defence gene activation (Van Breusegem et al. 2001).

The main messengers of SAR are salicylic acid (SA), jasmonic acid (JA), ethylene (C_2H_2) and nitric oxide (NO) (Fragnière et al. 2011). An excessive ROS production may cause negative effects on the plant cells. The antioxidant systems control the cellular ROS concentration to avoid their potential toxicity. CATs and peroxidases (POXs) are the most important enzymes which allow removing H_2O_2. Because SAR is a normal answer in plant defence, it can be artificially induced by pathogen pre-inoculations or using chemical inducers of acquired resistance (Kuć 1982), such as beta-aminobutyric acid (Cohen 2002), benzothiadiazole (Ryals et al. 1996) and 2,6-dicloisonicotinic acid (Kauss et al. 1992). It is known that SA-induced resistance to viruses in tobacco and *Arabidopsis thaliana* is mediated in part by a pathway that appears to involve signals transduced through changes in ROS (Singh et al. 2004). Indeed, SA impedes electron flow through the respiratory electron transport chain and enhances ROS levels in the mitochondria (Mayers et al. 2005). SA-induced resistance to *Tobacco mosaic virus* (TMV) is altered in transgenic tobacco plants

with altered levels of alternative oxidase (AOX), an enzyme that negatively regulates mitochondrial ROS levels (Gilliland et al. 2003). In *Arabidopsis thaliana*, as in tobacco, SA treatments inhibited the systemic movement of *Cucumber mosaic virus* (CMV). In addition, in squash SA-induced resistance to CMV and this was most likely due to inhibition of viral cell-to-cell movement. This means that the mechanisms of SA-induced resistance may differ markedly between host species (Mayers et al. 2005) and they are very poorly known.

3 Biostimulant Microorganisms and Their Importance in Sustainable Agriculture

Some microorganisms and the molecules they produce are able to biocontrol plant pathogens by inducing SAR and thus can be defined as biocontrol microorganisms (BCMs) (Vargas et al. 2008; Shoresh et al. 2010; Amaresan et al. 2012). Current biocontrol studies have confirmed the effectiveness of *Bacillus* spp., *Trichoderma* spp. and *Glomus* spp. in the plant protection not only against a wide range of pathogens fungi (Avis et al. 2008; Akrami et al. 2011; Hernández-Suárez et al. 2011) but also against bacteria (Avis et al. 2008; Segarra et al. 2009; Berić et al. 2012) and viruses (Wang et al. 2009; Luo et al. 2010; Wang et al. 2011; Elsharkawy et al. 2012), likely due to the induction of plant resistance mechanisms similar to SAR, hypersensitive response (HR) and induced systematic resistance (ISR) (Kaewchai et al. 2009). On the other hand, some fungal BCMs are able to promote plant growth and development, so acting as plant growth-promoting microorganisms (PGPMs), that in turn determines a higher tolerance of the plants against abiotic stresses, such as drought and salinity.

Both BCMs and PGPMs can be defined as "biostimulant microorganisms", able to foster plant growth and defence against pathogens throughout the crop life cycle, from seed germination to plant maturity. The study of the biochemical and molecular mechanisms involved in host–pathogen–antagonist interaction is essential for understanding the dynamics of infectious processes and can be useful for developing new strategies for the control of plant pathogens. At the same time, innovative methodologies and practices aimed to increase plant tolerance against abiotic stresses are required in sustainable agriculture.

The borderline between BCMs and PGPMs is not well defined. Indeed, BCMs, whose main action is to prevent or inhibit the growth of pathogens by SAR, exercise "indirect" benefits on plant growth by antibiosis based on the production of hydrolytic enzymes or inhibiting substances. These indirect effects have been clarified only in part, and even less is known regarding the "direct" effects of BCMs on the improvement of plant growth through production of siderophores and phytochelatins, which chelate metals and make them available to the roots. The most interesting PGPMs are those able to colonise the rhizosphere. This latter is particularly rich in nutrients and supports a microbial population that can exert positive effects on the

physiological state of the roots, on the absorption of nutrients and on plant tolerance to environmental stresses. A particular attention should be given to endomycorrhizal (*Glomus* spp.) and rhizosphere (*Trichoderma* spp.) coloniser, that could allow plants to achieve optimum yields.

The SAR represents a valid opportunity in plant natural protection. Therefore, the research activities should be oriented to the use of BCMs as inducers of SAR in agronomically important species against some of their most severe pathogens. Among BCMs, the most used in sustainable agriculture practices belong to *Bacillus* spp., *Trichoderma* spp. and *Glomus* spp. (Djonović et al. 2007; Samolski et al. 2009; Ambrico and Trupo 2011; Dichio et al. 2012; Bonneau et al. 2013; Li et al. 2013). Research data accumulated in the past few years have produced a completely novel understanding of the way by which bacteria and fungi interact not only with other microbes but also with plants and soil components. This has opened an avenue of new applications, in both agriculture and biotechnology that exploit the ability of biostimulant microorganisms to change plant metabolism and resistance to biotic and abiotic stresses (Woo et al. 2006).

4 *Bacillus*, *Trichoderma* and *Glomus spp*.

The bacteria belonging to *Bacillus* spp. are ubiquitous microorganisms, present in the soil and in the phylloplane, and they are also able to live as endophytes. They have been studied for their antagonistic activity and induction of plant resistance against stresses. In the last years, endophyte isolates of *B. subtilis* (ET-1), able to control several diseases caused by leaf and soil pathogens, have been identified (Felici et al. 2008; Ambrico et al. 2010; Ambrico and Trupo 2011). Many *Bacillus* isolates can promote plant vegetative development by producing several extracellular substances, so acting as plant growth-promoting microorganisms (PGPMs).

Trichoderma spp. and *Glomus* spp. are some of the most abundant fungi found in many soil types, able to colonise plant roots and plant debris (Harman et al. 2004). They are agriculturally and industrially important, being the major source of many commercial biostimulants and biofungicides. These fungi are rarely causes of plant diseases (Gams and Bissett 1998). On the contrary, many *Trichoderma* and *Glomus* species (e.g. *T. harzianum, T. viride, G. intraradices*) are strong BCMs against bacteria, fungi and nematodes, and for this reason more than 60 % of all registered biostimulants used for plant disease control are *Trichoderma*- and/or *Glomus*-based (Verma et al. 2007; Shoresh et al. 2010; Bharti et al. 2013; Estrada et al. 2013). Many studies considered the use of proteomic and functional genomic analysis in the attempt to obtain a complete picture of the changes that occur in the expressions of fungus, plant and pathogen when they interact each other, especially when an increase in disease resistance is generated (Grinyer et al. 2005; Woo et al. 2006; Yeoh et al. 2013; Leung et al. 2013). However, the mechanisms of the interaction *Trichoderma-/Glomus*-plant pathogen are very complex and includes not only the mycoparasitism but also competition for nutrients, release of extracellular hydrolytic

enzymes, antagonism against nematodes, colonisation of rhizosphere and phyllosphere, production of secondary metabolites that are toxic to plant pathogens, promotion of plant growth and root development and induction of systemic resistance against different pathogens (Harman et al. 2004; Mathivanan et al. 2008; Dutta and Podile 2010; Estrada et al. 2013).

The saprophytic fungi belonging to *Trichoderma* spp. can grow along the entire length of the root system along which it establishes a barrier against pathogen attack (Harman et al. 2004). Overall morphology and metabolism of plant inoculated with *Trichoderma harzianum* showed an increase in root growth and cell wall suberification in the exoderm and endoderm (Sofo et al. 2011, 2012) and the induction of the synthesis of antimicrobial phenolic compounds (Mathivanan et al. 2008). Furthermore, the cloning and functional characterization of a gene (*Sm1*) from *Trichoderma virens* that codes for a cerato-platanin has more recently allowed the identification of a novel proteinaceous nonenzymatic elicitor that triggers SAR in plants (Djonović et al. 2007). *Trichoderma* spp. are also important for their ability to synthesise peptaibols, a family of peptides with antibiotic function characterised by short chain lengths (<20 residues), C-terminal alcohol residues and high levels of nonstandard amino acids (Whitmore and Wallace 2004). Their antibiotic function arises from their membrane-insertion and pore-forming abilities, and it has been shown that peptaibols produced by *Trichoderma pseudokoningii* can induce programmed cell death in plant fungal pathogens (Shi et al. 2012).

The endomycorrhizal fungi belonging to *Glomus* spp. form a hyphal network that can obtain and transport nutrients, propagate the association and interconnect plants (Newman 1988). The production of plant-external hyphae varies according to the species and isolates of *Glomus*, can be influenced by soil properties and is an important determinant of mutualistic effectiveness (Kogel et al. 2006). The mycorrhization of plants by *Glomus* makes possible an enduring protection of cultivated plants against pathogens and a better use of nutrients, so improving plant tolerance to the diseases and to abiotic stresses and increasing plant productivity and quality in degraded soils (Datnoff et al. 1995; Augé 2001; Estrada et al. 2013).

It was recently discovered that plant mycorrhization with *Glomus* and soil colonisation by *Trichoderma* enhanced plant growth, in terms of total biomass and root development, by about 20 % and 30 %, respectively (Sofo et al. 2010). *Trichoderma harzianum* strain T-22 enhances root growth in both herbaceous (Fig. 5.1) and tree species (Fig. 5.2).

The ability of all these PGPMs in modulating plant defence mechanisms by the activation of the hypersensitive response (HR) and the induced systemic resistance (ISR) was demonstrated, but the details of this PGPM-plant molecular dialogue are poorly known, and many defensive compounds are likely to exist but remain to be identified. It seems that both SAR and ISR are intertwined molecularly and that a key role in plant defence mechanisms is played by molecules with signal functions, such as phytohormones (Vallad and Goodman 2004; Krouk et al. 2011). Moreover, the crosstalk between the different plant hormones, whose levels change after plant inoculation with PGPMs, results in synergetic or antagonistic interactions that play crucial roles in response of plants to abiotic stress, such as drought, salinity and

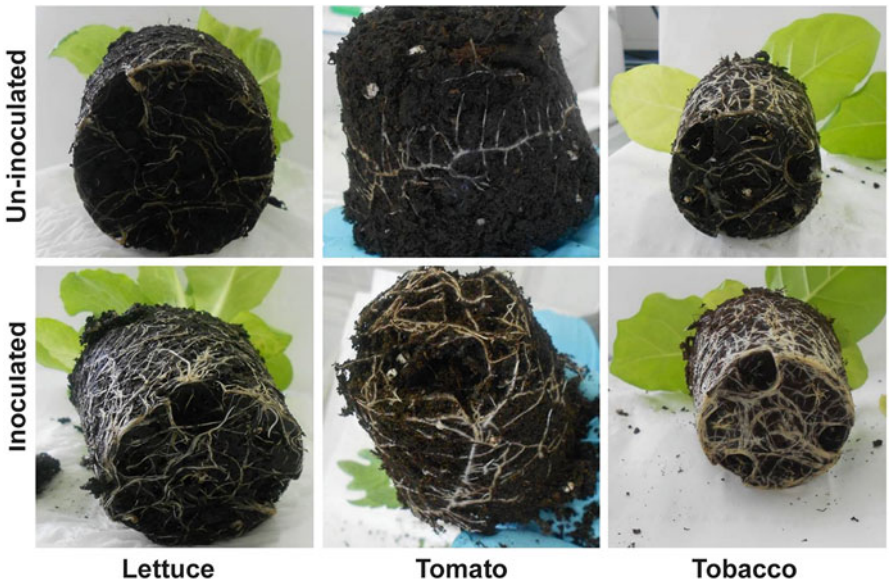

Fig. 5.1 Root growth in lettuce, tomato and tobacco seedlings observed after 25, 21 and 20 days, respectively, from the inoculation with *Trichoderma harzianum* strain T-22 (*below*) and in uninoculated controls (*above*)

toxic metals (Baroni et al. 2004; Peleg and Blumwald 2011). Thus, plant hormones play central roles in the ability of plants to adapt changing environments by mediating growth, development, nutrient allocation and source/sink transitions. Recently, the changes in phytohormone levels, particularly auxins and cytokinins have been demonstrated to be one of the direct mechanism by which *Trichoderma harzianum* promotes plant growth (Sofo et al. 2011). On this basis, the differences between BCMs and PGPMs seem to be increasingly blurred and their mechanisms of action appear to be overlapped.

5 Case Studies and Applications with Biostimulant Microorganisms

In BCM-inoculated plants, important physiological and biochemical parameters should be considered to individuate the degree of response against the pathogens under study. Notably, the integrity and functional status of the photosynthetic machinery, the assimilation, respiration and transport process and the mechanisms of photo-inhibition and photo-oxidation are of key importance in this kind of researches. Moreover, in the same plant systems, qRT-PCR-based gene transcripts analyses should be carried out to identify genes important for SAR induction, such as

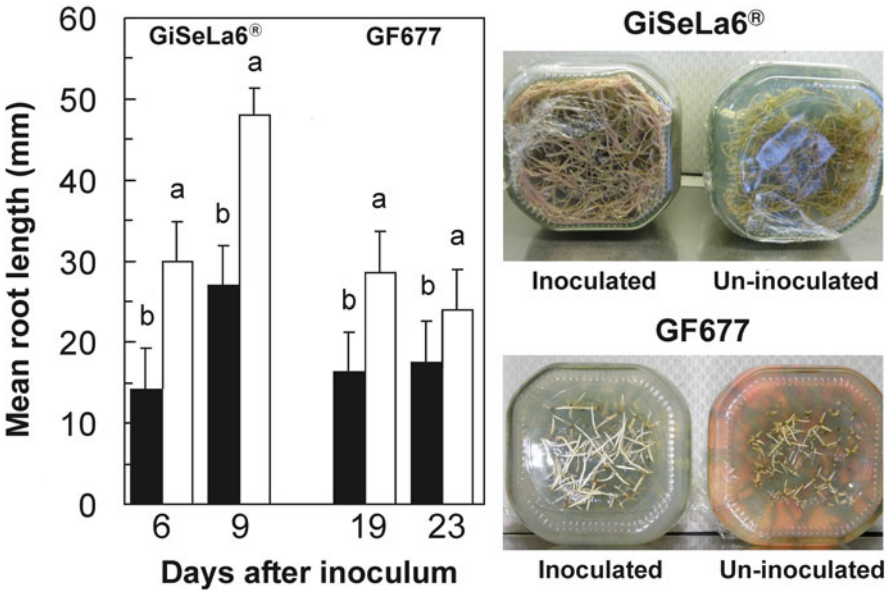

Fig. 5.2 (*On the left*) Mean root length (± standard error, $n = 100$) in in vitro cultured GiSeLa6® (cherry) and GF677 (peach) rootstocks inoculated with *Trichoderma harzianum* strain T-22 (*white columns*) and in uninoculated controls (*black columns*). For each treatment, mean values followed by a different lower-cased letter are significantly different at $P < 0.05$ according to Fisher's LSD test. (*On the right*) Root growth of GiSeLa6® and GF677 rootstocks inoculated with *Trichoderma harzianum* strain T-22 and in uninoculated controls observed after 9 days from inoculation. The medium was agarised Murashige and Skoog medium without vitamins and supplemented with indole-3-butyric acid

those associated with ROS (e.g. coding for SODs, CAT, POXs, etc.), PR proteins and peptides (e.g. chitinases, glucanases, ceratoplatanins, peptaibols, phytoalexins), phenylpropanoid (e.g. phenylalanine lyase, chalcone synthase) and phytohormones synthesis. Time course transcriptional analyses has to be performed in accordance to the progression of the infection and appearance of phenotypic and biochemical markers of damage. Genes known to be involved into metabolic processes underlying the plant–pathogen–antagonist interactions or the tolerance against abiotic stresses should be retrieved from public gene and EST databases. If not present in public databases, target genes from each plant species investigated can be amplified and cloned using sequence information from model species.

In plants subjected to different types of abiotic stress, comparative proteomics experiments should be carried out to identify specific proteins involved in plant resistance against pathogens, drought, salinity and other stresses. For this analysis, 2D-electrophoretic cells, protein fractionation and isoelectric focusing techniques and MALDI-TOF MS are commonly used. Accurate microscopic analyses should be carried out through electron (SEM and ESEM), epifluorescence and light microscopes in order to ascertain BCMs/PGPMs persistence and evaluate their

colonisation. Finally, comparative proteomics experiments are of primary importance to identify specific proteins involved in the common response (overlapping) against biotic and abiotic stresses.

6 Conclusions and Future Perspective

From an environmental point of view, the use of biostimulant microorganisms is an agronomic practice able to preserve natural resources and, due to reduced use of pesticides and fertilisers, to maintain soil fertility and safeguard human health.

The studies on biostimulant microorganisms can allow to discover new formulation of bioactive compounds. At the same time, the application of this innovative knowledge will foster the use of biostimulant microorganisms in agriculture, with evident benefits on soil fertility, natural resources saving, food safety and human health. In this way, biostimulant microorganisms can act on the plants through different pathways to improve crop vigour, yields and quality, increasing plant tolerance and recovery from biotic and abiotic stresses. This could ameliorate plant physiological status, facilitate nutrient assimilation, translocation and use and improve plant water balance, so increasing plant survival in the absence of pesticides and with a reduced chemical fertilisation. Furthermore, the antiviral effects of these biostimulant microorganisms and the associated biochemical and molecular mechanisms implicated are still scarcely known and could be of key importance in the biological pest control. Finally, the identification of elicitor-like substances produced by the studied BCMs and involved in defence responses against pathogens will be an important applied research field in the next future.

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Chapter 6
Cyclic Nucleotides and Nucleotide Cyclases in Plants Under Stress

Małgorzata Pietrowska-Borek, Tamara Chadzinikolau, and Sławomir Borek

1 Introduction

Adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) are very important molecules of signaling networks in prokaryotic and eukaryotic cells (Schaap 2005). In animal cells, both the signal function of cyclic nucleotides and their metabolism are well known (Beavo and Brunton 2002). In plants, the occurrence, metabolism, and function of these nucleotides have been discussed for almost 30 years. Now it is known that cyclic nucleotides participate in normal physiological processes during plant growth and development. Moreover, they play a significant role in plant signaling pathways activated in response to biotic and abiotic stresses.

2 Occurrence of cAMP and cGMP in Plants

The physiological role of cyclic nucleotides in higher plants is less well understood than in animal and prokaryotic cells and has been discussed for almost three decades. At the beginning of the 1970s, many papers were published in which the occurrence

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of cAMP in higher plants was described. However, all these reports were severely criticized because of the then lack of a sufficiently sensitive method to detect cAMP at a femtomolar level (Amrhein 1974). The skeptical appoach to the presence of cyclic nucleotides in plants was overcome by the use of mass spectrometric analysis, which has provided unequivocal evidence of the natural occurrence of cAMP, cGMP, and other cyclic nucleotides such cUMP, cCMP, cIMP, and c-dTMP in higher plants (Newton et al. 1984, 1989). Then began to appear more and more works describing the occurrence and function of cyclic nucleotides in higher plants. In barley (*Hordeum vulgare*) seedling roots, cAMP content was determined at 41.5 pmol g^{-1} fresh weight (Stroiński and Floryszak-Wieczorek 1985a). Concentrations of 36 pmol cAMP g^{-1} fresh weight in torenia (*Torenia*) stem segments (Ishioka and Tanimoto 1990) and 5 pmol cAMP g^{-1} fresh weight in suspension-cultured cells of common bean (*Phaseolus vulgaris*) have been described (Bolwell 1992). Gangwani et al. (1991) determined cAMP levels from 70 to 80 pmol g^{-1} fresh weight in axenic cultures of duckweed (*Lemna*), as assayed by both high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA). In rice (*Oryza sativa*) leaves, cAMP concentration was less than 1 pmol g^{-1} fresh weight (Komatsu and Hirano 1993). Witters et al. (2005), using immunocytochemical detection, proved the occurrence of cAMP in chloroplasts of tobacco (*Nicotiana tabacum*). In extract of tobacco BY-2 cells, the cAMP level was 22.79 pmol g^{-1} fresh weight (determined by RIA) (Richards et al. 2002). Recently, the level of cAMP has been determined at 14 nmol g^{-1} fresh weight in potato (*Solanum tuberosum*) plants growing in vitro and at 3 nmol g^{-1} fresh weight in suspension culture of *Arabidopsis* cells (Lomovatskaya et al. 2011). The role of cAMP in biochemical and physiological processes in plant cells is discussed in Sect. 6.6.

The first reports on the occurrence of cGMP in plants come from the 1970s and 1980s and similarly as for cAMP, stirred controversy. Only the study by mass spectrometry put an end to speculation and confirmed the presence of cGMP and other cyclic nucleotides in pea roots (Newton et al. 1989). In tobacco pith parenchyma cells, the cGMP level was estimated below 0.1 nmol g^{-1} dry weight (Lundeen et al. 1973). In Japanese red pine (*Pinus densiflora*), pollen cGMP was detected at the level of 60 pmol g^{-1} fresh weight (Takahashi et al. 1978). In roots of bean, the range of cGMP was 0.4–20 nmol g^{-1} fresh weight (Haddox et al. 1974); in medicinal plants (*Evodia rutaecarpa*, *Evodia officinalis*, *Zizyphus jujuba*), 10–50 nmol g^{-1} dry weight (Cyong and Takahashi 1982; Cyong et al. 1982); in maize (*Zea mays*) seedlings, 35–72 pmol g^{-1} fresh weight (Janistyn 1983); and in barley coleoptiles with enclosed leaves, 147–200 fmol g^{-1} fresh weight (Stroiński and Floryszak-Wieczorek 1985b). In spruce pine (*Pinus glabra*) needles which were exposed to nitric oxide (NO), an increase of cGMP level was observed (Pfeiffer et al. 1994). In extract of tobacco BY-2 cells, the cGMP level was 5.267 pmol g^{-1} fresh weight (Richards et al. 2002). Now it is clear that cGMP is present in various plant tissues (Penson et al. 1996; Durner et al. 1998; Donaldson et al. 2004). The role of cGMP in biochemical and physiological processes in plant cells is discussed in Sect. 6.6.

3 Metabolism of Cyclic Nucleotides in Plants

3.1 Synthesis of cGMP

Despite the fact that cGMP in plants was discovered later than cAMP, the knowledge regarding cGMP synthesis in plants is considerably greater than the state of the art in the question of cAMP metabolism. Cyclic GMP in prokaryotes and eukaryotes is synthesized from GTP by guanylate cyclase (GTP diphosphate-lyase (cyclizing; 3′,5′-cyclic-GMP-forming), EC 4.6.1.2) (Schaap 2005) (Fig. 6.1). Due to the very important role of cGMP in biochemical and physiological processes in plant cells (see Sect. 6.6), researchers were searching for molecules in higher plants which can catalyze cGMP synthesis. However, in plants they were somewhat elusive. One of the first reports of cGMP synthesis was the work of Newton and coworkers (1984), who studied guanylate cyclase-like activity in chloroplasts from common bean. Further, guanylate cyclase-like activity was found in plasma membranes in oat (*Avena sativa*) (Volotovsky et al. 2003). Recently more and more papers have shown that in plant cells, there occur components which have guanylate cyclase activity.

A molecule of guanylate cyclase (GC1) in plants was discovered for the first time by Ludidi and Gehring (2003) in *Arabidopsis* (AtGC1, At5g05930). This is a soluble protein which has guanylate cyclase activity in vitro. This first guanylate cyclase in higher plants was identified with a search motif deduced from an alignment of conserved and functionally assigned amino acids in the catalytic center of guanylate cyclase from lower and higher eukaryotes (Liu et al. 1997; McCue et al. 2000). These studies employed a search of a 14 amino acid long (e.g., SYGVVLLELLTGKR) (Fig. 6.2b) sequence (the original motif, Fig. 6.2c) catalytic center which may be sufficiently conserved to identify nucleotide cyclases in higher plants. Recently, a GC1 homolog was identified in *Pharbitis nil* and is involved in photoperiodic flower

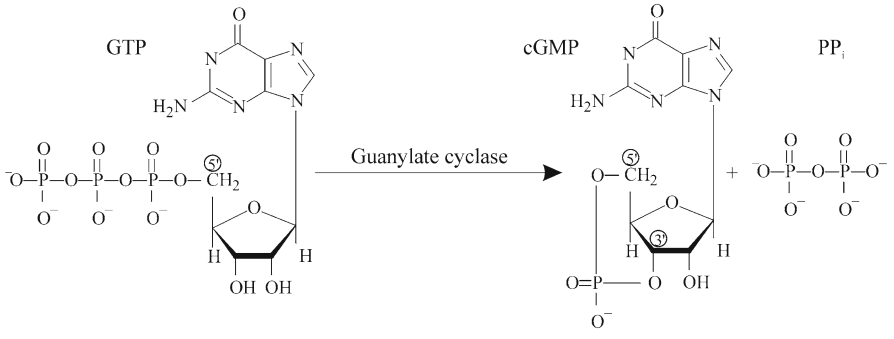

Fig. 6.1 Biosynthesis of guanosine 3′,5′-cyclic monophosphate (cGMP)

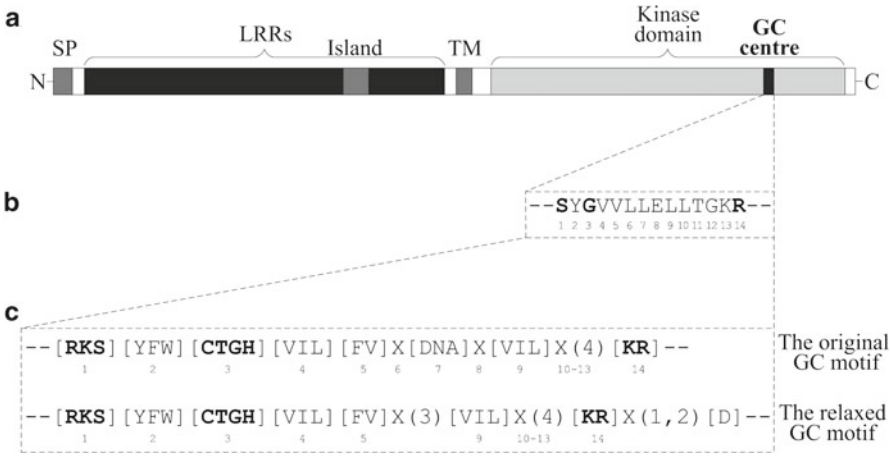

Fig. 6.2 Scheme of the *Arabidopsis* brassinosteroid receptor AtBRI1 (At4g39400) with guanylate cyclase catalytic domain (**a**), amino acid sequence of the original search motif modified by inclusion of L in position 7 (**b**), and guanylate cyclase motifs used in the identification of nucleotide cyclases in higher plants (**c**). Amino acids marked with bold are functionally assigned residues of the enzyme catalytic center. *GC* guanylate cyclase, *GC center* guanylate cyclase catalytic domain, *LRRs* leucine-rich repeats domain, *SP* signal peptide, *TM* transmembrane domain

induction (Szmidt-Jaworska et al. 2009). Other functional guanylyl cyclases were discovered by searching 14 amino acid motif (Fig. 6.2c). In those studies based on the relaxed search motif (Fig. 6.2c), several functional guanylate cyclases were identified in higher plants. In *Arabidopsis* there were over 40 such kinase-GC molecules identified. A lot of them are involved in regulation of plant growth and response to pathogen attack (Kwezi et al. 2007). Among the identified proteins with a 14 amino acid search motif was the brassinosteroid receptor AtBRI1 (Fig. 6.2a) that has guanylate cyclase activity in vitro. This brassinosteroid receptor (BRI1) was first identified as a leucine-rich repeat receptor-like kinase (LRR-RLK) located in the plasma membrane (Kwezi et al. 2007). Brassinosteroids are polyhydroxylated plant steroid hormones and are engaged in co-regulation of growth and development processes including embryogenesis, cell elongation, and vascular differentiation (Clouse 2002, 2011; Haubrick and Assmann 2006; Zhao et al. 2013). An extensive body of work indicated that brassinosteroids are able to enhance the ability of plants to cope with a variety of stresses including heavy metals, organic pollutants, drought, salinity, and extreme temperatures (Sharma et al. 2008, 2011; Peleg and Blumwald 2011; Ahammed et al. 2012; Cui et al. 2012). Moreover, brassinosteroids have been suggested as a potential hormone for phytoremediation application (Barbafieri and Tassi 2011). The fact that AtBRI1 possesses a functional guanylate cyclase domain within the cytosolic part of the molecule may indicate that cGMP is involved in some brassinosteroid-dependent processes. Recently, it was demonstrated that epibrassinolide can stimulate the increase in cGMP synthesis in freshly isolated leaf protoplasts (Irving et al. 2012).

A similar kinase-GC center was also confirmed in other LRR-RLKs. Those are cell wall associated kinase-like 10 (AtWAKL10, At1g79680) (Meier et al. 2010) and *Arabidopsis* pathogen peptide 1 receptor (AtPepR1, At1g73080) (Qi et al. 2010). It has been demonstrated that AtWAKL10 which has guanylate cyclase activity in vitro is consistently co-expressed with genes which are involved in early pathogen-defense responses in plants. This indicates that AtWAKL10 may also be involved in pathogen-induced cGMP generation (Meier et al. 2010). Another LRR-RLK is AtPepR1 (a plasma membrane receptor) and it is a receptor for AtPeps (peptide signaling molecules). Both of them are involved in pathogen-defense signaling cascades in plants. AtPeps belong to danger-associated molecular pattern (DAMP) (Huffaker et al. 2006; Huffaker and Ryan 2007; Ryan et al. 2007). AtPep1 binds to plant cell plasma membrane receptor AtPepR1, which in turn contains a putative guanylate cyclase domain and can synthesize cGMP. This cyclic nucleotide activates plant cyclic nucleotide-gated ion channel 2 (CNGC2), which is responsible for the cell membrane Ca^{2+} channel currents that facilitate downstream immune signaling, leading to pathogen-defense responses in plants (Ali et al. 2007). Structure and function of CNGCs are discussed in Sect. 6.4. Recent research has shown that guanylate cyclase activity of AtPepR1 provided a model for linking pathogen perception at the surface to intracellular Ca^{2+} signaling and immune responses in plants (Ma et al. 2009a; Qi et al. 2010).

Among LRR-RLKs, the phytosulfokine (PSK) receptor (AtPSKR1, At2g02220), which also can synthesize cGMP in vitro, was described (Kwezi et al. 2011). In these molecules a guanylate cyclase catalytic center is found within the intracellular kinase domain in the C-terminal and it is nested within the kinase catalytic domain (Irving et al. 2012). A natural ligand for AtPSKR1 is PSK-α, which can cause an increase in cGMP levels. Overexpression of AtPSKR1 in protoplasts raises endogenous basal cGMP levels (Kwezi et al. 2011).

The kinase-guanylate cyclases such as PSKR1, WALK10, and BRI1 are examples of moonlighting proteins with dual catalytic activity. Kinase-guanylate cyclases are highly unusual because they contain two catalytic centers in the same domain of the protein. The catalytic center of guanylate cyclase is embedded in the kinase domain adjacent to the P activation loop. Despite the fact that the active center of guanylate cyclase is encapsulated in the kinase domain, it is separate from the catalytic center of kinase. Therefore these molecules are moonlighting proteins rather than promiscuous enzymes (Irving et al. 2012). It is believed that promiscuous enzymes have a high degree of promiscuity (Khersonsky and Tawfik 2010). In the case of BRI1, even a mutation causing lack of kinase activity of this molecule had no effect on the guanylate cyclase center (Xu et al. 2008). Kinase activity of PSKR1 was reduced in the presence of cGMP. Probably cGMP modulates kinase function by allosterically binding in the guanylate cyclase catalytic center. The receptors PSKR1 and BRI1 are a novel class of receptor kinases with a moonlighting guanylate cyclase domain (Irving et al. 2012).

It is known that NO affects the cGMP level in tobacco cells (Durner et al. 1998; Pasqualini et al. 2009). This was the reason for searching for NO-dependent guanylate cyclase in plants. Based on heme-NO and oxygen-binding (H-NOX) domains

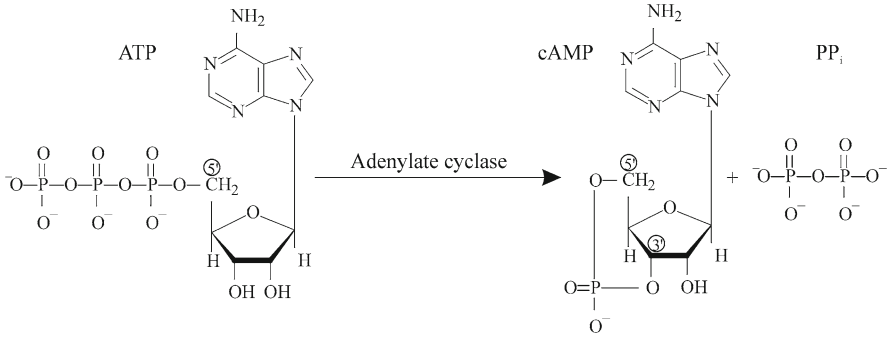

Fig. 6.3 Biosynthesis of adenosine 3′,5′-cyclic monophosphate (cAMP)

(Boon et al. 2005), a protein with a conserved heme-binding motif was identified. It was flavin-dependent monooxygenase from *Arabidopsis* (AtNOGC1, At1g62580), which also contains the 14 amino acid catalytic center which was found in experimentally tested guanylate cyclase in plants (Mulaudzi et al. 2011). It has been demonstrated electrochemically that this protein binds NO, has a higher affinity for NO than for O_2, and synthesizes cGMP in vitro in an NO-dependent way (Mulaudzi et al. 2011).

3.2 Synthesis of cAMP

Cyclic AMP in prokaryotes and eukaryotes is synthesized from ATP by adenylate cyclase (ATP diphosphate-lyase cyclizing, EC 4.6.1.1, Helmreich et al. 1976) (Fig. 6.3). Activity of adenylate cyclase was described in extracts from roots of alfalfa (*Medicago sativa*) (Carricarte et al. 1988), castor (*Ricinus max*) (Lusini et al. 1991), pea (*Pisum sativum*) (Pacini et al. 1993), and tobacco (Witters et al. 2004, 2005). Adenylate cyclase from alfalfa roots is a soluble protein with a molecular weight of about 84 kDa. Cations such as Ca^{2+} and Mg^{2+} are activators. In the presence of Ca^{2+}, the activity of adenylate cyclase in alfalfa roots was 204 pmol cAMP min^{-1} mg^{-1} protein. Adenylate cyclase activity was 15-fold higher in the presence of bovine calmodulin. Inhibitors are ethylene glycol tetraacetic acid (EGTA) and chlorpromazine (calmodulin inhibitor) (Carricarte et al. 1988). Other researchers described plasma membrane adenylate cyclase in castor roots (Lusini et al. 1991). Activity of this enzyme in the presence of $MnCl_2$ was about 20 pmol cAMP min^{-1} mg^{-1} protein. Contrary to adenylate cyclase from alfalfa, the enzyme from castor roots was sensitive to GTP, which suggests involvement of G proteins in its regulation (Lusini et al. 1991). Extract from pea roots showed synthesis of cAMP in the presence of ATP and Mg^{2+}. A plot of reaction velocity against substrate concentration was sigmoidal, indicating allostery. Concentration of 100 nM GTP

activates the enzyme, but 100 μM GTP inhibits it (Pacini et al. 1993). Cytoenzymological methods allowed the localization of adenylate cyclase in palisade parenchyma cells of excised tobacco leaf. It was shown that adenylate cyclase is located mainly at the chloroplast envelope (Witters et al. 2004, 2005).

The first evidence at the genetic level for cAMP enzymatic synthesis in plants is adenylate cyclase from maize pollen (Moutinho et al. 2001). PSiP (pollen-signaling protein) which is homologous to fungal adenylate cyclases was isolated from the maize cDNA library. This protein can generate cAMP which is involved in polarized pollen tube growth. Expression of this gene of PSiP in *Escherichia coli* (*cyaA* mutant) caused accumulation of cAMP in bacteria cells. However, activity of adenylate cyclase (the product of this gene) has not been confirmed in vitro (Moutinho et al. 2001). In *Arabidopsis* there are many predicted protein sequences homologous to PSiP, and those with the highest homologies correspond to two genes, *At3g14460* (*AtAC1*) and *At3g14470* (*AtAC2*). The proteins At3g14460 and At3g14470 are annotated as disease resistance proteins belonging to the nucleotide-binding site-leucine-rich repeat (NBS-LRR) family; they are involved in pathogen sensing and have a role in defense responses and apoptosis (DeYoung and Innes 2006). NBS-LRR proteins are also important in animal innate immune systems; however, in animals they seem to be involved in pathogen-associated molecular pattern (PAMP) recognition rather than recognition of pathogen effectors (Inohara et al. 2005). Expression of *At3g14460* and *At3g14470* in *Escherichia coli* cAMP mutant (*cyaA*) resulted in cAMP synthesis in bacteria. Gene expression studies using GUS staining and RT-PCR showed that *AtAC1* is expressed in root tips, inflorescence, and anther, while *AtAC2* is expressed in cauline, senescent roots, and inflorescence. T-DNA insertion mutants of *AtAC1* and *AtAC2* germinate under salt stress, suggesting the role of cAMP as a signaling intermediate in salinity tolerance (Jusoh et al. 2010).

The search for proteins with adenylate cyclase activity was started upon the search for proteins with guanylate cyclase activity in higher plants (Ludidi and Gehring 2003; Kwezi et al. 2007; Meier et al. 2010). Analysis of *Arabidopsis* proteome by BLAST was highly complicated because Prosite signatures for class one (EYFG [SA]X(2)LWXLYK) and two (YRNXW[NS]E[LIVM]RTLHFXG) of adenylate cyclases are not present in the *Arabidopsis* proteome even if researchers allow two mismatches (Gehring 2010). This was deduced from conserved and functionally assigned amino acids in the catalytic center of guanylate cyclase from lower and higher eukaryotes (Liu et al. 1997; McCue et al. 2000). After modification of the guanylate cyclase search motif, the relaxed amino acid sequence [RKS]X[DE]X(9,11)[KR]X(1,3)[DE] was obtained (Fig. 6.4c); it was used to search for adenylate cyclases in higher plants. In the modified adenylate cyclase search motifs, the amino acid residues conferring substrate specificity ([CTGH], position 3 in Fig. 6.2c) are substituted to [DE] (position 3 in Fig. 6.4c). Consequently, the core motif within the catalytic center consists of the functionally assigned residue that does the hydrogen bonding with the adenine (position 1), the amino acid that confers substrate specificity for ATP (position 3), and the amino acid that stabilizes the transition state from ATP to cAMP ([K,R], position 12–14). Additional diagnostic residues are the Mg^{2+}/Mn^{2+}-binding amino acid [D,E], usually 1–3 amino acids

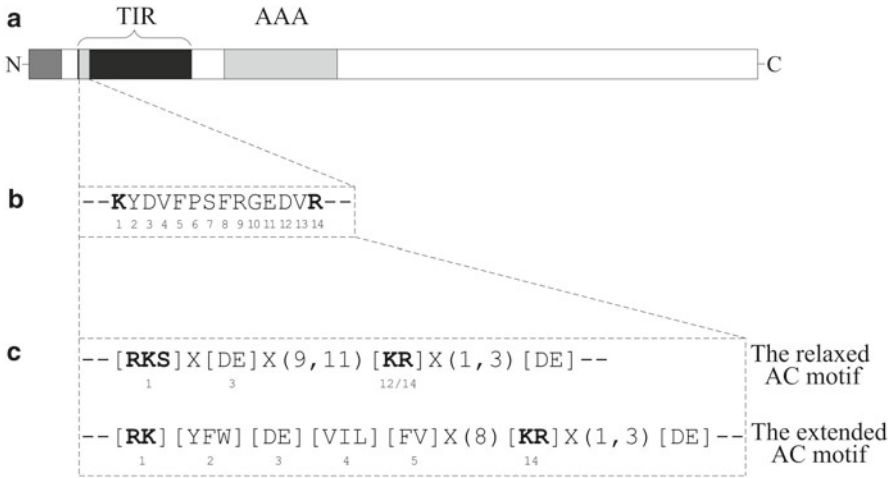

Fig. 6.4 Scheme of the *Arabidopsis* toll interleukin receptor nucleotide-binding site leucine-rich repeat protein (TIR-NBS-LRR, At3g04220) with adenylate cyclase catalytic domain (**a**), amino acid sequence of the original search motif (**b**), and adenylate cyclase motifs used in the identification of nucleotide cyclases in higher plants (**c**). Amino acids marked with bold are functionally assigned residues of the enzyme catalytic center. *AAA* ATPase associated with wide variety of cellular activities superfamily, *AC* adenylate cyclase, and *TIR* toll interleukin receptor

removed from the C-terminal of the transition state-stabilizing residue (Gehring 2010). Such a modified pattern ([RKS]X[DE]X(9,11)[KR]X(1,3)[DE]) (Fig. 6.4c) is present in maize adenylate cyclase (AJ307886.1), which is the only adenylate cyclase in plants experimentally proven at a genetic level and is also present in sorghum (*Sorghum bicolor*) ortholog (gb|EER90437.1) and in related *Arabidopsis* NBS-LRR class protein (At3g14460) (Gehring 2010).

Now in the TAIR database (The *Arabidopsis* Information Resource), there are three adenylate cyclases with a relaxed adenylate cyclase motif [RKS]X[DE] X(9,11)[KR]X(1,3)[DE]. These are At1g26190, At1g73980, and At2g11890. Unfortunately, their adenylate cyclase activity was not confirmed in vitro. In searching for candidates for adenylate cyclases, Gehring (2010) proposed to use a previously identified 14 amino acid long search motif employed in guanylate cyclase research. It is a deduced catalytic center search motif modified for specificity for ATP rather than GTP binding and with the C-terminal metal-binding residue ([RK] [YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE]) (Fig. 6.4c). Using this extended motif, there were identified nine putative adenylate cyclases in *Arabidopsis* (Table 6.1) (Gehring 2010). Among these proteins, two of them are F-box proteins (At3g28223 and At4g39756) which are associated with cellular functions such as signal transduction and regulation of the cell cycle (Craig and Tyers 1999). In plants many F-box proteins are represented in gene networks broadly regulated by microRNA-mediated gene silencing via RNA interference (Jones-Rhoades et al. 2006). The F-box domain is a protein structural motif of about 50 amino acids that mediates

Table 6.1 *Arabidopsis* proteins retrieved with the adenylate cyclase search pattern: [RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE] (Gehring 2010)

I.D.	Annotation
At1g25240	Epsin N-terminal homology
At1g62590	Pentatricopeptide (PPR) repeat-containing protein
At1g68110	Epsin N-terminal homology
At2g34780	Maternal effect embryo arrest 22
At3g02930	Chloroplast protein
At3g04220	TIR-NBS-LRR class
At3g18035	Linker histone-like protein, HON4
At3g28223	F-box protein
At4g39756	F-box protein

protein–protein interactions. It was first identified in cyclin F. Another protein among the nine putative adenylate cyclases is toll interleukin receptor nucleotide-binding site leucine-rich repeat protein (TIR-NBS-LRR, At3g04220) (Table 6.1, Fig. 6.4a–c). It turned out that this is a disease resistance protein (TIR-NBS-LRR class) family. It is a transmembrane receptor involved in signal transduction, the defense response, apoptosis, and the innate immune response, and contains an adenylate cyclase domain (Suzuki et al. 1990). The *Arabidopsis* genome contains approximately 200 genes that encode proteins with similarity to the nucleotide-binding site and other domains characteristic of plant resistance proteins. The observed diversity of the NBS-LRR proteins indicates the variety of recognition molecules available in an individual genotype to detect diverse biotic challenges (Meyers et al. 2003). Structurally similar to plant TIR-NBS-LRR class proteins is the protein of maize adenylate cyclase (AJ307886.1). Protein At3g04220 also contains a P-loop NTPase signature. This is a characteristic signature for signal transduction ATPases with numerous domains (STAND), and some STAND NTPases contain adenylate cyclase domains (Leipe et al. 2004).

3.3 Breakdown of cAMP and cGMP

Cyclic nucleotides, as activators of protein kinases and ion-channel modulators, are key regulators of many cellular processes (Newton and Smith 2004). Their immediate action is terminated through the activity of phosphodiesterases, a diverse family of enzymes that catalyze the hydrolysis of the 3′-ribose phosphate bond of cAMP and cGMP (Fig. 6.5). Thus, they are responsible for controlling cellular concentration of cAMP and cGMP by hydrolyzing them to 5′-NMP in the case of animals and a 3′-NMP/5′-NMP mixture in plants (Hofmann et al. 2002). Plant phosphodiesterases can be divided into two groups: phosphodiesterases that inactivate 2′,3′-cyclic nucleotides and phosphodiesterases that inactivate 3′,5′-cyclic nucleotides. Phosphodiesterase from pea seedling has an acidic pH optimum, does not require

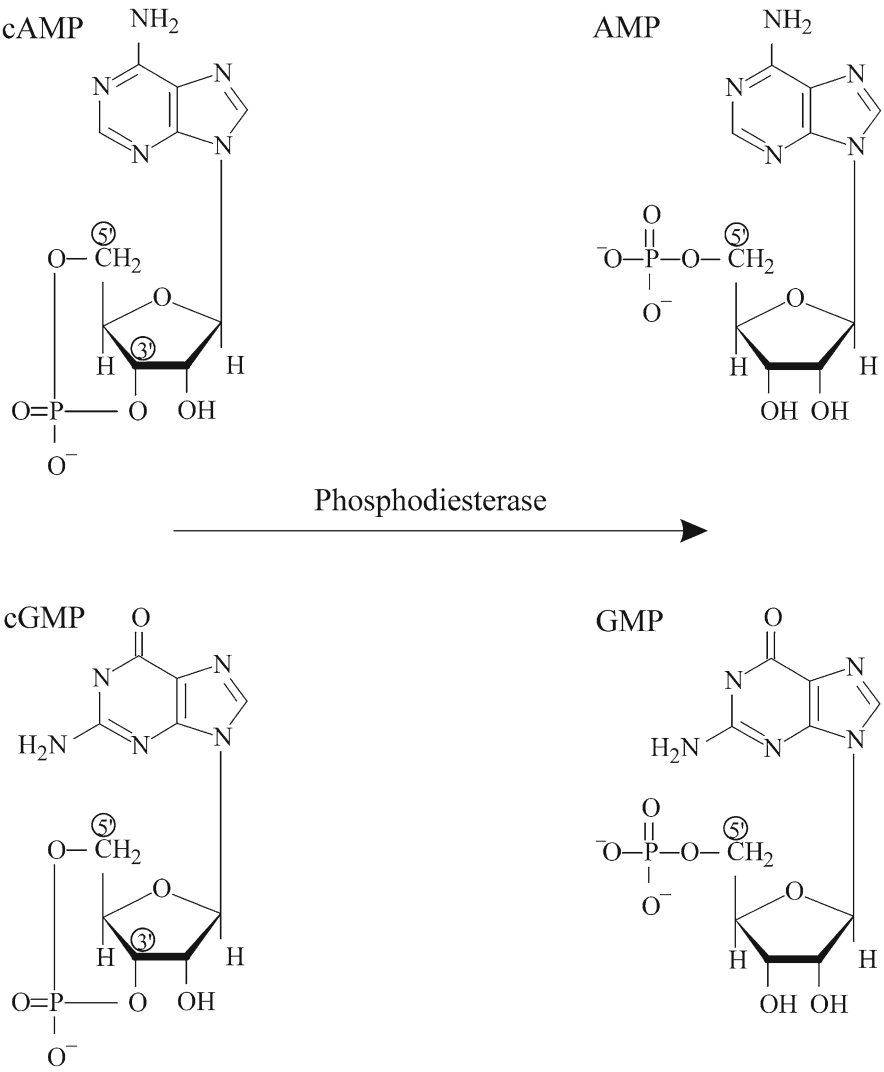

Fig. 6.5 Breakdown of adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP)

bivalent cations, and is insensitive to methylxanthines. Phosphodiesterase capable of hydrolyzing cAMP into AMP has substantially higher activity against 2′,3′-cAMP (RNA breakdown product) than against 3′,5′-cAMP (second messenger isomer) (Lin and Varner 1972).

Phosphodiesterases have been reported in the vacuole and in the extracellular compartments (Van der Rest et al. 2002). Tomato (*Lycopersicon esculentum*) extracellular phosphodiesterase, purified to homogeneity from the spent culture medium

of phosphate-starved cells, is required for complete degradation of extracellular RNA (Abel et al. 2000). Physical and catalytic properties of this enzyme are strikingly similar to those that have been studied earlier, during identification of activity of 3′,5′-cAMP phosphodiesterase in barley (Vandepeute et al. 1973), soybean (*Glycine max*) (Brewin and Northcote 1973), potato (Ashton and Polya 1975), sunflower (*Helianthus annuus*) (Junker et al. 1977), portulaca (*Portulaca*) (Endress 1979), pea (Chiatante et al. 1990), and carrot (*Daucus carota*) (Kurosaki and Kaburaki 1995). Phosphodiesterase described in lettuce (*Lactuca sativa*) is different from other 3′,5′-cyclic nucleotide phosphodiesterases that exhibit the activity of both the purine and pyrimidine cyclic nucleotide (Chiatante et al. 1988).

The subsequent studies have shown the presence of many isoforms with differences in distribution, specificity, and kinetics, which allowed the results of various research groups to be explained. Phosphodiesterase from common bean chloroplasts preferred 3′,5′-cyclic nucleotide as a substrate, and cCMP hydrolysis was stimulated in the presence of Fe^{3+} ions (Smith et al. 2001). The regulatory function of different calmodulin isoforms to phosphodiesterases has also been investigated. It was found that the 2 isoforms from soybean have different requirements in relation to Ca^{2+}, thereby differently affecting phosphodiesterase (Lee et al. 2000). In spinach (*Spinacia oleracea*), one of three phosphodiesterase isoforms had the highest activity with 3′,5′-cAMP and 3′,5′-cGMP and little activity with their 2′,3′-isomers. Additionally, this isoform was activated by Ca^{2+} and revealed sensitivity to endogenous effector protein (Brown et al. 1979; Dupon et al. 1987). This enzyme created complexes with acid phosphatases, ribonucleases, nucleotidases, and ATPases (Brown et al. 1980).

Cyclic AMP phosphodiesterase from *Nectria haematococca* (a plant pathogen) can be inhibited by several flavonoids. This suggests that the ability of specific flavones and flavanones to inhibit cAMP phosphodiesterase can elevate cAMP level and promote germination (Bagga and Straney 2000). Phosphodiesterase inhibitors such as Sildenafil citrate (Viagra™) and 3-isobutyl-1-methylxanthine, which enhance NO-induced cGMP production, synergistically diminish pollen germination with exogenously applied ATP (Reichler et al. 2009). Phosphodiesterase is also involved in vernalization and can serve as a receptor of low temperature in winter wheat (*Triticum aestivum*) (Fedenko et al. 2004).

Recently, in plants a new family of glycerophosphodiester phosphodiesterases (GPX-PDEs) was discovered, which have been previously described in bacteria, yeast, and mammals (Cheng et al. 2011). Plant GPX-PDE was first identified in carrot cells. Extracellular and intracellular enzyme activities were induced in phosphorus-deficient cell cultures of carrot, *Arabidopsis*, and sycamore (*Acer pseudoplatanus*) (Van der Rest et al. 2002). There is a wide range of substrates for GPX-PDEs such as glycerophosphocholine, glycerophosphoinositol, and glycerophosphoethanolamine. GPX-PDE catalyzes the hydrolysis of deacylated phospholipid glycerophosphodiester to glycerol-3-phosphate and the corresponding alcohol. These phosphodiesterases appear to have important functions in phosphorus scavenging and carbon stress.

Phosphodiesterases are involved in the degradation of phospholipids and nucleic acids, which constitute the majority of the fresh organic phosphorus inputs

to soil (Turner and Haygarth 2005). Initial hydrolysis by phosphodiesterase releases a phosphate monoester, which must then be hydrolyzed by phosphomonoesterase to release free phosphate for biological uptake. The low phosphodiesterase activity in acidic soils may be linked to the dominance of fungi or an effect of sorption on the enzyme.

4 Cyclic Nucleotide-Gated Ion Channels in Plants: Structure and Function

All ion channels are considered to be the most important membrane proteins intermediating in signal transduction and ion transport and play a crucial role in many physiological processes in living cells (Ward et al. 2009; Wang 2012). Cyclic nucleotide-gated ion channels (CNGCs) are found in the plasma membranes of various cell and tissue types of both the animal and plant kingdoms (Matulef and Zagotta 2003; Talke et al. 2003; Zelman et al. 2012) and recently also in prokaryotes (Nimigean et al. 2004; Kuo et al. 2007). Initially, these channels were discovered in vertebrate visual and olfactory signal transduction cascades (Zagotta and Siegelbaum 1996; Craven and Zagotta 2006) and then in the heart, brain, muscle, and other animal organs (Kaupp and Seifert 2002). Successively, the CNGC family and homologs of these proteins have also been identified in plants such as barley (Schuurink et al. 1996), tobacco (Arazi et al. 1999, 2000), *Arabidopsis* (Köhler et al. 1999; Köhler and Neuhaus 2000), and rice (The UniProt Consortium 2012).

Increasing numbers of studies on plant CNGCs provide information about their physiological role (Kaplan et al. 2007; Chin et al. 2009; Sherman and Fromm 2009; Dietrich et al. 2010; Zelman et al. 2012). In the *Arabidopsis* genome, 20 cation-conducting channels are members of the CNGC family (Mäser et al. 2001). To better understand this role in plant signal transduction pathways, many researchers have studied interactions between these channels and the cytosolic signaling molecule calmodulin, as well as secondary messengers such as cAMP, cGMP, and Ca^{2+} (Arazi et al. 2000; Köhler and Neuhaus 2000; Hua et al. 2003a). The effect of Pb^{2+} and Ni^{2+} on the growth of various mutants was also studied (Arazi et al. 2000). CNGC transgenics and mutants exhibit both cation sensitivity and tolerance (Sunkar et al. 2000; Chan et al. 2003; Gobert et al. 2006; Ma et al. 2006). The expression of cloned plant CNGCs in heterologous systems allows electrophysiological analysis of the recombinant proteins (Leng et al. 2002; Wang 2012). Thanks to such research we know that CNGCs may be involved in a broad array of mechanisms impacting plant growth, development, and response to plant defense and environmental stresses (Chan et al. 2003; Gobert et al. 2006; Yoshioka et al. 2006; Borsics et al. 2007; Frietsch et al. 2007).

The operation of these nonselective cation channels may be the result of the cyclic nucleotides binding and either depolarization or hyperpolarization events (Hua et al. 2003b). CNGCs specifically localized to the plasma membrane,

endoplasmic reticulum, and Golgi cisternae (Christopher et al. 2007; Yuen and Christopher 2010) may also be targeted to the chloroplast (Sherman and Fromm 2009) and be components of vacuole membranes (Yuen and Christopher 2013).

These ligand-gated cation-conducting channels provide putative pathway for Ca^{2+} and may allow the diffusion of monovalent cations such as Na^+ or K^+ (Ma et al. 2010). Functional analysis in heterologous systems showed that CNGCs characterized freely conduct K + and Na + apart from CNGC2 which discriminates against Na^+ (Leng et al. 2002; Hua et al. 2003a, b). Moreover, the latest research of Abdel-Hamid et al. (2011) suggests that *Arabidopsis* cyclic nucleotide-gated ion channel (AtCNGC) 11 and 12 are not involved in K^+-dependent physiological responses. Probably AtCNGC2 may also be selective for other monovalent cations (Li^+, Cs^+, and Rb^+). Only 1, 2, 11, 12, and 18 plant CNGC isoforms have been experimentally verified to conduct Ca^{2+}, whereas AtCNGC3 does not conduct those cations (Gobert et al. 2006). It is different from the native isoforms 2, 4, 11, and 12 that are involved in signaling cascades related to plant pathogen-defense responses (Ma et al. 2010).

Plant CNGCs have a complex structure with various subunits and domains (Chin et al. 2009; Zelman et al. 2012), which play a critical role in their function. CNGCs are usually presented in the form of several regions. Starting from the N-terminus, there are six transmembrane domains (S1–S6) with the pore region (P) between the fifth and sixth domains (Fig. 6.6) that determines the specificity of ion permeation. The fourth domain has similarity to the receptor type Shaker (Rehmann et al. 2007). Further regions are the most conserved region CNBD (cyclic nucleotide-binding domain) containing the phosphate-binding cassette that binds the sugar and phosphate moieties of the cyclic nucleotide ligands (Cukkemane et al. 2011), and adjacent to CNBD the "hinge" region, which is believed to be engaged in direct contact with the cyclic nucleotide (Young and Krougliak 2004). Plant CNGC polypeptides also contain a CaMBD (calmodulin-binding domain), but in contrast to animal CNGC, the functional CaMBD resides at the C-terminus (Arazi et al. 1999; Köhler et al. 1999, Sherman and Fromm 2009). Some studies have shown that calmodulin blocks plant CNGC ion conductance. This protein may compete with cyclic nucleotide for binding to the channel and therefore prevent cyclic nucleotide activation (Ali et al. 2007; Li et al. 2005). Other results implicate a more complex CNG channel regulation (Fischer et al. 2013). While several members of the CNGC family employ the αC-helix of the CNBD to bind CaM (Kaplan et al. 2007), in the CNGC20 has been mapped a functional Ca^{2+}-dependent CaM-binding site C-terminally to the CNBD, which belongs to the isoleucineglutamine (IQ) class of binding sites (Fischer et al. 2013). CNGC20 is the first example for the IQ domain-mediated function in a plant transport protein, which is conserved among plant CNGCs and is present in several members of the glutamate receptor family of cation channels and in phosphate transporters of *Arabidopsis* (de Castro et al. 2006).

Cyclic nucleotides bind to the CNBD domain, causing a conformational change leading to opening of the channel. In some cases cGMP was found not to act as an activating ligand (Lemtiri-Chlieh and Berkowitz 2004). More often the cAMP activates this channel. There occurs an influx of monovalent and divalent cations into the cell, which activates downstream signaling components that result in a physiological

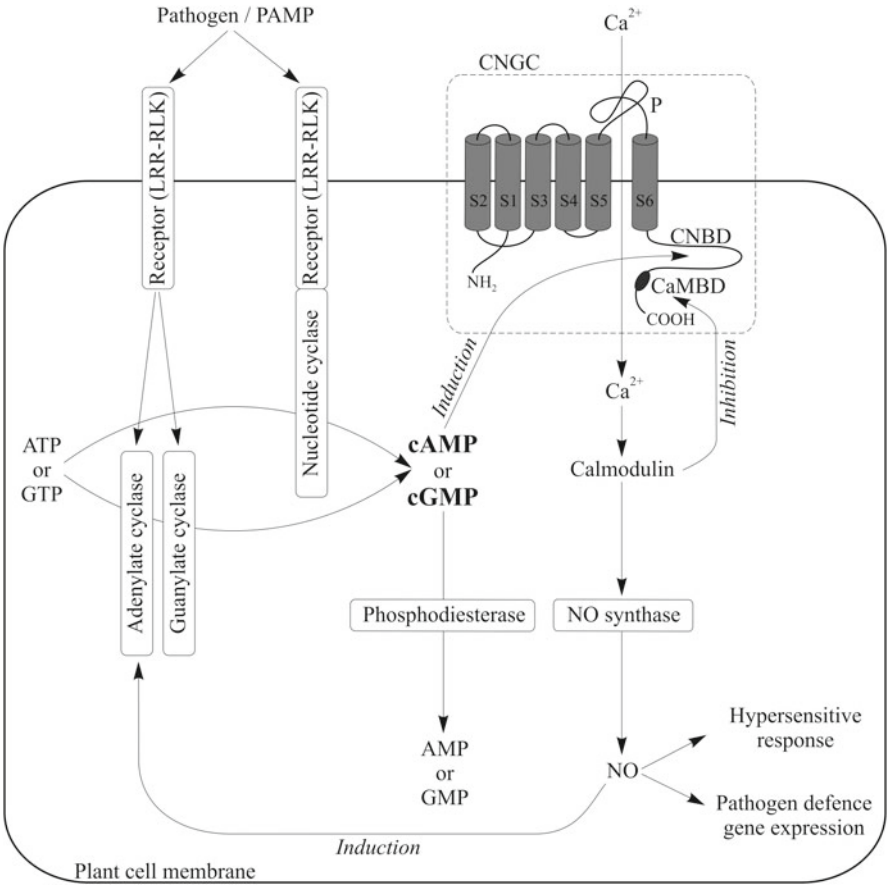

Fig. 6.6 Schematic representation of possible early events in the plant immune response-signaling cascade and structure model of cyclic nucleotide-gated channel (CNGC). *CaMBD* calmodulin-binding domain, *CNBD* cyclic nucleotide-binding domain, *LRR-RLK* leucine-rich repeat receptor-like kinase, *NO* nitric oxide, *P* pore loop, *PAMP* pathogen-associated molecular pattern, *S1–S6* α-helical transmembrane segments

response. Binding of Ca^{2+} activates calmodulin, allowing it to bind to the CaMBD, which leads to NO and H_2O_2 production, gene expression, and ultimately closing of this channel as a negative feedback mechanism (Chin et al. 2009; Ma et al. 2010).

When plants are exposed to pathogens, CNGC-mediated Ca^{2+} influx results in a hypersensitive response (Fig. 6.6). Calmodulin and calmodulin-like protein influence pathogen response signaling downstream by interacting with enzymatic proteins and transcriptional regulators. Overexpression of soybean calmodulin in tobacco resulted in enhanced resistance to a range of pathogens (Heo et al. 1999), whereas tobacco or tomato calmodulin silencing lines indicated increased

susceptibility to virulent bacterial or fungal pathogens (Bouché et al. 2005; Takabatake et al. 2007). Cytosolic Ca^{2+} elevation could lead presumably to NO synthase (NOS)-dependent NO generation (Ma et al. 2008) or reactive oxygen species production through Ca^{2+}-dependent protein kinase (CDPK) signaling. These CDPKs have been shown to phosphorylate NADPH oxidase, which generates H_2O_2 during hypersensitivity reactions (Kobayashi et al. 2007).

CNGCs play a prominent role in regulating various developmental pathways. For example, AtCNGC2 is a positive regulator both in early seedling development and in early stages of senescence (Köhler et al. 2001). It turns out that AtCNGC2 is important for cell elongation in stamens and pistils, thus being important for pollen tube guidance and fertility (Chaiwongsar et al. 2009). Frietsch et al. (2007) also demonstrated the role in pollen tube development. AtCNGC18 is not expressed in the leaves and roots of young seedlings but primarily in pollen grains. For other AtCNGCs (7, 8, 16, and 18), the situation was similar (Bock et al. 2006). Another pollen-expressed AtCNGC16 was critical for pollen fertility under conditions of heat stress and drought (Tunc-Ozdemir et al. 2013). A knockout of CNGC6 in *Arabidopsis* resulted in plants with vegetative tissues showing a decreased tolerance to heat stress. A comparable disruption of a heat stress response was not observed in knockouts of a CNGC-b in the moss *Physcomitrella patens* and its putative ortholog in *Arabidopsis* (CNGC2) (Finka et al. 2012). Rather, these mutant plants showed hyperactivation of a heat stress response at lower temperatures. Moreover, it was demonstrated that AtCNGC10 antisense lines exhibit significant changes in growth and metabolism, characterized by a reduction in leaf surface area, impaired gravitropic responses of significantly shorter roots, reduced laminar thickness and palisade columnar cell length, as well as accumulation of increased amounts of starch in leaves compared to wild type (Borsics et al. 2007).

CNGCs, as nonselective cation transporters, also represent a possible entry pathway for heavy metal ions (CNGC1 and 10). A few cyclic nucleotide target proteins have been connected with uptake and homeostasis of heavy metals such as Ni^{2+} and Pb^{2+} (Arazi et al. 1999; Sunkar et al. 2000). CNGCs are also involved in responses to salt stress (Maathuis et al. 2003). AtCNGC3 contributes the uptake of monovalent cations including Na^+ in root tissue. Seedling of *cngc3 Arabidopsis* mutant were more tolerant compared to the wild type and grew better at high KCl concentrations (Gobert et al. 2006). Besides AtCNGC3, also AtCNGC10 acts in cation transport during salt stress (Guo et al. 2008).

CNGCs have also been identified as important regulators of pathogen resistance. Recent studies suggest that CNGC11 and 12 are positive regulators of responses to pathogen (Yoshioka et al. 2006). The first line of induced defense is the immune system, which is activated by pathogen-associated molecular pattern (PAMP) molecules (Moeder et al. 2011) or microbe-associated molecular pattern (MAMP) components (Zhang and Zhou 2010). cAMP and cytosolic Ca^{2+} elevation was demonstrated in leaves of *Arabidopsis* within a few minutes after inoculating with *Pseudomonas syringae* (Ma et al. 2009b). Mutations in several CNGCs have been associated with altered plant responses to virulent and avirulent pathogens, including *Pseudomonas syringae* (Dietrich et al. 2010). AtCNGC2 mutant (*dnd1*) and

AtCNGC4 mutant (*dnd2/hlm1*) as well as barley mutant (*nec1*) exhibit spontaneous cell death formation and disease resistance (Balagué et al. 2003; Jurkowski et al. 2004; Rostoks et al. 2006). These *dnd1* and *dnd2* plants constitutively express pathogen-related (PR) genes (Vlot et al. 2008), accumulate salicylic acid (SA), and exhibit dwarf appearance (Yu et al. 2000). One SA-independent signaling pathway is controlled by a mechanism dependent on jasmonates (JA) and/or ethylene (ET). There is an extensive cross talk between SA and JA/ET signaling, which is usually thought to be antagonistic (Glazebrook 2005). Plants use these various signaling pathways depending on the type of pathogen (Spoel et al. 2007). Others, equally new reports, show that CNGC19 and CNGC20 may serve a similar molecular function in the response to both biotic and abiotic stress. Yuen and Christopher (2013) suggest that group IV-A CNGCs mediate plant responses to salinity and pathogen infection by facilitating the movement of Ca^{2+} cations between the central vacuole and the cytosol.

5 Cyclic Nucleotide-Dependent Protein Kinases in Plants

Among the first proteins to be found regulated by cyclic nucleotides were the cAMP-dependent kinases (PKA) and cGMP-dependent kinases (PKG) activated by cAMP and cGMP, respectively. These kinases phosphorylate an array of cellular targets including other kinases, phosphatases, gene transcription factors, and an ever-growing list of ion channels (Lemtiri-Chlieh et al. 2011).

There are reports on cAMP-dependent kinases (PKA) whose activity was changed in the presence of cyclic nucleotides in maize, duckweed (*Lemna paucicostata*), and coconut palm (*Cocos nucifera*) (Janistyn 1989; Kato et al. 1983; Komatsu and Hirano 1993). The team of Polya (1991) partially purified enzyme from petunia (*Petunia*) petals, which phosphorylated a synthetic substrate for PKA, namely, Kemtide (LRRASLG). This Kemtide kinase was not affected by cAMP and Ca^{2+}-calmodulin. The presence of cAMP-dependent kinase was also confirmed in wheat etioplast and chloroplasts and in broad bean (*Vicia faba*) stomatal cells (Newton and Smith 2004). Other research conducted on bean showed that forskolin and micromolar concentrations of cAMP increased phosphorylation; however, in these studies, kinase was not isolated (Friedrich et al. 1999). The PKA inhibitor K252a partially inhibited abscisic acid (ABA) induction of β-glucuronidase activity in tobacco cells transformed with a construct containing a promoter sensitive to ABA (rd29A-GUS) (Liu et al. 2002). ABA induced PKA activity (PKABA1) that inhibited induction of amylase synthesis in the barley aleurone layer (Gomez-Cadenas et al. 2001).

Cyclic GMP-dependent protein kinase has been purified from tissues of *Pharbitis nil* (Szmidt-Jaworska et al. 2003). The activity of isolated polypeptide was stimulated by micromolar concentrations of cGMP. The PKG enzyme activity was also observed in oat (Dubovskaya et al. 2002). The binding of ^{3}H-labeled cGMP to

subcellular fractions of the structural components was dependent on the conditions of incubation and irradiation of growing plants. The strongest bond was found in the cytosolic fraction. The protein of this fraction has two binding sites, with different affinity for cGMP, and binding was increased by red light and Ca^{2+}. Other work indicates the existence of proteins in plant cells, similar to the kinase (AtWAKL10) which has two domains: the guanylate cyclase activity and cGMP-dependent kinase. It was found that this protein is involved in plant defense mechanisms as a result of infection (Meier et al. 2010).

In the sequence of PKA, the cyclic nucleotide-binding site occurs (Newton and Smith 2004). The full cDNA sequence of cyclic nucleotide-binding proteins has been isolated from tobacco and *Arabidopsis*. There have also been reports of the presence of gene-encoding proteins having a sequence characteristic of yeast and animal cyclic nucleotide-dependent kinases (Hammond and Zhao 2000a, b). The *SBPK* gene from germinating pollen of *Solanum berthaultii* encodes a protein of very high homology to cAMP- and cGMP-dependent kinase (Liu et al. 1999). Inactivation of gene expression through the use of antisense expression resulted in plants' production of a high proportion of modified pollen grain (Moutinho et al. 2001; Tsuruhara and Tezuka 2001). Bioinformatic analysis indicated the presence in the plant genome genes which contain both cyclic nucleotide-binding domain (CNBD) and the kinase domain (Krupa et al. 2006). The protein kinases which are unique to A. thaliana but not to O. sativa and vice versa have been identified.

The family of protein kinases named AGC is a subgroup of Ser/Thr protein kinases with similarity in their catalytic kinase domains to cAMP-dependent protein kinase A, cGMP-dependent protein kinase G, and phospholipid-dependent protein kinase C (Hirt et al. 2011; Garcia et al. 2012). The *Arabidopsis* genome encodes 39 members of the AGC protein kinase family, which are involved in various signaling pathways including auxin signaling (Bögre et al. 2003; Roberts and Offringa 2008; Rademacher and Offringa 2012). One of these kinases, OXI1, is shown to be required for reactive oxygen species-mediated responses such as root hair elongation and for disease resistance to oomycete *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae* bacteria. OXI1 activity was induced by H_2O_2, wounding, cellulase, and different elicitor treatments mimicking pathogen attack (Rentel et al. 2004; Anthony et al. 2006; Petersen et al. 2009). Homologs of *Arabidopsis* AGC kinases have been characterized in other plant species. These kinases are involved in different biological processes, including death of tomato cells (Devarenne et al. 2006), nodule formation of *Medicago truncatula* (Pislariu and Dickstein 2007), blue-light perception (Lariguet and Dunand 2005), as well as disease resistance of tomato (Hammond and Zhao 2000a, b) and rice (Matsui et al. 2010). Plant AGC protein kinases take part in auxin transport (Bai et al. 2005, Santner and Watson 2006, Morita and Kyozuka 2007). Moreover, they participate in immune responses and regulate pathogen-induced mitogen-activated protein kinase (MAPK) cascades (Kim et al. 2002; del Pozo et al. 2004).

6 Cyclic Nucleotides and Nucleotide Cyclases in Plant Stress Responses

Cyclic nucleotides have only recently been accepted as important secondary messengers in higher plants. Both cAMP and cGMP are reported to be involved in a number of physiological processes. Cyclic AMP has been shown to play a role in stimulating protein kinase activity in rice seedling leaves and roots. It was observed that phosphorylation of three proteins (with molecular masses of 55, 50, and 40 kDa) was significantly stimulated by cAMP. This suggests that cAMP-dependent protein kinase in the rice leaf might be involved in cell regulatory systems through phosphorylation (Komatsu and Hirano 1993). Cyclic AMP also plays a possible role in activation of phenylalanine ammonia lyase by its phosphorylation in suspension-cultured cells of French bean (Bolwell 1992).

Cyclic AMP has also been postulated to be involved in plant cell cycle progression, because there was evidence of cAMP fluctuations during the cell cycle in tobacco BY-2 cells. Furthermore, inhibition of cAMP biosynthesis by indomethacin at the beginning of the G1 phase arrested cell cycle progression at the G1/S phase (Ehsan et al. 1998, 1999).

Accumulation of endogenous cAMP was observed in conditioned common broomrape (*Orobanche minor*) seeds. It has been shown that cAMP level is regulated by gibberellin and that cAMP may act as a mediator in gibberellin signaling during common broomrape seed germination (Uematsu et al. 2007). A similar relationship was also observed in germinating phacelia (*Phacelia tanacetifolia*) seeds (Uematsu and Fukui 2008). Cyclic AMP may play a key role in both light signaling and the regulation of photosynthesis as well as responses to temperature (Thomas et al. 2013).

The second messenger in response to the plant hormone gibberellic acid could also be cGMP. In the barley aleurone layer exposed to gibberellic acid, the level of cGMP was increased, and induction of α-amylase gene transcription occurred (Penson et al. 1996). Bastian et al. (2010) strongly suggest gibberellic acid and cGMP-dependent transcriptional regulation. A link between gibberellic acid and increase in cGMP level was also observed in *Arabidopsis* tissue (Isner and Maathuis 2011). There was found a rapid and significant effect of the hormones, viz., abscisic acid, auxin, and jasmonic acid on cytoplasmic cGMP level. In contrast, brassinosteroids and cytokinin did not evoke a cGMP signal (Isner et al. 2012). Both gibberellic acid and abscisic acids are involved in the modulation of *Arabidopsis* seed germination by cGMP (Teng et al. 2010).

Cyclic GMP is involved in auxin-induced adventitious cucumber root growth. In this process NO acts as a secondary messenger in the IAA-mediated pathway. NO induces adventitious root formation through the activation of guanylate cyclase (Pagnussat et al. 2003). Plants with symbiotic nodules contained a high level of cAMP in the soybean root nodules but low levels of cAMP and cGMP in leaves (Terakado et al. 1997). Studies on pea (cv. Izusen) and soybean (cv. Enrei) and its supernodulating mutant *En6500* showed involvement of cAMP and cGMP in the regulation of root nodule formation (Terakado et al. 2003).

Both cAMP and adenylate cyclase are located in the developing tobacco chloroplasts (Witters et al. 2004, 2005). Studies conducted on lower plants showed that cAMP is involved in chlorophyll synthesis (Berchtold and Bachofen 1977), photosynthetic activity (Segovia et al. 2001; Gordillo et al. 2004), and blue (Iseki et al. 2002) light signaling. In red macroalga (*Porphyra leucosticte*), the cAMP level was relatively low when cells were incubated in darkness, or exposed to red or far-red light, but white light induced a pronounced increase in cAMP concentration. Under increasing white light irradiance, the cAMP level closely followed the increase in photosynthetic oxygen evolution rate. These results suggest a direct relationship between photosynthesis and cAMP accumulation (Segovia et al. 2001). All these data suggest participation of cAMP in signaling pathways in photosynthetic activity also in higher plants. Cyclic GMP regulates flowering in *Pharbitis nil*. The level of cGMP and activity of guanylate cyclase in *Pharbitis nil* were affected by light (Szmidt-Jaworska et al. 2004, 2008a, b). Cyclic GMP as a mediator participating in photoperiodic flower induction may govern this process by the phosphorylation mechanism via its influence on cGMP-dependent protein kinase activity (Szmidt-Jaworska et al. 2009).

Cyclic nucleotides can be involved in stomatal movement. Cyclic AMP was involved in stomatal movement in *Arabidopsis* (Curvetto et al. 1994) and cGMP in stomatal movement in *Commelina communis* (Pharmawati et al. 1998, 2001). A transient accumulation of cGMP in tobacco was caused by NO (Durner et al. 1998), and it was reported that NO is a signaling component of the abscisic acid (ABA)-induced stomatal closure and that both ABA- and NO-induced closure require the synthesis and action of cGMP (Neill et al. 2002; Dubovskaya et al. 2011). Cyclic GMP modulates stomatal opening induced by natriuretic peptides and immunoreactive analogues. Moreover, cGMP may affect K^+ fluxes during stomatal movement (Pharmawati et al. 2001). Joudoi et al. (2013)) showed that cGMP and its nitrated derivative 8-nitro-cGMP play important roles in the signaling pathways that lead to stomatal opening and closure.

Cyclic GMP effects on postembryonic developmental of *Arabidopsis* lateral root and also mediates acropetal auxin transport and basipetal auxin transport in the root (Li and Jia 2013). Participation of NO in cGMP synthesis was also observed in NO-treated spruce (*Picea abies*) needles (Pfeiffer et al. 1994). In addition, the specific inhibition of guanylate cyclase activity blocked the NO-induced cell death in *Arabidopsis* cells, and this inhibition is reversed by 8-Br-cGMP (cell membrane-permeable cGMP analog) (Clarke et al. 2000).

Cyclic GMP is involved in several processes such as phytochrome signaling, where the antagonism of cGMP and Ca^{2+} regulates the chloroplast development and anthocyanin biosynthesis (Bowler et al. 1994a, b; Neuhaus et al. 1997). Cyclic GMP induces expression of chalcone synthase, an enzyme involved in anthocyanin synthesis. In soybean cell suspension, cGMP can stimulate the induction of genes encoding chalcone synthase and ferredoxin $NADP^+$ oxidoreductase and can initiate anthocyanin biosynthesis (Bowler et al. 1994a). Cyclic GMP can act as a common regulator for the transcriptional activation of different enzymes of the flavonoid biosynthetic pathway and can affect anthocyanin accumulation (Suita et al. 2009).

In turn, accumulation of flavonoids has been observed in the plant response to various stress factors (Dixon and Paiva 1995).

Cyclic nucleotides can be important molecules in plant responses to abiotic stress. In *Arabidopsis* plants cAMP or cGMP increased plant survival in salinity stress. In *Arabidopsis*, cAMP plays a role in ion transport (Anderson et al. 1992; Trewavas 1997) and regulates ion channels (Bolwell 1995). Cyclic GMP inhibits Na^+ uptake and its accumulation (Maathuis and Sanders 2001; Essah et al. 2003; Rubio et al. 2003). The smaller amount of Na^+ in tissues of cyclic nucleotide-treated plants may contribute to improving plant salinity tolerance. Addition of cAMP or cGMP to *Arabidopsis* seedlings improves their salinity tolerance by negatively regulating voltage-independent channels in the root hairs (Maathuis and Sanders 2001). In addition, it was observed that salt and osmotic stress rapidly increases cGMP level in *Arabidopsis* seedlings (Donaldson et al. 2004). Exposure of *Arabidopsis* roots to 8-Br-cGMP (membrane-permeable cGMP analog) induces changes in abundance of many transcripts involved in metabolism, gene expression, signaling, and defense. Under these conditions, nonselective ion channels and cation/proton antiporters were found to be affected. Cyclic GMP modulates the influx and efflux of monovalent cations such as Na^+ and K^+ in *Arabidopsis* roots (Maathuis 2006). In tobacco protoplast, cGMP leads to a rapid influx of Ca^{2+} probably through Ca^{2+} channels (Volotovsky et al. 1998). Moreover, cGMP may affect nonselective ion channels in *Arabidopsis* root plasma membranes (Maathuis and Sanders 2001). More recently, an increase of the cAMP content and adenylate cyclase activity was observed in *Arabidopsis* seedlings treated with cadmium (Pietrowska-Borek et al. 2012).

Cyclic nucleotides can be important molecules in plant responses to biotic stress. Synthesis of cAMP was confirmed in alfalfa cell cultures after exposure to the glycoprotein elicitor of the phytopathogenic fungus *Verticilium alboatrum* (Cooke et al. 1994). In cultured carrot cells, the increased level of cAMP coincided with the early stages of the response to phytoalexins. It mediated the production of 6-methoxymellein and the activation of calcium uptake into cells (Kurosaki et al. 1987; Kurosaki and Nishi 1993). In cultured French bean cells, cAMP concentration increased from 5 to 18 pmol g^{-1} fresh weight upon fungal elicitor treatment, but this increase was transient, returning to almost baseline within 1 h (Bolwell 1992). Jiang et al. (2005) showed that pathogenic fungi *Verticillium dahliae* toxins increased the levels of endogenous cAMP in *Arabidopsis* (up to 9–10-fold higher than in the control plants). It was evidenced that cAMP mediated in defense responses in *Arabidopsis* to this pathogen by regulation of the production of salicylic acid and by induction of pathogenesis-related protein 1 gene (*PR1*) transcription. Exogenously applied 8-Br-cAMP (membrane-permeable cAMP analog) caused an accumulation of salicylic acid in *Arabidopsis* seedlings (Jiang et al. 2005). An increase of the cAMP content and adenylate cyclase activity was observed in *Hippeastrum* bulbs as a defense response against mechanical wounding and pathogen attack (Grzegorzewska et al. 2012). In cell culture of *Cupressus lusitanica*, cAMP is engaged in β-thujaplicin accumulation and in ethylene biosynthesis, which play an important regulating role in yeast elicitor-induced β-thujaplicin

accumulation (Zhao et al. 2003). More recently, it was indicated that the cAMP signaling pathway is involved in the plant response to *Fusarium verticillioides* (Choi and Xu 2010). Cyclic GMP also plays a very important role in plant defense against pathogens, including induction of the expression of a phenylalanine ammonia lyase gene in tobacco cells (Durner et al. 1998) and the transcription of the defense genes in tobacco (Pasqualini et al. 2009). Accumulation of cGMP was observed in *Arabidopsis* leaves following *Pseudomonas syringae* infection (Meier et al. 2009).

7 Methods for Determination of cAMP and cGMP in Plants

The first data on the occurrence of cyclic nucleotides in higher plants were considered controversial. Difficulties in the assay of cyclic nucleotides in the plant material resulted from low sensitivity of the methods used in those experiments. These methods have been adopted from the research of cyclic nucleotides in animal tissues, which contain significantly more cyclic nucleotides than plant cells, and extracts from animal cells do not contain secondary metabolites. The critical reviews were interrupted by using highly sensitive methods such as mass spectrometry (Newton et al. 1980, 1984; Newton 1996), hyphenated liquid chromatography, and electrospray mass spectrometry. In these methods, it is possible to quantify cyclic nucleotides as low as 25 femtomoles in plant extracts (Witters et al. 1996, 1997; Ehsan et al. 1998).

Several methods have been developed to measure cAMP and cGMP. Among them are enzymatic methods (Kuo and Greengard 1970), the widely applied protein-binding assay method (Gilman 1970), and the immunoassay method. Gilman's (1970) method is based on the competitive replacement of the unlabeled nucleotide, which is present in the complex containing cNMP-binding protein in the experimental sample with 8-^{3}H-cNMP. The immunoassay technique is based on immunological detection of ^{125}I-iodinated tracers. Nowadays, the commonly used methods are radioimmunoassay (RIA) (Rosenberg et al. 1982) and enzyme immunoassay (EIA) (Harper and Brooker 1975). RIA is based on the primary binding of the standard antigen with antibodies. In the next stage, the unlabeled antigen is added to the generated complex, which competitively supplants the radioactive label. On the basis of a decrease in the radioactivity observed, a measurement is made according to the quantity of the bound antigen. EIAs, which do not require radioactive markers, have been developed. This method is also based on the principle of competitive replacement of cNMP–enzyme conjugate bound with antibodies with cNMP present in the sample (Lomovatskaya et al. 2005, 2011). Another method which allows detection of cAMP and adenylate cyclase is the combination of cytoenzymological and immunocytochemical techniques (Witters et al. 2005). Cellular cGMP in plant cells and tissues can be measured by using the endogenous fluorescent reporter FlincG. In this method, cGMP was detected in real-time in vivo and noninvasively (Isner and Maathuis 2011).

8 Conclusions and Future Perspective

Summarizing the data, cAMP and cGMP can be directly involved in many plant growth and developmental processes and in numerous plant responses to biotic and abiotic stresses. The function of cAMP and cGMP in plants ranges from the modification of cyclic nucleotide-gated ion channels to the regulation of transcription of many cyclic nucleotide responsive genes. To understand the role of cyclic nucleotides and adenylate cyclases in plant functions and in plant stress responses, it will be necessary to perform simultaneous analysis of ecophysiological, physiological, biochemical, and metabolic data obtained from wild-type, mutant, and knockout lines.

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Chapter 7
Breeding and Transgenic Approaches for Development of Abiotic Stress Tolerance in Rice

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1 Introduction

Rice is supporting life of greater number of people living in Asia since it was domesticated between 8,000 and 10,000 years ago (Greenland 1997). It is a source of income for more than 100 million householders around the world (IRRI 2002). Rice is a staple food of almost half the world's population and is a main crop grown by more than 50 % of world's farmers. This crop has shaped the cultural, social, and economic development in Asia. Rice is critical for survival of large population; however, its production is often disturbed by several abiotic factors such as water, high salt, and temperature. Additionally, changing climate scenario has further accelerated the frequent occurrence of abiotic stresses, particularly drought and high temperatures. Nearly 22 % of the agricultural land is saline globally (FAO 2004), while drought-affected areas are now expanding which is anticipated to upsurge further (Burke et al. 2006).

Abiotic stresses remain the utmost challenge to crop production, estimated to reduce yields loss of more than 50 % for most crop plants (Boyer 1982; Bray et al. 2000). Abiotic factors badly affect the growth and productivity of plants by triggering array of morphological, physiological, biochemical, and molecular responses. In most of the times, multiple abiotic stresses affect plants growth in combination. For instance, heat stress and drought are usually encountered together that can further be exacerbated by mineral toxicities. In such cases, plants exhibit overlapping biochemical and molecular response to different stress factors. It is understood that plants have various mechanisms that facilitate them to thrive against environmental factors. However, these mechanisms are not very well understood in majority of the agriculturally important plants. Hence, deciphering the fundamental mechanisms of

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plant abiotic stress responses and the improvement of tolerant crops has gained much attention in last few years.

Abiotic stress induces altered expression of several genes in plants, resulting in increased or decreased accumulation of certain metabolites and proteins, playing an important role in adjusting the plants to stress conditions. Transcriptomics of abiotic response in plants revealed that although several genes were differentially expressed in response to different abiotic factors, they probably regulate similar pathways of stress response (Ozturk et al. 2002). Genes associated with the key steps of the abiotic stress response have been identified and characterized using functional genomics approaches. More precisely, the finding of ABA receptors, advancement in current knowledge of the gene regulation at transcript and protein level, and further research on hormone interactions under environmental stimuli have abridged the understanding of plants response to stress at molecular level.

A major bottleneck towards development of abiotic stress-tolerant crops through breeding or transgenic approaches has been to understand the changes at biochemical, molecular, and cellular level that take place during to stress. There have been numerous efforts to breed rice crop using traditional approaches for improved stress tolerance; however, a very limited success could be gained. Landraces and related wild species of rice demonstrate significant level of abiotic stress tolerance; however, the wild variation has not been exploited to the extent in modern rice varieties. The identification of molecular markers using advanced genomic tools has accelerated the breeding programs for abiotic stress tolerance in rice. The use of emerging high-throughput technologies to identify key genomic loci regulating stress response and phenotyping for allelic variation in the wild and landrace has further accelerated the attempts towards development of abiotic stress tolerance. The outcomes of these genomic technologies can be utilized through conventional breeding or genetic transformation depending on circumstances. The physiological and biochemical functions of several genes have been explored in *Arabidopsis* and rice which have encouraged using beneficial genes for genetic improvement of different crops by molecular breeding or transgenic approaches.

Transgenic approach has certain advantages over traditional breeding program, as manipulation of genes is possible into novel permutations. Here, genes can be transferred to a new species where they are not present naturally and also can be expressed ectopically. The technology has proven that major crop species including rice can be transformed with genes from any biological sources. Several efforts have been made towards transferring some of the important genes imparting stress tolerance to crop species. Transgenic plants can be used as sources of new variation in germplasm and can be further utilized in various breeding programs for development of stress-tolerant cultivars. In addition, the technology has facilitated towards understanding the genes functions in vivo which helps to characterize the gene and protein networks imparting abiotic stress tolerance. The book chapter provides a compilation of traditional and molecular breeding approaches of abiotic stress tolerance in rice. A brief account of transgenic technology used for development of abiotic stress-tolerant rice is also given.

2 Conventional Breeding Approaches for Abiotic Stress Tolerance

The main objective of conventional plant breeding for abiotic stress tolerance is to increase the favorable alleles that contribute towards stress tolerance in a plant genome. The stress resistance genes could be brought to the cultivars from germplasm collections including wild relatives or cross-compatible relatives of crops which exist in extreme stress conditions. The conventional plant breeding methods for both self- and cross-pollinated crops have been described in various text books (e.g., Allard 1999). In the commonly used pedigree method, selecting desirable plants begins in the early segregating generations with high heritability of major genes governed traits. However, for traits of low heritability, like abiotic stress tolerance governed by poly or minor genes, selection is possible only in later generations when a line becomes homozygous. The screening under artificially created stress conditions or naturally existing stress environments is an essential selection criterion for identification of stress tolerant lines. Thus, breeding for abiotic stress tolerance involves considerable time of 5–10 years for identification of elite stress-tolerant lines and is very expensive. Some of the conventional breeding methods which are commonly practiced for abiotic stress tolerance in rice are listed below:

1. Selection: screening for stress-tolerant cultivars under targeted environments.
2. Exploiting variation already present in the existing genotypes/varieties by backcross breeding and pedigree method of breeding/single seed descent method.
3. Interspecific hybridization to raise tolerance level of sensitive varieties.
4. Generating variation within existing crops using recurrent selection.
5. Wide hybridization—breeding with stress: tolerant wild relatives.
6. Ideotype breeding: breeding for specified individual traits which ultimately contribute towards increasing the genetic yield potential.
7. Mutation breeding: breeding by utilizing variations created by naturally or artificially induced mutations.

Traditional breeding has contributed significantly to abiotic stress tolerance, and a few abiotic stress-tolerant rice varieties have been identified/developed and released in different countries (Table 7.1). But, unlike biotic stress resistance where a single gene conferred resistance can effectively combat the pest/disease, abiotic stress tolerance is complicated due to the involvement of minor or poly genes.

2.1 Limitations of Conventional Breeding Approaches

- Applying uniform level of stress over a field is one of the major limitations in screening large number of breeding lines for abiotic stress tolerance.
- Great skills and more investments are needed to stimulate required stress conditions for breeding for abiotic stress tolerance.

Table 7.1 Traditional and recently released rice varieties for abiotic stress tolerance by conventional breeding

Abiotic stress-tolerant rice varieties	Country
Drought	
Vandana, Bala, Dehula, Sahbhagi dhan	India
Azucena, Sahod Ulan, Salumpikit	Philippines
Morebereken	Guinea
Nam Sagui 1	Thailand
N22(Nagina 22), Sukha dhan 1, Sukha dhan 2, Sukha dhan 3,Tarharra 1	Nepal
Salinity	
Pokkali, Cheriveruppu, Nona Bokra, SR26B, Damodar, Getu, CSR23	India
Ketum bar	Indonesia
Khao seetha	Thailand
Soc Nau	Vietnam
BRRI dhan 47, BINA dhan	Bangladesh
Salinas 1/NSICRc 182, NSICRc 172	Philippines
Submergence	
FR13A, Kurkaruppan, FR13B	India
Goda heenati, Thavalu	Sri Lanka
Heat	
Kasalath	India
N22	Nepal
Cold	
Koshihikari	Japan
Silewah	Indonesia
Padi Labou Alumbis	Malaysia

- Germplasm accessions with abiotic stress tolerance may not be directly useful to farmers. The genes conferring abiotic stress tolerance must be introgressed into improved cultivars for releasing it for cultivation which takes about 8–10 years.
- The target environment for screening abiotic stress tolerance is unlikely to pose combination of stresses for developing stress-tolerant varieties.

Therefore breeding for abiotic stress tolerance is more tedious, time consuming, and expensive. Alternatively, by deploying marker-assisted selection (MAS) strategy, breeders could select stress-resistant plants via linked markers which were not possible earlier by conventional breeding approach.

3 Molecular Breeding Approaches for Abiotic Stress Tolerance

Conventional breeding efficiency and precision could be improved manifolds by using DNA markers via marker-assisted selection (MAS) (Collard and Mackill 2008). The marker-assisted selection is a process of selecting desirable plants via makers

linked to gene(s) of interest. In rice, several types of DNA markers are being used like hybridization-based markers such as restriction fragment length polymorphisms (RFLPs) and PCR-based markers such as random amplified polymorphic DNA markers (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRS) or microsatellites, and single nucleotide polymorphisms (SNPs). With the help of these molecular markers, now it is feasible to track both major genes governed traits and quantitative traits. The main advantages of using molecular markers in plant breeding as described by Jena and Mackill (2008) are listed below:

1. Time saving: DNA isolation can be done from any plant part like seeds, leaves, etc. In rice good quality DNA can be obtained from any plant tissue. A larger number of crosses can be avoided by knowing the trait information via linked markers before plant maturity.
2. DNA markers are consistent in their expression and are not influenced by environmental factors.
3. They are highly efficient in identification of desirable homozygous lines in early segregating generations itself via codominant markers. Codominant markers are able to differentiate homozygous and heterozygous plants by amplification pattern.
4. Cost-effective: Using DNA markers, breeders avoid evaluating a large number of crosses in hectares of land, and thus it saves space and labor costs.
5. DNA markers linked to polygenic traits allow breeders to select polygenic traits or QTLs as a single Mendelian factor which was previously not possible by conventional breeding.
6. Biosafety: Screening for pest and disease resistance is possible without inoculating or contaminating plants with pest or disease cultures either in the field or green house.

3.1 The Key Molecular Breeding Strategies for Improving Abiotic Stress Tolerance in Crop Plants

Varshney et al. (2011) described the following molecular breeding strategies:

- The most important step is the development of large numbers of molecular markers for abiotic stress tolerance and simultaneous development of marker platforms like SSRs (simple sequence repeats) and SNPs (single nucleotide polymorphisms) for genotyping.
- Use of genetically and phenotypically divergent parents for developing initial mapping populations.
- Identification of QTLs or markers associated with abiotic stress tolerance traits with the help of linkage mapping or association mapping.
- The validation of identified QTLs/markers in different mapping populations or in tolerant genotypes.

- Selection of appropriate molecular breeding strategy like marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS), or genome-wide selection (GWS) to develop superior abiotic stress-tolerant cultivars.

In this section, breeding and molecular breeding strategies for specific abiotic stresses have been summarized.

3.2 Drought

Drought is one of the major abiotic stresses limiting rice production, especially in rainfed areas across Asia and sub-Saharan Africa. Due to increasing water scarcity and global climate change, drought tolerance is considered to be one of the major objectives of breeding programs of rice which is a highly water-demanding crop. Depending on the timing and severity of drought in relation to the crop developmental stage, drought can be categorized into three types: vegetative, intermittent, and terminal (Chang et al. 1979; Fischer et al. 2003; Kamoshita et al. 2008). Of these three types, the occurrence of terminal drought stress at the reproductive stage (3 weeks before anthesis) during the cropping season directly affects grain yield and has devastating consequences (Fukai et al. 2001; Lanceras et al. 2004; Tsubo et al. 2006; Venuprasad et al. 2009). The drought stress intensity can be classified as moderate if the yield reduction is 30–65 % as compared with the irrigated control, severely stressed if it exceeds 65 %, and mildly stressed at less than 30 % (Verulkar et al. 2010).

The conventional breeding approach for drought tolerance involves selection of crop plants for yield and yield-contributing traits in drought-affected environments. Through selection of secondary traits of drought tolerance like root characters, leaf water potential, relative water content, and osmotic adjustment (Fukai et al. 1999; Price and Courtois 1999; Jongdee et al. 2002; Pantuwan et al. 2002), the expected yield levels could not be achieved. However, few studies explained that under well-managed field conditions, grain yield levels need to be compared under drought stress and non-stress conditions; hence direct selection for grain yield under drought stress can be practiced to achieve increased yield. Thereafter more emphasis has been given for direct selection of grain yield instead of secondary traits for drought tolerance (Kumar et al. 2008; Venuprasad et al. 2008). Through direct selection of grain yield under drought stress conditions, many promising lines were identified for both rainfed upland and lowlands (Verulkar et al. 2010; Mandal et al. 2010). Kumar et al. (2008) stated that many drought tolerance trials fail to impose severe stress conditions and thereby not able to select drought-tolerant lines precisely. Ouk et al. (2006) explained that the slow progress in drought tolerance breeding is mainly due to lack of effective selection criteria and low heritability of grain yield under drought stress.

Recent research developments in biotechnology and availability of advanced genomic tools enhanced interest in drought tolerance breeding among plant breeders. Drought is one of the difficult traits to be controlled by conventional plant breeding

approach and most ideal trait to be improved through marker-assisted selection. The MAS for improving drought tolerance in rice is important and useful since the naturally occurring drought stress is sporadic in time and intensity (Jongdee et al. 2002). For effective implementation of MAS in a rice-breeding program for drought tolerance, the targeted gene(s)/QTLs must have a large effect on grain yield under stress and be consistently expressed in different genetic backgrounds. Therefore, it is imperative to identify background-independent drought-tolerant QTLs from rice germplasm resources and corresponding tightly linked molecular markers for developing drought-tolerant cultivars by MAS.

The major steps in QTL mapping for drought tolerance (Price et al. 2002a) are given below:

1. Identify parents which possess extreme phenotypes for drought tolerance (ideally one of the parents should have high drought tolerance, while the other should be highly drought susceptible).
2. Cross the selected parents to produce segregating progenies (F_2, selfed progenies advanced through single seed descent, i.e., recombinant inbred lines, backcross-derived lines, advanced backcross lines, near isogenic lines, and double haploids) of 70–300 plants or lines (100 lines is generally considered the lower limit).
3. Phenotype the population along with their parents for drought tolerance in 2–3 diverse locations/seasons.
4. Screen the parents for marker polymorphism using PCR-based markers, preferably SSR markers (commonly 70–200 markers).
5. Using the markers polymorphic between the parents, screen all the segregating plants for identification of their genotype with respect to each polymorphic marker.
6. Construct a genetic map based on genotype data using computer programs like Mapmaker or Joinmap.
7. Identify markers (genomic regions) which display association with trait phenotype (drought tolerance/susceptibility) using interval mapping techniques like Mapmaker/QTL, QTL Cartographer, or PLABQTL. Also, it is necessary to identify how much proportion of target trait is explained by each associated QTLs/markers.
8. Validate the marker-trait associations in alternate mapping populations. Also, validate how much each of the QTL explains trait phenotype.
9. Once major QTLs and their linked markers are identified and validated, marker-assisted introgression of the QTLs into elite genetic background by recombination breeding or backcross breeding can be practiced.

Similar to conventional breeding, in the molecular breeding also, initial efforts were devoted to identify secondary traits responsible for drought tolerance like root characters and osmotic adjustment, etc. (Yadav et al. 1997; Kamoshita et al. 2000; Babu et al. 2003). Because QTLs for secondary traits are not directly linked to yield increase under drought, thus marker-assisted selection of QTLs linked to secondary traits have not been much encouraging in improving yield under drought conditions.

Table 7.2 QTLs identified for drought tolerance in rice

QTL	Chr	Marker interval/linked markers	Cross	Population	Environment	Trait targeted[a]	References
qDTY3.1 *qDTY2.1*	3	RM 416 RM 324	Apo × Swarna	$F_{4:5}$	Dry-season screening	GY GY	Venuprasad et al. (2009)
QTL 12.1	12	RM28048–RM511	Vandana × Way Rarem	F_3	Greenhouse	GY	Bernier et al. (2009)
qDTY1.1	1	RM11943–RM431	N22 × Swarna, N22 × IR64, and N22 × MTU1010	$F_{3:4}$	Dry-season screening	GY	Vikram et al. (2011)
qDTY2.3	2	RM1367–RM573	Vandana × Way Rarem	$F_{3:5}$	Upland severe stress	GY GY	Dixit et al. (2012)
qDTY3.2	3	RM523–RM545				HI PH	
qDTY3.2	3	RM7332–RM523					
qDTY1.1	1	RM315–RM431					
qDTY3.2	3	RM569–RM517	IR77298-5-6-18/2 × Sabitri	BC_1	Lowland severe stress	GY GY	Yadawa et al. (2013)
qDTY3.2	3	RM569–RM517				GY DFF	
qDTY3.2	3	RM569–RM517					
qDTY3.1	3	RM517–RM411					
qDTY12.1	12	RM28166–RM28199	IR74371-46-1-1 × Sabitri	$BC_1F_{3:5}$	Lowland reproductive stage	GY	Mishra et al. (2013)
QFgp9	9	RM242–RM201	IR 64 × Tarom Molaei	BC_2F_8	Dry season	FGP GYP	Wang et al. (2013)
QSf11	11	RM286–RM332	Teqing × Tarom Molaei			SF PN	
QGyp3c	3	RM130–RM570					

[a]Thousand-grain weight (TGW), panicle number per plant (PN), filled grains per panicle (FGP), seed fertility (SF), grain yield per plant (GYP), grain yield (GY), harvest index (HI), plant height at maturity (PH), days to 50 % flowering (DFF)

The QTL mapping for grain yield and yield-related traits pertaining to drought tolerance has enhanced the understanding about the genetic basis of this complex trait (Lanceras et al. 2004; Xu et al. 2005; Gu 2007; Yue et al. 2008; Venuprasad et al. 2009). QTLs mapped for drought tolerance are listed in Table 7.2. However, very few QTLs are actively targeted in breeding programs because most QTL have not been validated in different genetic backgrounds and environments (Price et al.

2002b; Li et al. 2005; MacMillan et al. 2006; Yue et al. 2008), which hampers the pace of drought tolerance breeding.

A study by Bernier et al. (2007) reported the detection of a major QTL with larger effect for drought tolerance (*qDTY 12.1*) in a Vandana/Way Rarem population under field conditions. This drought-tolerant QTL (*qDTY 12.1*) has been mapped between two SSR markers RM28048 and RM511 of 10.2 cM regions of chromosome 12 and contributing for 51 % of genetic variance. A major QTL from a cross of CT9993/IR62266 under lowland drought stress was mapped on chromosome 1 for grain yield which explains 32 % of the total genetic variance (Kumar et al. 2007). Venuprasad et al. (2009) also identified a major QTL for grain yield under drought stress on chromosome 3 which accounts 36 % of the genetic variance. Vikram et al. (2011) identified a major QTL named *qDTY1.1* for grain yield under drought stress in three mapping populations, viz., N22/Swarna, N22/IR64, and N22/MTU1010 on rice chromosome 1 flanked by SSR markers RM11943 and RM431. In a combined 2-year analysis, *qDTY1.1* showed additive genetic variance of 29.3 %, 24.3 %, and 16.1 % of mean yield in N22/Swarna, N22/IR64, and N22/MTU1010, respectively, under drought stress. In general, QTLs from specific genetic background disappear completely in different genetic backgrounds (Collins et al. 2008). The QTL *qDTY1.1* is the first reported genetic background-independent and consistent QTL expressed under both drought stress and non-stress conditions (Vikram et al. 2011). Another three genetic background-independent QTLs (*QTgw7, QSf7, and QFgp11*) reported by Wang et al. (2013) have also been effectively utilized in MAS—for drought tolerance.

In the past two decades, though QTL analysis in rice played an important role in identifying genomic regions responsible for drought tolerance, MAS-derived drought-tolerant varieties could not reach the farmers' fields yet. The challenge of breeding for drought-tolerant rice cultivars, especially at the reproductive stage, can be met with designed QTL pyramiding (DQP) (Wang et al. 2013) by pooling the favorable large effect drought tolerance QTLs into a widely adapted rice cultivars for both lowland and upland conditions. The major QTL (*qtl.12.1*) with larger effects on grain yield under water stress with three more other QTLs (*qtl3.1, qtl1.1, qtl 9.1*) for drought tolerance are being pyramided for significant increase of drought tolerance in rice varieties. Further, phenotyping techniques are being improved to establish platforms for large-scale, precise measurements of yield and related traits under drought.

3.3 Submergence Tolerance

In Asia, over 22 million hectares of rice area is under the threat of submergence that ranges from flash flood to deepwater situations (Mackill et al. 2012). These stress conditions due to submergence are highly unpredictable, and they can last for short duration (7–14 days) to several months that results in partial or complete failure of the crop. In case of rice, short-term flooding commonly known as flash flooding is

the most common situation. The submergence stresses observed in rice crop are categorized as:

- Submergence at the time of germination
- Flash flooding during vegetative stages
- Flooding at vegetative and reproductive stage, leading to stagnant water

FR13A, FR13B, Goda Heenati, Thavalu, and Kurkaruppan are some of the local rice landraces that were used as sources of submergence tolerance in most of the breeding and genetic studies (Vergara and Mazaredo 1975; HilleRisLambers and Vergara 1982). At IRRI, these landraces were used for development of few semi-dwarf submergence-tolerant varieties (Mohanty and Chaudhary 1986; Mackill et al. 1993, 1996). However, these submergence-tolerant varieties could not be widely adopted by rice farmers owing to some undesirable traits like poor grain quality and lower yields acquired from donor landraces (Mackill et al. 1996, 2012).

Several studies conducted in the past to understand the genetic control of submergence tolerance in rice suggested both simple and quantitative inheritance for tolerance (Suprihanto and Coffman 1981; Mohanty and Khush 1985; Sinha and Saran 1988; Haque et al. 1989). A highly submergence-tolerant *indica* cultivar FR13A reported to withstand up to 2 weeks of complete submergence (Xu and Mackill 1996; Harushima 1998; Xu et al. 2000; Chen 2002). Xu and Mackill (1996) identified a major QTL associated with submergence tolerance in the cross of IR4093126 (an *indica* tolerant line) X PI543851 (a sensitive *japonica* line) which contributed for 70 % of the phenotypic variation in the mapping population and designated this QTL as *Sub1* (submergence tolerance on chromosome 1). The most submergence-tolerant line, namely, FR13A, contributed to the donor line for this *Sub1* QTL (Mackill et al. 1993). Similarly, the other minor QTLs affecting this submergence tolerance trait were mapped on chromosomes 1, 2, 5, 6, 7, 10, and 11 (Nandi et al. 1997; Toojinda et al. 2003). Interestingly, Singh et al. (2011) reported that *Sub1* does not confer any tolerance to flooding-induced stagnant water. Several other QTLs identified for submergence tolerance are presented in Table 7.3.

3.3.1 Marker-Assisted Backcrossing of *Sub1* Locus

Since *Sub1* is a major locus explaining ~70 % of the phenotypic variation, several attempts were made to transfer this locus to different cultivars. In 2003, IRRI initiated a program to introduce the *Sub1* QTL into six mega-varieties, like Swarna, Samba Mahsuri, BR11, IR64, CR1009, and TDK1, using marker-assisted backcrossing (MABC) approach. The main objective of the MABC program for *Sub1* was to transfer only the small segment of chromosome 9 harboring the *Sub1A* gene while simultaneously recovering all the genetic background of the mega-varieties used as recurrent parents (Collard and Mackill 2008; Mackill et al. 2012). Using this approach, a small genomic region containing the *Sub1* locus was introgressed into each of the six modern high-yielding varieties within 2–3 years. More recently, introgression of *Sub1* into two more varieties, PSB Rc18 of Philippines and Ciherang

Table 7.3 Submergence tolerance-related QTLs identified in rice

QTL	Chr	Flanking markers	Cross	Population	Targeted trait	References
qSUB 1 q	9	RM219–RM464A	PI543851 × IR4093126 Swarna × (IR49830-7) FR13A	BC_3F_2	Survival under submergence	Xu and Mackill (1996) and Neeraja et al. (2007)
qSUB1.1	1	MDC17–RM12168	IR72 × Madabaru	$F_{2:3}$	Survival under submergence	Septiningsih et al. (2012)
qSUB2.1	2	RM6318–RM2578				
qSUB9.1	9	RM23911–RM23966				
qSUB12.1	12	RM511–RM463				

of Indonesia, was also completed. The size of the introgressed fragment ranged from 1.2 to 6.3 Mb (Neeraja et al. 2007; Septiningsih et al. 2009; Iftekharuddaula et al. 2011). During submergence, *Sub1* introgressed lines showed a yield advantage of 1–3.5 t/ha based on the duration and flood conditions. There was a general delay in flowering in all the genotypes after submergences as expected, and the surviving plants took additional time to recover and resume normal vegetative growth (Singh et al. 2009). Several varieties containing *Sub1* locus were developed and some of them were released in different countries (Table 7.4). In addition to the *Sub1* locus, another novel major QTL locus *qSub1.1* on chromosome 1 for submergence tolerance with 52 % phenotypic variance has been identified from new sources (Septiningsih et al. 2012). The pyramiding of these major and minor QTLs is in progress to improve submergence tolerance.

3.3.2 Multiple Abiotic Stress Tolerance Along with *Sub1* Tolerance

The ideal rice genotype for coastal areas needs tolerance to both salinity and submergence tolerance for wider adaptability. To develop rice varieties for salt and submergence tolerance, Marcado et al. (2010) crossed the progenies obtained from the crosses of IR66946-3R-178-1-1 X Cheriviruppu (salinity tolerant) and IR82809-237 X IR82810-407 (submergence tolerance) to derive lines of both submergence and salinity tolerance. These derived lines were subjected to 2 weeks of submergence under saline conditions of EC 12 dS m-1. The survived plants were genotyped for the presence of *Saltol* and *Sub1 QTLs,* and homozygous individuals were advanced up to F_6 generation. They reported that combining *Saltol* and *Sub1* in one genetic background seems feasible without any negative impacts on agronomic traits, and this will help the breeders to develop wider adoptable varieties for coastal regions. The introgression of *Sub1* gene into drought-tolerant varieties is

Table 7.4 *Sub1* introgression lines evaluated in South and Southeast Asia (Ismail et al. 2013)

Name	IR designation	Status
Swarna-Sub1	IR 05 F102	Released in India and Indonesia (2009), Bangladesh (2010), and Nepal and Myanmar (2011)
Samba Mahsuri-Sub1	IR 07 F101	Released in Nepal (2011). Under final evaluation in India and Bangladesh
IR64-Sub1	IR 07 F102	Released in the Philippines and Indonesia in 2009. Under evaluation in India and Bangladesh
CR1009-Sub1	IR 07 F291	Under advanced evaluation in India
BR11-Sub1	IR 07 F290	Released in Bangladesh (2010). Under final evaluation in India
Ciherang-Sub1	IR 09 F436	Released in Indonesia (2012). Under advanced evaluation in India and Bangladesh
TDK1-Sub1	IR07F289	Under advanced evaluation in Lao PDR
PSB Rc18-Sub1	IR 09 F437	Under evaluation in the Philippines
BT7 -Sub1		Under advanced stage of evaluation in Vietnam

under progress (Verulkar et al. 2010). Under IRRI collaborative project, several popular Indian rice varieties are being introgressed with *Sub1*, *Saltol,* and QTLs for drought, viz., *DTY 1.1*, *DTY 2.2*, *DTY 3.1,* and *DTY 9.1,* and their combination based on ecology (Thomson et al. 2010b; Vikram et al. 2011). Currently, under the IRRI-India collaborative STRASA (Stress-Tolerant Rice for Africa and South Asia) project, a top priority is given to develop new varieties with *Sub1* and stagnant floodwater tolerance with the hypothesis that *SNORKEL* genes or QTL_S identified from deepwater rice can be deployed for enhancing stagnant floodwater tolerance.

The study of submergence tolerance using *Sub1* locus is the first successful example of introgression of a major QTL for abiotic stress tolerance in rice with direct benefits to farmers. The success of *Sub1* will pave a way for molecular breeding strategies of crop improvement program for abiotic stress tolerance.

3.4 Salt Tolerance

Salinity is the most widespread soil problem limiting rice production throughout the world. Approximately 30 % of rice-growing area in the world is affected by salinity (Prasad et al. 2000; Takehisa et al. 2004). Salinity and other abiotic stresses have made millions of hectares of humid regions of South and South East Asia unfit for rice cultivation (Boje-Klein 1986). Though early vegetative and reproductive stages of rice are considered to be sensitive to salinity, it can survive well under saline conditions by leaching salts from top soils due to standing water in the rice fields. In general, rice is a recommended crop for desalinization of saline lands. Salinity problem is always associated with mineral deficiencies and toxicities. Due to poor quality irrigation, water inland rice crop is subjected to alkalinity, P and Zn deficiencies. On the other hand, acidity and P, Zn, and Al toxicities are common problems

in coastal regions by tidal water intrusions (De Datta et al. 1993). Also, most of the saline soils are prone to other abiotic stresses like submergence and drought. Therefore, improving salt tolerance is one of the major objectives of rice-breeding program.

Through the conventional breeding programs, notable success has been made in developing high-yielding, salt-tolerant rice varieties, utilizing the available salt tolerance, widely adapted sources such as Pokkali and Nona-Bokra as well as rice salt-tolerant wild relatives. The reviews of rice breeding for salt tolerance by several researchers (Akbar et al. 1986; Maas and Hoffman 1977; Mori and Kinoshita 1987; Gregorio et al. 1997) explained various reasons for slow progress in salinity tolerance breeding like complex tolerance mechanisms, lack of adequate screening techniques, limited knowledge on genes involved in salt tolerance, low heritability and less expressive nature of the salt tolerance trait, low selection efficiency, lack of understanding about salinity, and environmental interactions. This could be overcome by the use of molecular marker technology to analyze the QTLs associated with salt tolerance especially in identification of specific regions of chromosomes that will help significantly to improve the efficiency of selection in the breeding programs (Ammar et al. 2007).

In rice, QTL analysis for salt tolerance (Wang et al. 2012) on sodium uptake, potassium uptake, and ion balance is used for genetic analysis through MAS. For salt tolerance of rice, a large number of QTLs have been reported by many researchers (Table 7.5). Bonilla et al. (2002) mapped the most important salt tolerance QTL *Saltol* on chromosome 1 which accounts for high potassium and low sodium absorptions and a low sodium/potassium ratio under salinity stress. The *Saltol* locus is considered to be a promising QTL for marker-assisted selection of salt tolerance trait of rice.

3.4.1 Marker-Assisted Backcrossing Scheme for *Saltol*

To develop a marker-assisted backcross breeding strategy for *Saltol* locus, Thomson et al. (2010a) reported the most useful SSR markers by testing a large number of molecular markers in the *Saltol* region. These markers include RM8094; RM3412; telomere flanking markers like RM1287 and RM10694; and centromere flanking markers such as RM493 and RM10694 in the salt tolerance QTL region. This study also suggested markers for the negative selection of *Saltol* region, with the help of SSR markers located above QTL by RM490, RM562, and below QTL region by RM7075. The MABC program for salt tolerance was developed using FL478 as donor.

Salt tolerance QTL *SKC1* was cloned using map-based cloning approach which encodes for sodium transporter and maintains the potassium homeostasis under salt stress conditions (Ren et al. 2005). The QTLs identified in different salt-tolerant varieties are given in Table 7.5. With the available knowledge on QTL cloning and fine mapping of *Saltol*, several other salt tolerance QTLs would certainly accelerate the development of salt-tolerant varieties in rice.

Table 7.5 QTLs identified for salt tolerance in rice

QTL	Chr	Marker interval	Cross	Population	Environment	Targeted trait	References
Saltol	1	RM23–RM140	IR29/Pokkali	RIL	Hydroponic screening	Seedling shoot Na-K ratio	Bonilla et al. (2002)
QTL 1	1	RM84–RM259	CSR27 × MI48	F_3	100 mM NaCl	Seedling stage	Ammar et al. (2007)
QTL 2	2	RM572–RM294					
QTL 3	3	RM5320–RM3648					
QTL 4	4	RM3648–RM280					
QTL 43	3	RM563–RM186					
QTL 45	5	RM233B–RM334					
qSTR6	6	RM3727–RM340	Tarommahali × Khazar	$F_{2:3}$	Hydroponic screening	Na^+/K^+ ratio Dry mass of shoot	Sabouri et al. (2009)
qSTR-3a	3	RM1022–RM6283					
qSTR-3b	3	RM6832–RM7389					
qDM-3	3	RM1022–RM6283					
qDM-8	8	RM4955–RM152					
qSKC-1	1	RM8094–RM10825	IR29/Pokkali	RIL	Under controlled conditions	Shoot K^+ concentration	Thomson et al. (2010a, b)
Saltol 1-1	1	RM8094–RM493	BR40 × NSIC Rc106	$F_{2:3}$	Salinity stress of 12 dSm-1 (EC)	Seedling stage	Islam et al. (2011)
Saltol 8-1	8	RM25–RM210					
Saltol 10-1	10	RM25092–RM25519					
qSKC1	1	RM7341–RM7419	Jiucaiqing × IR26	RILs, $F_{2:9}$	100 (mM) NaCl treatments	Shoot K^+ concentration	Wang et al. (2012)

3.5 Heat Stress

Owing to changing climate and increased average temperature of the globe, heat stress has been a major factor of disaster which affects the crop cultivation and productivity. In rice, a high temperature of 5 °C beyond threshold level at flowering can induce floret sterility and higher yield losses (Osada et al. 1973; Stake and Yoshida 1978; Matsushima et al. 1982), with the maximum yield loss up to 80 % (Li 2003). A recent study indicated that the rice grain yields have declined by 10 % for every 1 °C increase in night temperature (Peng et al. 2004). Global annual average day and night temperature for the period between 1979 and 2003 has increased by 0.35 and 1.13 °C respectively. The trend of this global climate warming will continue (Salinger 2005; IPCC 2007) and constitutes a more serious menace for rice production (Battisti and Naylor 2009). In addition, it is estimated that there are approximately 4 mha of rice under potential threat of high temperature worldwide, and the area covers 14 main rice production areas in Asia, Africa, and United States (Mutsuo 1990). Therefore, new varieties with improved heat tolerance are a desperate need for sustainable rice production.

In the conventional heat stress breeding, a major drawback is lack of reliable screening methods and efficient selection criteria to identify heat-tolerant plants. The most commonly used method for selecting heat-tolerant lines is to grow breeding lines in the targeted hot environments and identifying lines with high yield potential (Ehlers and Hall 1998). The glasshouse screening in a controlled temperature environment may facilitate more reliable results than field-level screenings. In rice, heat-tolerant *indica* and *japonica* genotypes were identified by many researchers (Matsui et al. 1997, 2001; Prasad et al. 2006).

Conventional breeding for heat stress tolerance in crop plants has not been very successful because of lack of suitable gene sources in sexually compatible gene pools and complex nature of the heat stress trait. Through molecular breeding approach, efforts have been made to carry out molecular mapping of heat tolerance QTLs at booting, flowering, and grain filling to ripening stages in rice. A total of 52 main effect QTLs and 25 epistatic ones capable of explaining 2.27–50.11 % of phenotypic variance have been identified on loci covering all 12 chromosomes (Table 7.6). The SSR markers RM3735 on chromosome 4 and RM3586 on chromosome 3 were reported to be linked to heat stress tolerance trait (Zhang et al. 2009). The most significant heat-tolerant QTL that can explain up to 50 % of phenotype variance is from Kasalath, an *indica* landrace cultivar from India (Zhu et al. 2006). However, genes that govern the heat tolerance with qualitative effects on both plant growth and development have not yet been identified. Wei et al. (2013) identified HT54 (an *indica* cultivar) that could tolerate high temperature up to 48 °C for 79 h at seedling stage, and its heat tolerance was controlled by a dominant major locus designated as *OsHTAS* (*O. sativa* heat tolerance at seedling stage). This locus was mapped on chromosome 9 within an interval of 420 kb between markers of InDel5 and RM7364. This could be a useful marker for MAS of heat stress tolerance in rice.

Table 7.6 Identified QTLs for heat tolerance in rice

QTL	Chr	Cross	Population	Environment	Trait	References
QTLs HS	1,2,3,4,5,7,8,11	IR-64 × Azuncena	DH population	Field and greenhouse	Seed setting	Cao et al. (2003)
QTLS HS	1,4,7	Nipponbare × Kasalath	BIL	Treated with high temperature	Grain filling stage	Zhu et al. (2005)
QTLs HS	2,3,5	Zhongyouzao 8 × Toyonishiki	$F_{6\text{-}8}$RIL	Field CK Greenhouse HT	Spikelet fertility	Zhang et al. (2008 a, b)
QTLs HT	2,3,8,9,12	T219 × T226	$F_{6\text{-}8}$ RIL	Natural CK Growth chamber HT	Spikelet fertility	Chen et al. (2008 a, b)
QTLs HT	1,2,3,4,8,10,11	Bala × Azucena	F_6 RIL	Growth chamber	Spikelet fertility	Jagadish et al. (2010)

3.6 Cold Tolerance

Among the various abiotic stresses, cold stress is also a dominant limiting factor in rice production in the temperate and tropical high lands globally (Jena et al. 2012). Two major subspecies of *O. sativa,* viz., *japonica* and *indica,* can be distinguished based on their tolerance to cold stress. The *indicas* are more susceptible to cold stress than the *japonicas*. Cold tolerance of *indica* germplasm can be improved by crossing with *japonica* germplasm. Temperature in the range of 15–19 °C during the reproductive stage may lead to spikelet sterility. Low temperature affects microspore development and leads to the production of sterile pollen grains (Satake 1976). Spikelet fertility of rice decreases due to temperature-sensitive booting and reproductive tissues, like premeiotic mother cells, microspores, and pollen grains (Nishiyama 1982; Dai et al. 2002). Low temperatures at the booting stage lead to young microspore degeneration, hypertrophy, tapetal cells disintegration, and diminishing supply of nutrients from the anther walls to the pollens (Hayase et al. 1969; Nishiyama 1976; Satake 1989).

Rice breeders have made efforts to develop more cold-tolerant cultivars. Several cold-tolerant varieties were identified by screening more than 17,000 accessions from IRRI gene bank during 1974–1977, and later their cold tolerance was tested at the booting stage by Satake and Toriyama (1979). It showed that tropical *japonicas*, Silewah and Padi Labou Alumbis, are cold tolerant. Some genetic studies have revealed the complex nature of cold tolerance trait in rice. Nishimura and Hamamura (1993) showed that cold tolerance trait is under dominant digenic control at the reproductive stage, but Nagasawa et al. (1994) identified that cold tolerance trait is governed by polygenes. However, with the help of reported molecular markers and

QTLs, several studies were conducted on different mapping populations for cold tolerance and the identified QTLs are presented in Table 7.7. Saito et al. (1995) identified two QTLs for cold tolerance on chromosomes 3 and 4 at the booting stage in Norin-Pl8 mapping populations. Two cold-tolerant genes (*Ctb1* and *Ctb2*) were identified and fine mapped on the chromosome 4 in the 56 kbp region (Saito et al. 2001). By using different mapping populations like F_2, BC_5F_3, and doubled-haploid populations (Li et al. 1997; Dai et al. 2004; Xu et al. 2008b), several QTLs for cold tolerance were identified. QTLs for cold tolerance were mapped at the reproductive stage on chromosomes 1, 2, 3, 5, 6, 7, 9, and 12 using recombinant inbred lines (RILs) mapping population (Andaya and Mackill 2003a, b). Cold tolerance QTLs on chromosomes 1, 6, and 7 from wild rice introgression lines were identified by Liu et al. (2003). Suh et al. (2010) identified three QTLs for percent seed set under cold stress by using a RIL population derived from a temperate *japonica* crossed with tropical *japonica*. Kuroki et al. (2007) and Ye et al. (2010), using different F_2 populations, mapped two QTLs for cold tolerance, namely, *qCTB8* and *qLTSPKST10.1* on chromosomes 8 and 10.

The cold tolerance mechanism and genetics are still not well understood, probably due to different phenotyping methods followed by researchers and a lack of effective cold tolerance QTLs expressed under various crop growth stages during stress conditions. Several morphological as well as yield traits are affected by cold stress during different growth stages of rice. A reliable phenotyping method for cold tolerance was developed by Suh et al. (2010) through cold-water irrigation during all growth stages in the field and imposing cold-air temperature in the greenhouse. The availability of improved phenotyping method for identification of QTLs/genes for cold tolerance may hasten the development of cold-tolerant rice varieties.

3.7 Major Limitations of Molecular Breeding Approaches for Abiotic Stress Tolerance

Although several QTLs have been identified and validated for abiotic stress tolerance in rice, their utility in development of superior cultivars has been limited success except the identification of a major QTL *Sub1* locus for submergence tolerance which is the first successful introgression of QTL in development of superior cultivars with direct impact on farmers' field. The limited success in molecular breeding for abiotic stress tolerance in rice may be due to the following points:

- The nature of abiotic stress is very complex and changing climatic scenario makes it more complex.
- Usually abiotic stress tolerance is measured using traits like grain yield under stress which is a complex trait driven by different mechanisms and processes; therefore by selecting a single QTL or gene is not sufficient to develop stress-tolerant cultivars through molecular breeding approach.

Table 7.7 Identified QTLs for cold tolerance in rice cultivars

QTL	Chr	Flanking markers	Cross	Population	Environment	Trait	References
qCtb1 and *qCtb2*	4	–	Norin-PL8 × Kiara397	BC_1F_5	Cool water irrigation	Booting stage	Saito et al. (2001)
qCT-7	7	S778	Koshihikari × Adhikari	DH	Running cold-water system	% floret sterility	Takeuchi et al. (2001)
qCT-7	7	OPO20				Culm length	
qCTS12a	12	RM101–RM292	M-202 × IR50	F_6	PGV36 (Controlled Environments Ltd., Winnipeg)	Seedling stage	Andaya and Mackill (2003a, b)
qCTB2a	2	RM324–RM301				Booting stage	
qCTB3	3	RM156–RM214					
qCTB-1-1	1	L169–R1841	Kunmingxiaobaigu × Towada	F_2	Field experiment	Booting stage	Dai et al. (2004)
qCTB-10-2	10	G1010–RM239					
qSCT-3	3	RM156–RM16	Lemont (japonica) × Teqing (indica)	F_{12}(SSD)	Paper-roll tests (growth chamber)	Early seedling stage	Zhang et al. (2005)
qSCT-7	7	RM336–RM10					
qSCT-11	11	RZ53–RM202					
qSCT-3	3	RM156–RM16					
qSCT-11	11	RM202–RM209					
qSCT-1	1	XNpb87-2-C955	Asominori × IR24	RIL	Cold treatment	Three-leaf seedling stage	Jianga et al. (2008)
qSCT-5	5	R569–G1458					
qSCT-6	6	G2028–XNpb386					

qCTB-1-1	1	RM1282–RM3148	Towada×KMXBG	BC_5F_3	Cold environment	Booting stage	Xu et al. (2008b)
qCTB-4-1	4	RM518–RM6770					
qCTB-4-2	4	RM7200–RM821					
qCTB-5-1	5	RM7452–RM7271					
qCTB-5-2	5	RM19106–RM31					
qCTB-10-1	10	RM3590–RM24918					
qCTB-10-2	10	RM2125–RM2887					
qCTB-11-1	11	RM1812–RM332					
qLTSPKST10.1	10	S10010.9–S10014.4	Reiziq×Lijiangheigu	BC_2F_1	Glasshouse ($30/19^0$ C) day/night	Booting stage	Ye et al. (2010)
qPSST-3	3	RM569–RM231	Geumobyeo× IR66160-121-4-4-2	F_8	Cold-water treated plot	Percent seed set	Suh et al. (2010)
qPSST-7	7	RM3767–RM1377					
qPSST-9	9	RM24427–RM24545					
qCTB8	8	RM5647–RM5434	Hokkai-PL9× Hokkai287	F_2 and F_7	Cool water irrigation	Booting stage	Kuroki et al. (2007)

- Lack of accurate, precise, and high-throughput phenotyping facilities especially in developing countries limits our understanding towards abiotic stress tolerance.
- Abiotic stress tolerance traits are mainly governed by small effect QTLs or epistatic QTLs, whereas developing superior stress-tolerant cultivars becomes a formidable task by MABC, where larger backcross populations are required to pyramid small effect QTLs.

Recent molecular breeding techniques like marker-assisted recurrent selection (MARS) and genome-wide selection (GWS) or genomic selection (GS) could be a reliable alternative to overcome the abovementioned problems (Varshney et al. 2009; Tester 2010). However, currently not much progress has been made for abiotic stress tolerance in rice using MARS and GWS approaches.

4 Transgenic Approaches of Abiotic Stress Tolerance

The introgression of genes or QTLs through breeding approaches is a time-taking process and often carries along undesirable traits from the donor parents due to complexity of stress tolerance traits. It is important, therefore, to look for alternative strategies to develop stress-tolerant crops. The transgenic development by the overexpression or silencing of useful genes can be a viable and alternate option to facilitate the efforts towards abiotic stress-tolerant rice development. Also, this has particular advantage when desirable gene is from cross species or other organisms. Further, transgenic provides an option to control the expression of genes in a particular time, tissues, and also the level of expression of the transgene for their best function. This is particularly important as in most of the cases, expression of genes is required in either a specific tissue or growth stage or stress. Various genetic engineering tools have been explored to improve stress tolerance in plants (Allen 1995). There is a plenty of literature available on abiotic stress-associated genes and utilization of those genes for abiotic stress tolerance (Grover and Minhas 2000; Langridge et al. 2006; Gao et al. 2008; Bhatnagar-Mathur et al. 2008; Kolodyazhnaya et al. 2009; Jewell et al. 2010; Qin et al. 2011; Singh et al. 2012; Todaka et al. 2012; Mizoi and Yamaguchi-Shinozaki 2013).

4.1 Utilization of Genes and Promoters for Abiotic Stress Tolerance in Rice

An elaborate account of genes expressed under abiotic stress in various crop species has been given (Langridge et al. 2006; Wahid et al. 2007; Bhatnagar-Mathur et al. 2008). Transgenic rice developed using several such genes for abiotic stress is listed in Table 7.8. Briefly, to achieve the abiotic stress tolerance, following genes have been utilized for transgenic rice development.

Table 7.8 Transgenic rice lines transformed with genes for abiotic stress tolerance

Transgene	Source organism	Trait improved	References
HVA1	Barley	Drought tolerance	Xu et al. (1996) and Babu et al. (2004)
CBF3/DREB 1A	*A. thaliana*	Drought and salinity tolerance	Oh et al. (2005)
SNAC 1	*O. sativa* L.	Drought tolerance	Hu et al. (2006)
HvCBF4	*H. vulgare* L.	Drought, salinity and low temperature stress tolerance	Oh et al. (2007)
Os LEA-3-1	*O. sativa* L.	Drought tolerance	Xiao et al. (2007)
OsCc1	*O. sativa* L.	Drought tolerance	Oh et al. (2009)
TaSTRG	*Triticum aestivum* L.	Salt and drought tolerance	Zhou et al. (2009)
TSRF1	*L. esculentum* L.	Osmotic and drought tolerance	Quan et al. (2010)
OsDREB2A	*O. sativa* L.	Drought and salt tolerance	Cui et al. (2011) and Mallikarjuna et al. (2011)
SbDREB	*Sorghum bicolor*	Drought tolerance	Bihani et al. (2011)
OsSDIR1	*O. sativa* L.	Drought and salt tolerance	Zhang et al. (2008a)
codA	Rice	Salt stress tolerance	Mohanty et al. (2002)
OsWRKY11	Rice	Drought tolerance	Wu et al. (2008)
HRD		Drought tolerance	Karaba et al. (2007)
ZFP252	Rice	Salt stress tolerance	Xu et al. (2008a)
p5cs	Moth bean	Salinity and water deficit stress tolerance	Su and Wu (2004)
OsTPP1	Rice	Salt and cold stress tolerance	Ge et al. (2008)
SOD2	*Schizosaccharomyces pombe*	Salt tolerance	Zhao et al. (2006)
Sod1	*Avicennia marina*	Oxidative and salt stress tolerance	Prashanth et al. (2008)
SNAC2	Rice	Salt stress tolerance	Hu et al. (2008)
wft2, and *wft1*	Wheat	Chilling tolerance	Kawakami et al. (2008)
pdc1	Rice	Submergence tolerance	Quimlo et al. (2000)
OsASR1 or OsASR3	Rice	Drought and cold stress tolerance	Joo et al. (2013)

4.1.1 Genes Associated with Osmolytes Accumulation

Among the group of genes associated with abiotic stress response are genes involved in osmolytes accumulation. The upregulation of compatible solutes (osmolytes) helps in osmoprotection through maintaining cell turgor. In addition, these solutes help in stabilization of membranes and/or proteins through antioxidation and

chaperoning processes (Yancey et al. 1982; Bohnert and Jensen 1996; Lee et al. 1997; Hare et al. 1998; McNeil et al. 1999; Diamant et al. 2001; Jewell et al. 2010). Well-characterized osmolytes include proline, glycine betaine, polyamines, mannitol, galactinol, and trehalose. Among these osmoprotectants, glycine betaine, proline, and mannitol are present naturally in plants, while other solutes such as choline-O-sulfate, dimethylsulfoniopropionate (DMSP), D-ononitol, and trehalose are rarely present. Rontein et al. (2002) described the classification and function of these osmolytes in plants. Since the beneficial effects of osmoprotectants are generally not species-specific, it is easy to engineer foreign osmoprotectants into plants for development of stress tolerance. Several efforts have been made to engineer genes associated with the accumulation of compatible solutes in order to develop abiotic stress tolerance (Vinocur and Altman 2005). Transgenic rice with the modulated expression of pyruvate decarboxylase 1 gene was developed that demonstrated a strong correlation of increased enzyme activities with submergence tolerance (Quimlo et al. 2000).

4.1.2 Late Embryogenesis-Abundant Protein Genes

LEA (late embryogenesis-abundant) proteins have been suggested to be associated with desiccation tolerance. They perform important role in water status stabilization, cytosolic structures protection, sequestration of ions, proteins renaturation and transport, prevention of membrane leakage, and stabilization of membranes/proteins. These protein genes are found universally in plants. They have also been found in other organisms, such as the bacteria, different species of nematodes, rotifers, and cyanobacteria (Hundertmark and Hincha 2008). They are called "late embryogenesis abundant" because they are more abundant during late embryogenesis than during mid-embryogenesis. These proteins are involved in acquisition of desiccation tolerance under water stress, temperature, and salt (Sivamani et al. 2000; Bartels et al. 2007). These proteins induced in many desiccation-sensitive plants during drought stress are also present in the biomass tissue of resurrection plants (Bartels et al. 2007). LEA proteins show high hydrophilicity and heat stability in solution due to specific amino acid composition. Plant LEA proteins are divided into different groups based on the appearance of conserved sequence motifs or amino acid composition (Zhang et al. 2000; Wise 2003). A survey of the biochemistry, biophysics, and bioinformatics of the LEA proteins was well presented in a review (Tunnacliffe and Wise 2007). They highlighted several possible functions of LEA proteins in antioxidation and membrane/protein stabilization during drought, either by direct interaction or by acting as molecular shields. A total of 34 rice LEA (*OsLEA*) genes have been identified (Wang et al. 2007). The annotation of rice genes encoding LEA, dehydrin, or seed maturation proteins has been done by the TIGR community. The increased expression of transgene encoding LEA proteins has potential to improve abiotic stress tolerance of transgenic crops. In a study, rice transgenics transferred with *HVA1* gene of barley demonstrated increased tolerance to drought (Sivamani et al. 2000; Rohila et al. 2002). The same gene was expressed under a constitutive rice promoter and a stress-inducible promoter in the

background of Basmati rice that resulted in increased stress tolerance (Rohila et al. 2002). The transgenic expression of wheat genes PMA80 and PMA1959 enhanced the dehydration tolerance of rice (TNG67), though the reported water use efficiency (WUE) was low as compared to other reports of wheat cultigens (Cheng et al. 2002).

4.1.3 Reactive Oxygen Species and Associated Genes

Reactive oxygen species (ROS) are ubiquitous molecules involved in signalling pathways associated with plant stress and development. In response to various abiotic and biotic stresses, ROS such as superoxide, perhydroxyl radical, hydrogen peroxide, hydroxyl radical, alkoxy radical, peroxyl radical, singlet oxygen, and organic hydroperoxide gets accumulated that ultimately imposes oxidative stress, exacerbating cellular damages. Antioxidants involved in plant response to degrade ROS include catalase, ascorbate peroxidase (APX), superoxide dismutase (SOD), and glutathione reductase enzymes. Also, some nonenzymatic proteins, osmolytes, and amphiphilic molecules such as ascorbate, glutathione, carotenoids, and anthocyanins have antioxidative functionality (Wang et al. 2003). Strategies for abiotic stress tolerance have been attempted through transgenic development by overexpressing enzymes associated with oxidative protection. In one of the approaches, catalase gene of *Escherichia coli* was used for transgenic development to improve salt tolerance of rice cultivar, Nipponbare. These transgenic rice plants showed high tolerance to salt and could grow for more than 14 days in the presence of 250 mM NaCl (Nagamiya et al. 2007). In another such attempt, to develop drought-tolerant rice plants, manganese superoxide dismutase (MnSOD) of *Pisum sativum* was transformed into rice chloroplasts under the control of an oxidative stress-inducible promoter SWPA2 (Wang et al. 2005).

4.1.4 Transporter Proteins and Membrane Lipid-Associated Genes

Transport of water and ions occurs in plants through transcellular and intracellular pathways. The key proteins located at either tonoplast or plasma membrane known as aquaporins are involved in root water uptake (Aroca et al. 2012; Postaire et al. 2010). It facilitates transport of glycerol, small molecules, gas, and water, through membranes and, therefore, has important function in maintaining water homeostasis (Bartels et al. 2007). Attempts were made to overexpress an aquaporin in transgenic *Arabidopsis* and tobacco plants to develop tolerance to various abiotic stresses (Jang et al. 2007). Role of root-specific aquaporins in cold stress acclimatization in rice has been characterized (Ahamed et al. 2012).

Lipid biochemistry of the membranes can be altered through transgenic approaches to improve photosynthesis during stress conditions (Grover and Minhas 2000). Phospholipases C and D are associated with ABA-mediated signal transduction and stress perception in plants (Bartels et al. 2007; Wang et al. 2008a). The overexpression of PLD resulted in controlling stomatal movements, and response to water stress was demonstrated in transgenic tobacco (Sang et al. 2001).

4.1.5 Heat Shock Proteins

Heat stress leads to synthesis and accumulation of specific proteins known as heat shock proteins (HSPs) (Nakamoto and Hiyama 1999; Schoffl et al. 1999). These proteins primarily known for chaperone functions in plants play crucial role during heat stress (Torok et al. 2001) and also during drought stress through plant cell protection mechanisms. Approaches for enhanced thermo-tolerance by transgenic expression HSPs have been attempted in several plant species (Malik et al. 1999; Li et al. 2003; Katiyar-Agarwal et al. 2003). In a study on model organism *Saccharomyces cerevisiae,* increased resistance to salt, drought, and heat stress was achieved by constitutively expressing HSP24 from *Trichoderma harzianum* (Liming et al. 2008). Using a similar strategy, small heat shock protein gene (sHSP17.7) was overexpressed in the rice cultivar "Hoshinoyume" that resulted into enhanced tolerance to drought stress (Sato and Yokoya 2008). sHSPs encode a protein which was shown to act as molecular chaperones.

4.1.6 Genes Associated with Signal Transduction Pathways

The signalling pathways of abiotic stress hold a key to answer several complex questions associated with stress tolerance development to increase crop yields under suboptimal conditions. Genes associated with signal perception and signal transduction in response to abiotic stress have been of research interest. Signalling molecules may be common to various abiotic factors such as water, salinity, and temperature (Shinozaki and Yamaguchi-Shinozaki 1999). Although numerous signal transduction pathways operating for gene regulation during stress are known, ABA is a well-studied messenger molecule acting in one of the signal transduction mechanisms. It is a plant hormone that plays a critical role in response to various abiotic stress signals. ABA-independent signal transduction pathways also occur in plants. Signal response through phosphorylation/dephosphorylation is a well-studied mechanism to convey the response from receptor to the activator molecules of a gene expression and regulation pathway (Xiong and Ishitani 2006). The primary signalling molecules of abiotic stress response include Ca^{2+} sensors, phospholipid-cleaving enzymes, ROS, and mitogen-activated protein kinases (MAPK). Reduction of the cell's sensitivity to abiotic stress factors through altering these signal transduction components could be a viable option to develop stress tolerance using transgenic approach (Grover et al. 1999).

4.1.7 Transcription Factors

Transcription factors (TFs) are master regulators encoded by the early genes of stress response that activate downstream genes associated with delayed response (Zhu 2002). Several TFs play critical role in the plant response to abiotic stress (Vinocur and Altman 2005; Bartels and Sunkar 2005; Todaka et al. 2012).

These regulatory proteins interact with several other proteins involved in DNA and RNA metabolism to facilitate or hamper the gene expression. Hence, TFs have ability to control the expression of array of downstream target genes associated with pathways of abiotic stress response. One such category of plant TFs is DREBs (dehydration responsive element binding proteins) which belongs to AP2/ERF TFs. These proteins are regulators of expression of group of abiotic stress-associated genes, thus imparting a major role in stress tolerance phenotype of plants. Two major DREB regulons are known to function in stress response of plants; the DREB1/C-repeat binding factor (CBF) functions during the cold stress response, while DREB2 regulon plays important role during heat and osmotic stress (Mizoi et al. 2011). Almost ten genes of DREB1 type have been identified in rice genome, among which expression of *OsDREB1A* and *OsDREB1B* is upregulated by cold stress (Dubouzet et al. 2003). The induced expression of *OsDREB1A* and *OsDREB1B* showed enhanced tolerance to cold, drought, and high salinity in transgenic rice (Ito et al. 2006). Further, expression level of *OsDREB1F* gets induced by abiotic stresses and also ABA application (Wang et al. 2008b). The overexpression of *OsDREB1G* improved drought tolerance (Chen et al. 2008a, b). Similar to DREB1, four homologues of DREB2 have been reported in rice genome, among which *OsDREB2A* and *OsDREB2B* are regulated by drought, salinity, and heat stresses (Matsukura et al. 2010). Expression of *OsDREB2B* is induced by cold stress also.

ABA-dependent gene expression is regulated by the ABRE (ABA-responsive element) binding protein (AREB) or ABRE binding factor (ABF) regulon that plays an important role during abiotic stress (Fujita et al. 2011). These transcription factors are bZIP proteins that recognize and bind to the ABRE sequence motif to activate ABA-dependent gene expression (Choi et al. 2000; Uno et al. 2000). *OsABF2* of rice is a bZIP-type TF that showed induced expression under abiotic stresses and ABA application (Hossain et al. 2010). The induced expression of *OsABI5* in transgenic rice showed high sensitivity to salt stress, while antisense expression of same gene resulted in increased stress tolerance (Zou et al. 2008).

NAC proteins constitute one of the largest plant-specific TF families, with 149 members in rice (Xiong et al. 2005). These proteins show a specific feature of highly conserved DNA-binding domain at N-terminal and a variable C-terminal domain (Hu et al. 2008). The NAC regulon participates in important transcriptional networks associated with abiotic stress responses (Nakashima et al. 2009). Expression of *OsNAC5* is enhanced during dehydration, cold, and ABA treatments (Takasaki et al. 2010). The other transcription factor families involved in abiotic stress response in rice include MYB, MYC, WRKY, and Cys2His2 zinc finger (Todaka et al. 2012). Transgenic expression of *OsWRKY11* under the control of a HSP101 promoter displayed drought tolerance in rice (Wu et al. 2008). In order to improve drought tolerance in transgenic rice, ZFP252, a TFIIIA-type zinc-finger protein gene, was overexpressed (Xu et al. 2008a). Similarly, submergence tolerance was reported in rice by overexpression of an ethylene response factor-like gene Sub1A (Xu et al. 2006). Likewise, overexpression of a heat shock transcription factor (*OsHsfA7*) enhanced salt and drought tolerance in transgenic rice (Liu et al. 2013).

4.1.8 Plant Promoters Used for Abiotic Stress-Tolerant Transgenic Rice

While developing transgenic rice for abiotic stress tolerance, gene expression will have to be controlled such that the desired protein/RNA is confined to specific target cells, tissue, and organ or to a particular developmental stage. Similarly, silencing of a targeted gene has to be regulated in a time- and tissue-dependent manner. Promoters offer a fundamental key to control gene expression, and there have been serious efforts in isolating and characterizing abiotic stress-induced plant promoters. The most commonly used promoters in generating transgenic plants are constitutive promoters such as CaMV35S, actin, and ubiquitin. However, since these promoters are constitutive in nature, the transgene expression occurs in almost all tissues and growth phases of plants which may not be desirable in most of the cases. Sometimes, the gene expression under these constitutive promoters may lead to growth abnormalities in plants which may be due to over-synthesis of a particular protein that may disturb the molecular homeostasis of a cell and drive most of the plant energy towards undesirable activities. Hence, in order to bring specificity in gene expression in a particular tissue and time, choice of promoters will be the key factor to develop stress-tolerant transgenic plants. In fact, stress-inducible promoters have been explored to develop transgenic plant (Katiyar-Agarwal et al. 1999). The promoters such as RD29A/COR78/LTI78 of drought, salt stress, and cold-inducible genes contain two major *cis*-acting elements, ABRE and DRE (Yamaguchi-Shinozaki and Shinozaki 1994, 2005). The *Arabidopsis* rd29A promoter includes both DRE and ABRE elements. The rd29B promoter contains only ABREs. Two *cis*-acting ABRE sequence motifs are important DNA elements regulating the expression of ABA-responsive RD29B gene of *Arabidopsis* (Uno et al. 2000). Under the control of stress-inducible rd29 promoter, cDNAs encoding DREB1A and DREB1B transcription factors were transformed in *indica* rice cultivars BR29 and IR68899B. Improved dehydration tolerance was observed in the plants harboring *Arabidopsis DREB1A*, while *DREB1B* was more effective in development of salt stress tolerance (Datta et al. 2012). Using the stress-inducible *ABRC1* promoter of *HAV22*-form barley, stress-regulated expression of *Arabidopsis* CBF1 was obtained in transgenic tomato (Lee et al. 2003).

5 Conclusions and Future Perspective

Abiotic stresses such as drought, salinity, heat stress, cold stress, submergence, and stress-associated nutrient deficiencies are major constraints in rice production. There have been sincere efforts by plant breeders to develop stress-tolerant rice cultivars. Despite several attempts using traditional breeding approaches, an expected success could not be achieved due to complexity of trait and several other problems discussed in this chapter. With the intervention of marker-assisted selection, high-throughput genotyping, and next-generation genomic tools, breeding strategies could be utilized more effectively to develop abiotic stress tolerance.

Further, identification of new genes and QTLs is accelerating the efforts in this direction. In addition, transgenic technology offers great potential to develop abiotic stress-tolerant rice cultivars. The technology has been well utilized in several countries to develop many crop plants with useful traits. If the transgenic technology can be used smartly, it can certainly complement the breeding efforts to meet the challenges of abiotic stress tolerance development in rice.

The development of rice genotypes with improved abiotic stress tolerance will undoubtedly have an important impact on global food security. An intensive effort utilizing the biochemical, physiological, molecular, and cellular studies of abiotic stress response is essential for linking the knowledge gaps on the effects of the genes, regulatory RNAs, and proteins in a short and long term. Understanding the network of different stress signal transduction pathways will be important to identify the key genes responsible for abiotic stress tolerance in rice. After the intervention of high-throughput genomic tools, an array of stress-related genes have been identified and been transferred to rice using breeding and transgenic strategies to enhance stresses tolerance. With the growing knowledge of QTLs or candidate genes for abiotic stress tolerance in rice, pyramiding of QTLs/genes can add the effect of useful genes together for a particular trait or more than one trait by using MAS approaches. Marker-assisted backcrossing is an important tool to introgress useful alleles into agronomically superior germplasm. Transgenic rice plants with enhanced abiotic stress tolerance offers an attractive alternative to conventional breeding approaches for the genetic improvement of rice cultivars. Moreover, transgenic development is the only viable option to utilize the gene pool from different species, wild relatives, and organisms other than plants. The creativity and innovations in traditional and molecular breeding approaches and the wise application of gene transfer technologies hold the key to attain the goal of abiotic stress tolerance development in rice.

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Chapter 8
Mineral Bioavailability Through Mutation Breeding in Pulse Crops: A Review

Mohammad Imran Kozgar, Mohd Rafiq Wani, Samiullah Khan, and Parvaiz Ahmad

1 Introduction

The question posed by researchers and thinkers "Can we feed the world and sustain nutritional balance in the diet at an affordable price with the available technologies?" (Jain and Suprasanna 2011) has to be answered at practical level and becomes very important when the human population is expected to reach 12 billion by 2060. Food insecurity is becoming the major constraint in the progress of various nations including India. With burgeoning human population, the ghost of hunger are making its impact among millions of people all around the globe and threatening the human kind with its full zeal. At present, conditions related to food insecurity and malnutrition is of great concern (Figs. 8.1 and 8.2) as revealed by the report of Food and Agriculture Organization (FAO 2009). Malnutrition conditions affect more than 40 % of the world's population with respect to various micronutrients (Jain and Suprasanna 2011).

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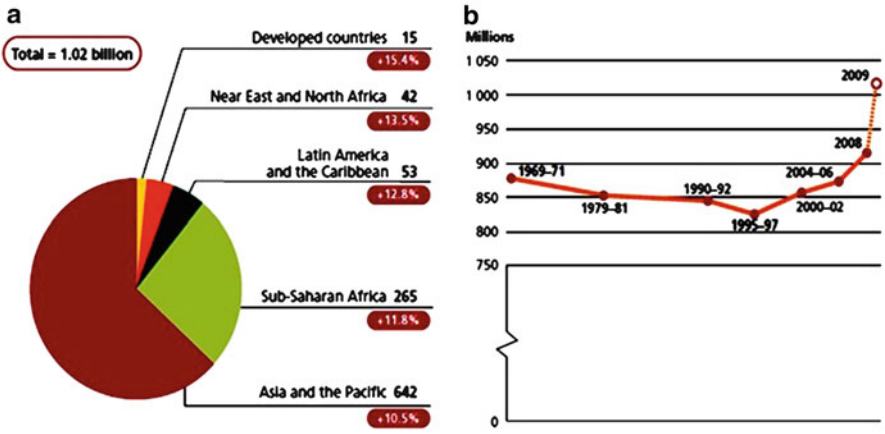

Fig. 8.1 **(a)** Undernourishment in 2009, by region (millions) and increase on 2008. **(b)** Number of undernourished in the world, 1969–1971 to 2009 (Source: FAO 2009: The state of food insecurity in the world)

Although, several world food summits were organized during the past decades, but the number of hungry people, concomitant to undernourishment, increased and now exceeds one billion (Swaminathan 2010). Revealing the position of India, the malnutrition conditions due to food insecurity have jumped to 42.5 % (NFHS 3 2006) as compared to 35 % of sub-Sahara, even if India's economy is the tenth largest, while that of sub-Sahara is on the 79th position in the world economic market. The results show that the number of people undernourished are very much higher in Asia and pacific regions, to which India is contributing a lot. The low yield of production by diverse kinds of plants is directly linked to the hunger and malnutrition conditions. Mutation breeding can be a vital and handy tool for increasing the crop production and creates the diverse genetic diversity among the crops like pulses. India is a great producer of pulse crops, and these crops have high potential to reduce the hunger and malnutrition conditions to some extent, once handled in sustainable manner following breeding strategies (Latham 1997). Creating genetic diversity is a prerequisite for any plant breeding programs (Rao and Hodgkin 2002; Paudel 2008), and most of the major pulse crops like chickpea, as being the self-pollinating crop, lack sufficient genetic diversity (Rao and Hodgkin 2002). Mutation breeding methodologies are able to create most of the genetic diversity among such self-pollinating crops as revealed by various research reports (van Harten 1998; Micke 1999; Kharkwal 1999; Maluszynski 2001; Jain 2002; Toker et al. 2007, 2011; Toker 2009; Wani et al. 2011; Husain et al. 2013) and shorten the time to be taken for the development of cultivars via induced mutations as compared to hybridization technique (Toker et al. 2011; Mba 2013).

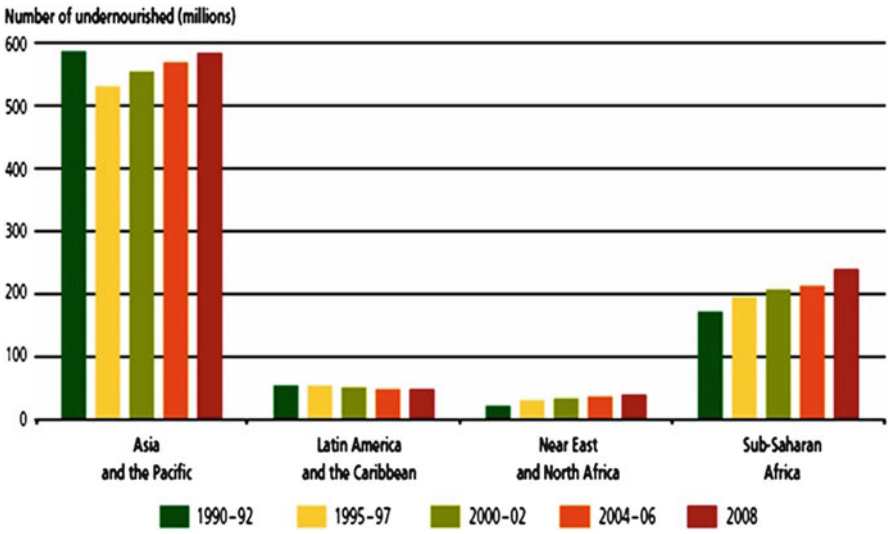

Fig. 8.2 Undernourishment on the rise throughout the world: number of undernourished in the selected regions, 1990–1992 to 2008 (Source: FAO 2009: The state of food insecurity in the world)

2 Impact and Uses of Mutation Breeding

Induced mutation has played a pivotal role to curb the world food insecurity and malnutrition as new edible crop varieties with improved crop productivity and mineral bioavailability have been directly accessed by people at local positions (Kharkwal and Shu 2009). Mutation breeding creates the variation within a crop variety and offers the possibility of inducing desired attributes that either cannot be found in nature or have been lost during evolution (Novak and Brunner 1992). Micke (1999) advocated that the mutation approach is a superior method of crop improvement especially in those cases, where the required amount of variation could be produced rapidly. For the improvement of plants, mutation breeding combines several advantages by upgrading a specific character without altering the original genetic makeup of the cultivar and is a well-functioning branch of plant breeding supplementing to conventional methods in a favorable manner (Gottschalk 1986; Toker et al. 2007; Hadi and Fuller 2013). By the end of 2012, more than 3,200 varieties in more than 175 plant species derived from mutagenesis programs have been officially released worldwide as listed in FAO/IAEA Mutant Varieties and Genetic Stocks Database (MVGS), including 410 mutant varieties of pulses. The mutant varieties of major pulse crops released through mutation breeding techniques in India with potential characteristics are given in Table 8.1. The induction of mutations for generating high yielding mutants could be a novel strategy to

Table 8.1 Mutant varieties of some major pulse crops released from India with the type of mutagen used, year of release and main characteristic features

Mutant Variety	Mutagen used	Year	Characteristics
Cicer arietinum L. (chickpea)			
Pusa 547	Gamma rays (600 Gy)	2006	High yield, good cooking quality, tolerance to *Fusarium* wilt, stunt virus and root rot
BGM 547	Gamma rays	2005	High yield, bold grain size, attractive golden-brown color and moderate resistance to wilt, root rot, stunt, and *Helicoverpa armigera*
Pusa 417 (Girnar)	Gamma rays (600 Gy)	1985	High yield, short, semierect, profusely branched, high pod number, 110–130 days to maturity, wilt resistance, moderate resistance to stunt virus, collar root, foot rot, root rot, low pod borer, and nematode damage
Pusa 413 (Atul)	Gamma rays (600 Gy)	1985	High yield, wilt resistance, moderate blight resistance, resistance to stunt virus, foot root, root rot, semierect, higher number of branching, more than 2 grains/pod, 130–140 days to maturity and plant architecture
Pusa 408 (Ajay)	Gamma rays (600 Gy)	1985	High yield, blight resistance, semierect, 140–155 days to maturity and plant architecture
Kiran	Neutrons	1984	Erect plant type, increased pod number, high yield, early maturity, and salt tolerance
Lens culinaris Medik. (lentil)			
Rajendra Masoor 1	Gamma rays (100 Gy)	1996	Tolerance to low temperatures, early maturity, and good for late sowing
S-256 (Ranjan)	Irradiation	1981	High yield and spreading type
Cajanus cajan L. (pigeon pea)			
TJT-501	NA	2009	High yielding, early maturity, and tolerance to *Phytophthora* blight
TT-401	NA	2007	High yield, tolerance to pod borer and pod fly damage
TAT 10	Hybridization with two fast neutrons (25 Gy) raised mutants	1984	Medium-large-sized grain, extra early maturity (115–120)

(continued)

Table 8.1 (continued)

Mutant Variety	Mutagen used	Year	Characteristics
TAT 5	Fast neutrons	1984	Increased seed size (50 %), high TGW, and early maturity (140 days)
Co 5	Gamma rays (160 Gy)	1984	Early maturity, photoperiod insensitivity, and drought tolerance
Trombay Vishakha-1	Fast neutrons	1983	Increased seed size with all desirable traits of parent variety T-21
Co 3	EMS (0.6 %)	1977	High yield, bold seeded, higher shelling, field dormancy for 15–20 days
Pisum sativum L. (garden pea)			
Hans	Chemical mutagen EI	1979	Early maturity, high yield, and better seed quality
Phaseolus vulgaris L. (kidney bean)			
Pusa Parvati	Irradiation of seeds with X-rays (70, 140, 210 Gy)	1970	Early maturity (40–45 days), bushy type with attractive round meaty light-green pods, high yield (45 % more)

NA not available

combat hunger problem in Indian regions with other associated desirable characteristics for some of the major pulse crops like chickpea, lentil, pigeon pea, garden pea, and kidney bean.

The latter two countries in Table 8.2 (China and Japan) of Asiatic region are practically utilizing the useful applications of mutation breeding. Japan, although, being a smaller country in terms of various parameters related to agriculture like land, number of people involved, geographical diversity, and soil type as compared to India, has been able to utilize mutation breeding mainly because of cooperation and coordination with other countries and has been a pioneering country to develop the Forum for Nuclear Cooperation in Asia (FNCA), where mutation breeding is flourishing with compact coordination among the participating countries. The FNCA Mutation Breeding Project aims to contribute increased food production in Asia, improve the food quality, and encourage the utilization of radiation for developing new mutant varieties of crops that can better resist drought, insects, and diseases by using mutation breeding (FNCA 2011). FNCA is a Japan-led cooperation framework for peaceful use of nuclear technology in Asia. The cooperation consists of FNCA meetings and the project activities with the participation of Australia, Bangladesh, China, Indonesia, Kazakhstan, Korea, Malaysia, Mongolia, the Philippines, Thailand, and Vietnam, and unfortunately India is not the part. This is a point of great concern for the national developmental programs, where the index of hungry and undernourished people is increasing day by day as per the reports of various national and international surveys.

Table 8.2 Number of mutant varieties of major cereal and pulse crops released in the world, India, China, and Japan

Crop	World	India	China	Japan
Cereals				
Wheat	252	4	162	7
Rice	816	59	290	222
Maize	96	0	47	0
Barley	309	13	7	10
Rye	2	0	0	1
Millet	30	0	20	0
Oats	23	0	0	0
Total[a]	1,546	76	526	240
Pulses				
Chickpea	21	6	0	0
Faba bean	20	0	0	1
Lentil	13	2	0	0
Pigeon pea	7	7	0	0
Mung bean	36	16	0	0
Urd bean	9	8	0	0
Garden pea	34	1	1	0
Kidney bean	59	1	1	0
Groundnut	71	25	33	0
Soybean	170	7	79	30
Azuki bean	3	0	2	1
Total[a]	443	73	116	32
Grand total (cereals + pulses)	1,989	149	642	272

[a]For major mentioned crops only

3 Global Micronutrient Conditions

Micronutrient (mineral element) deficiency is widespread in developing and developed countries and found in more than two billion people worldwide (Tulchinsky 2010). Pulse crops are excellent source of mineral elements like Fe, Zn, Mn, and Cu and even vitamins and proteins; however, the mineral bioavailability from pulses is poor, and thus their value as source of micronutrients is diminished (Messina 1999). The database about facts on global hunger and malnutrition have been compiled by various sources, and from last 6 to 7 years, based on the figures given below, there seems to be no respite in the alarming situations.

3.1 Global Hunger

1. 1.02 billion people do not have enough to eat—more than the populations of the USA, Canada, and the European Union (Source: FAO news release, 19 June 2009).

2. The number of undernourished people in the world increased by 75 million in 2007 and 40 million in 2008, largely due to higher food processes (Source: FAO news release, 9 December 2008).
3. Around 907 million people in developing countries alone are hungry (Source: The State of Food Insecurity in the World, FAO 2008).
4. Asia and the Pacific region are home to over half the world's population and nearly two thirds of the world's hungry people (Source: The State of Food Insecurity in the World, FAO 2008).
5. More than 60 % of chronically hungry people are women (Source: The State of Food Insecurity in the World, FAO 2006).
6. 65 % of the world's hungry live in only seven countries: India, China, the Democratic Republic of Congo, Bangladesh, Indonesia, Pakistan, and Ethiopia (Source: The State of Food Insecurity in the World, FAO 2008).

3.2 Child Hunger

1. More than 70 % of the world's 146 million underweight children under age of 5 years live in just ten countries, with more than 50 % located in South Asia alone (Source: Progress for Children: A Report Card on Nutrition, UNICEF 2006).
2. 10.9 million children, under 5 years of age, die in developing countries each year. Malnutrition- and hunger-related diseases cause 60 % of the deaths (Source: The State of the World's Children, UNICEF 2007).
3. The cost of undernutrition to national economic development is estimated at US$20–30 billion per annum (Source: Progress for Children: A Report Card on Nutrition, UNICEF 2006).
4. One out of four children, roughly 146 million in developing countries, is underweight (Source: The State of the World's Children, UNICEF 2007).
5. Every year WFP feeds more than 20 million children in school feeding programs in some 70 countries. In 2008, WFP fed a record 23 million children (Source: WFP School Feeding Unit).

3.3 Malnutrition

1. It is estimated that 684,000 child deaths worldwide could be prevented by increasing access to vitamin A and zinc (Source: WFP Annual Report 2007).
2. Undernutrition contributes 53 % of 9.7 million deaths of children under five each year in developing countries (Source: Under five deaths by cause, UNICEF 2006).
3. Lack of vitamin A kills a million infants each year (Source: Vitamin and Mineral Deficiency, A Global Progress Report, UNICEF).
4. Iron deficiency is the most prevalent form of malnutrition worldwide affecting an estimated 2 billion people. Eradicating of iron deficiency can improve national

productivity levels by as much as 20 % (Source: World Health Organization, WHO Global Database on Anemia).
5. Iron deficiency is impairing the mental development of 40–60 % children in developing countries (Source: Vitamin and Mineral Deficiency, A Global Progress Report, p2 UNICEF).
6. Vitamin A deficiency affects approximately 25 % of the developing world's preschoolers. It is associated with blindness, susceptibility to diseases, and higher mortality rates. It leads to the death of approximately 1–3 million children each year (Source: UN Standing Committee on Nutrition, World Nutrition Situation 5th Report 2005).
7. Iodine deficiency is the greatest single cause of mental retardation and brain damage. Worldwide, 1.9 billion people are at the risk of iodine deficiency, which can easily be prevented by adding iodine to salt (Source: UN Standing Committee on Nutrition, World Nutrition Situation 5th Report 2005).
8. WFP-supported deworming reached 10 million children in 2007 (Source: WFP Annual Performance Report 2007).

4 Induced Mutations in Architecture of Plants for Mineral Bioavailability

The bioavailability of mineral elements in the tissues mainly depends on the architecture of plants vis-à-vis absorption processes, transportation mechanisms, and accumulation of absorbed mineral elements at/in desired plant traits. The uptake of mineral nutrients is also dependant on the physiochemical and biological properties of the soil (Frossard et al. 2000) and on the gene-environment interactions. It has been reported in many studies that plants take up most of the nutrients in the form of free ions like Zn^{2+} and Fe^{2+}. Henceforth, there are two stages involved while nutrients are absorbed in the plant tissues, and they are (1) release of free ions from the solid phase of the soil into solution and (2) plant architecture which governs the uptake (Frossard et al. 2000). In countries like India, the bioavailability of micronutrients such as Fe, Zn, Mn, and Ca in plant foods is quite low, which ultimately are the focal causes of metabolic disorders related to these nutritional factors (Bohn et al. 2008). It has been reported that malnutrition affects more than 40 % of the world's population and is very common in developing nations (Jain and Suprasanna 2011). One of the reason of malnutrition is poor bioavailability of mineral elements (Ramakrishnan 2002), the others being inadequate food intake and poor diet quality.

In order to improve the nutritional status, the bioavailability of micronutrients in the plant sources has to be enhanced, and induced mutation is a good functional branch which may fit the role in a very sustainable manner as has been reported by various authorities (Overturf et al. 2003; Raboy 2009; White et al. 2009; Jain and Suprasanna 2011). Of the mutant varieties released across the world, more than 776 mutants have been induced for nutritional qualities (Jain and Suprasanna 2011). Enhancing the mineral contents in the crop tissues of mutants in early generations

Table 8.3 Mineral compositions of mature major pulse crop seeds

	Mineral elements (mg/100 g) of raw seeds						
Major pulse crops	Ca	Fe	Mg	P	K	Na	Zn
Chickpea	105	6.24	115	366	875	24	3.43
Faba bean	103	6.70	192	421	1062	13	3.14
Mung bean	132	6.74	189	367	1246	15	2.68
Urd bean	138	7.57	267	379	983	38	3.35
Lentil	56	7.54	122	451	955	6	4.78
Pigeon pea	130	5.23	183	367	1,392	17	2.76
Garden pea	25	1.47	33	108	244	5	1.24
Kidney bean	186	3.40	188	304	1316	18	1.90

Source: USDA National Nutrient Database for Standard Reference, Release 25 (2012)

treated by mutagenic treatments has also been reported recently (Kozgar et al. 2012). It has been already reported that increased micronutrient (mineral elements) density in seed destined for human consumption may alleviate micronutrient deficiencies in human population around the world (Rengel et al. 1999), but still the practical applicability has not been enforced with full zeal. Gregoria (2002) has also supported the fact that it is possible to combine the high micronutrient traits with high yield, unlike protein content and yield that are negatively correlated through breeding strategies. Several mutant genes that significantly enhance the nutritional values of crops have been successfully introduced into various crop varieties through induced mutagenesis programs (Jain and Suprasanna 2011). The mutant genes have been successfully introduced in various commercial varieties which has enhanced their nutritive values (Ranalli 2012).

4.1 Major Micronutrients in Pulses and Their Accumulation Through Induced Mutagenesis

Enhancement in the amount of the mineral/micronutrient bioavailability for food consumption in plants is a challenge, particularly in developing countries (Frossard et al. 2000). The micronutrients available in plant derived foods have their own important value and play an imperative role in the human physiological development by taking part in their metabolic pathways at different stages of life. Pulse crops are seat of various mineral elements and some of the major micronutrients present in the most consumed pulse crops in reference to Indian scenario are given in Table 8.3.

In order to improve the mineral nutritional value, there are three approaches to engineer the crops for filling the desired attribute, and these are (1) to elevate the concentration of minerals in appropriate tissues such as endosperm, (2) to elevate the levels of compounds that enhance mineral nutrition, and (3) to decrease anti-nutrient factors like phytic acid (Raboy 2000; King 2002; Concepcio´n Mendoza 2002).

The three approaches may be filled up by applying fertilizers to the soil or a crop (Rengel et al. 1999), by means of plant breeding (Graham and Welch 1996; Graham et al. 1999), and by genetic engineering (Grusak and Dellapenna 1999).

Induced mutagenesis, as a part of plant breeding, has been pivotal to improve the mineral bioavailability mainly by applying the third type of approach, viz., decreasing anti-nutrient factors, which has been graded to offer great promise for trace elements, especially among the populations that are primarily dependant on plant derived diets (Concepcio´n Mendoza 2002) like pulse crops. The two low phytic acid mutants (*lpa*) have been isolated in cereals like maize, rice, and barley. The mutant variety from soybean has also been isolated with low phytic acid contents (Raboy et al. 2000, 2001; Raboy 2002; Gilman et al. 2013). The role of induced mutagenesis in enhancing the micronutrients became more important as it has shown that the variation in mineral elements, like that of Fe in the seeds, is due to its genetic component and the environmental effects have the less impact (Gregorio et al. 1999).

5 Constraints in Cultivating the Pulse Crops

There are different types of problems which are making hindrance in the cultivation of pulse crops. Kumar et al. (2011) has outlined six types of constraints which are coming in the way of pulse production, and they are:

1. *Ecological and environmental constraints*. Due to adverse weather conditions like irregularities in rainfall, temperature, and excess moisture, acidity, alkalinity, and salinity of the soil got elevated.
2. *Basic research constraints.* Although, All India Pulse Improvement Project was started very early in 1965, but the emphasis was only on the production aspect with basic research completely missing.
3. *Agronomic constraints.* It includes improper sowing time, low seed rate, inadequate intercultural operation, limited use of organic matter and culture, insufficient use of fertilizers, and inadequate irrigation.
4. *Plant protection constraints.* Mutation breeding technique has given some rescue for the development of disease resistant cultivars, but the major breakthrough is still awaited.
5. *Seed constraints.* Availability of high yielding seeds is very less, especially in pulse crops.
6. *Other constraints.* In spite of high prices of grain legumes in the country, they are considered neglected and low economic return crops by the farmers. This is mainly due to the presence of many types of biological and other types of pests which are feeding on different parts of the pulse crops. The problems of storage are also prevailing in these crops.

All these constraints need to be eliminated by well-planned strategic policies, rules, laws, and cooperation among the related agencies and institutes.

5.1 Strategic Developments

The development of mutation breeding techniques for self-pollinating crops like pulses is to be encouraged as these crops are unable to show/generate the high genetic diversity, which is prerequisite for any breeding program. Cereal crops, in other way, can flourish by other breeding methods like cross-fertilization and inter-varietal crosses. With the advent of new molecular and biotechnological techniques like EMAIL (Endonucleolytic Mutation Analysis by Internal Labelling), TILLING (Targeting Induced Local Lesions in Genomes), IBT (Ion Beam Technology), and High Hydrostatic Pressure (HHP), the induction of mutation of desired characteristics in any particular crop has been now possible somewhat and with certain accuracy. The techniques of tissue culture for creating somaclonal variants have also reached to new heights in the genomic era. The authorized and legal agencies have to work in coordination so that the full utilization of the techniques could be availed and the constraints could be cleared at ease. Laws, regarding conservation of land for agricultural purposes, should be implemented; otherwise in future, the land for cultivation of crops would only be available in dreams. All these programs and techniques like microarray and real-time PCR have to be associated with mutagenesis in new areas of functional genomics, and allied fields may help in the discovery of genes in order to elucidate the function and mechanisms of gene action (Latado et al. 2012; Ramel et al. 2013).

6 Conclusions and Future Perspective

Due to less genetic diversity and self-pollinating nature of number of pulse crops, induced mutation breeding is of paramount importance. Mutation breeding technique has been serving the human kind in many forms, and a lot of cultivars all around the world have been developed which are enriched in desired characteristics like disease resistant, cold resistant, heat resistant, and high yielding, but the breakthrough in Indian subcontinent in this particular area is still awaited which can be implemented when the legal authorities make amendments in policies through which the mutation breeding technique can flourish well. The cooperation to FNCA and getting its membership is of utmost importance for the peaceful means and to reduce the hunger in collaborative research works. In order to break the yield plateau in crops like pulses concomitant with an increase in micronutrient availability, the collaborative work between agriculturists, human society, decisive policy bodies, and stakeholders, keeping in view the ecological balance, has to be enhanced overall. With the amalgam to genomic techniques (forward as well as reverse one), the mutation breeding strategy will enrich the agriculture output, and there is lot to work on this aspect. To plug the gap of experimental work to the practical aspect, persisting in Indian scenario at large scale, the researchers working on mutation breeding in cognizance with government agencies should lead from the front and frame the food productivity, harvesting, processing, packaging, and distribution rules and policies for common welfare of the mankind.

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Chapter 9
Abiotic Stress and Control of Yield in Cereals

Bhinu V-S Pillai and Sreekala Chellamma

1 Introduction

Globally, there is an increasing demand for coarse grains such as maize, rice, and wheat to meet the supply gap for food, feeds, and energy, created by rising human populations, and agricultural and bioenergy needs. As such, maize production has to more than double to compensate for the supply gaps, especially in developing nations (Pingali and Pandey 2001). Short-duration or early-maturing genotypes are useful, because they secure harvests against fluctuating weather conditions and ensure an early supply of needed grain. Early-maturing maize genotypes typically take 130 days to reach physiological maturity, according to CIMMYT (Magorokosho et al. 2009). Nevertheless, under optimum growing conditions in Southern Africa, early-maturing maize genotypes yield between 15 and 30 % less than late-maturing ones, probably due to either limited source efficiency or sink capacity (Lee and Tollenaar 2007). Despite this, early-maturing genotypes are deployed in drought-prone and marginal environments, which affect grain supply. For instance, more than 80 % of maize genotypes grown in the Southern Africa farming system, many of which are in marginal and drought-prone areas, are either early-maturing open-pollinated hybrids or early-maturing open-pollinated varieties (Pswarayi and Vivek 2008). The challenge, therefore, is to improve the grain yield of widely adopted early-maturing maize genotypes, while maintaining their earliness, to secure harvests.

Given the world food requirements, increasing human population, diminishing farmland, and adverse climatic conditions, critical questions are being asked about how to revamp global agriculture and understand the reasons leading to a feeling of insecurity regarding food and why is farming increasingly looked upon as a disincentive? Responding to such a need, the plant biotechnology industry is pursuing

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efforts to develop crops that deliver increased and stable yields under normal growth and stress conditions. It appears that after tackling the initial wave of insect resistance and herbicide resistance, the plant biotechnology industry is gearing to tackle abiotic stress. Such a pursuit encompasses efforts to reduce the yield gap existing between the apparent yield potential and the actual yield potential via breeding and transgene technology applications. This necessitates extensive knowledge not only about the abiotic stress but also the plant's response, both phenotypic and biochemical, to a particular stress. Phenotypic characterization, field design, consistent procedures, and standardized experimental conditions are needed to perform studies to enhance our knowledge. These data need to be integrated with computational biology (genomics, modeling) and breeding efforts (mapping, transgenic breeding, etc). We also need to capture the outcome of multidisciplinary genomics knowledge to identify potential gene targets to develop conceptual ideotypes to meet the future demand of the plant-biotechnology industry.

Several genes that regulate abiotic stress response have been identified, for example, in *Arabidopsis* plants. The completion of *At* genome sequencing in 2000 and the development of several high-throughput molecular platforms have accelerated molecular characterization of several physiological and biochemical processes involved in abiotic stress tolerance in plants. The fundamental mechanisms that underlie the plant abiotic responses are indeed conserved among plant species, and therefore, the knowledge gained in model plants such as *Arabidopsis* or haplotypes can be exploited to improve stress tolerance in crop plants. Though several examples of ectopic expression of key genes involved in stress response that can induce stress tolerance in different crops have been demonstrated, generating crops that have improved tolerance under field conditions is still a challenge. It is important to safeguard critical parameters, such as yield when devising strategies to tackle stress; at no cost should these key attributes be compromised. If it is due to our limited understanding of the stress physiology, or the choice of plant models or the manner in which we generate data (e.g., greenhouse vs. field), or other unknowns, it is encouraging to note that we may be inching closer to cracking the stress tolerance problem.

2 Abiotic Stress and Seed Development

One of the major challenges faced by agriculture industry is a solution towards managing crop production under conditions of varying levels and timing of abiotic stress. A lot of efforts were directed towards understanding the physiology of stress at early stages of development; however, knowledge on the physiological impact of abiotic stress at later stages of development is limited (Dolferus et al. 2011). It is possible that stress that is mild enough to not show any impact on vegetative stages might result in significant impact on yield components such as grain yield. Grain number and grain yield are both resulting from similar processes within the plant system (Sinclair and Jamieson 2006); however these are constrained by a crop's ability to gather resources.

2.1 Abiotic Stress

Abiotic stress is defined as environmental conditions that impair plant growth and result in yields below normal levels. In essence, the stress could be resulting from changes in or between the atmosphere and plants causing a reduction in growth and yield potential. Several forms of abiotic stress are known, and some of the types of abiotic stress experienced by plants involve toxicity to metals, excessive salinity, drought, temperature extremes (high or low), and nutrient deficiency, among others. Given the multitude of stress factors and the diverse influence, the area of stress response in plants and the concomitant impact on yield is gaining considerable significance in the agrarian space. Up to 70 % decrease in plant survival, biomass production, and yield were reported in literature due to major abiotic stresses like drought, heat, cold, and high salinity (Ahmad et al. 2012, 2010; Chellamma and Pillai 2012; Thakur et al. 2010; Mantri et al. 2012).

Plants being complex organisms, prolonged exposure to abiotic stress leads to altered plant metabolism and damage to cells and biomolecules. Through complex interactions between organelles and cellular components and via exchange of transcription factors, plant cells are able to modulate gene expression and developmental response to abiotic stress. The nature of molecular responses of the plant cells to their environment though complicated defines how the stress is tackled (or not). This chapter discusses how abiotic stress impacts plant physiology, in particular their reproductive development that translates to yield and quality loss in cereal crops.

2.2 Abiotic Stress Tolerance

Plants have developed advanced and efficient technologies to mitigate biotic and abiotic stress during the course of evolution. Exposure of plants to abiotic stress results in induction of several signaling cascades. This involves activation of ion channels and production of reactive oxygen species (ROS) and kinase cascades. In addition, hormones such as salicylic acid, ethylene, jasmonic acid, abscisic acid, and osmolytes are also induced as a result of stress signaling cascades (Perez-Clemente et al. 2013). These signaling pathways are known to induce expression of defense genes (Jaspers and Kangasjarvi 2010), thus enabling the plant to mitigate the effect of stress. However, agricultural species are less tolerant to adverse environmental conditions compared to many wild relatives that have gone through natural adaptation. Improved understanding of abiotic stress tolerance has led to crop improvement through development of tolerant crops and optimal use of genetic resources, land resources, and wild relatives of several crop plants. These accomplished via molecular breeding, genetic engineering, and integrated breeding result in superior and stress-tolerant cultivars. For instance, the response to drought has been widely discussed over three decades. According to Levitt (1972) plants use

stress avoidance and stress tolerance mechanisms. At low to medium stress levels, plants sense the stress and the plant tissue avoids low water potential and decrease water content by maintaining a balance between water intake and water loss via stomatal transpiration or uses the osmotic response system, cell wall hardening, etc. to maintain high water content even with a low water potential. When the stress effect is high or severe, plants engage proline (Ahmed et al. 2010), protective ion solutes, scavenging reactive oxygen and other measures to tolerate or mitigate stress. Depending on how the plant senses severity of water stress, one or more responses occur.

Molecular breeding approaches could involve the development of genomic resources such as molecular markers, namely, SNPs, SSRs, and marker genotyping platforms, followed by development of mapping populations involving two diverse parental lines or germplasm showing potential for abiotic stress tolerance. Linkage mapping efforts identify QTLs or associated markers related to abiotic stress tolerance such as reduced photorespiration rate, leaf water retention, high rates of leaf photosynthesis, stomatal conductance, osmotic flux, plant canopy, and root development, which are validated through breeding programs. Conventional breeding approaches have been used for improved abiotic stress responses. However, a review of several reports suggests limited success (Richards et al. 2010). The slow progress could be attributed to complexities introduced by GxE (Genotype × Environment) interactions, focus on yield rather than specific traits contributing to stress tolerance, and limitations involved in transferring traits through hybridization as opposed to novel technologies (Tester and Bacic 2005), despite advancements in the sequencing world (Yu et al. 2002; Goff et al. 2002; The International Rice Genome Sequencing Project IRGSP 2005; Tuskan et al. 2006; Paterson et al. 2009; Schnable et al. 2009; Schmutz et al. 2010).

Genetic engineering approaches on the other hand include identification and use of genes encoding effector proteins, signal proteins, promoters responding to various stresses, and transcription factors with intent to, for example, regulate stomatal function and improve water use efficiency. Genetic diversity screening is another platform, where functional genomics approaches are used towards discovering genes responsible for abiotic stress (Sutton 2009). Cereals being in the grass family have extensive genetic diversity, and this can be exploited towards comparative genomic approaches and developing stress tolerance in cereal crops through transgenic approaches (Mizoi and Yamaguchi-Shinozaki 2013). Successful development of plant transformation systems, in most cereals, makes it easier to exploit approaches of genetic manipulation for improvement of stress-tolerant research (Tester and Bacic 2005). Even though molecular breeding approaches can be followed for transfer of identified genes to desired cereal species, genetic engineering provides advantages in combining different elements at the same time.

Genomic approaches for stress tolerance involve understanding a gene function. Several high-throughput technologies have been employed in the recent past (Chain et al. 2009; Feuillet et al. 2010), and many signaling cascades were identified (Nakagami et al. 2006; Suarez-Rodriguez et al. 2007; Qiu et al. 2008; Jaspers and Kangasjarvi 2010). Genomic technologies revealed multigenicity of plant stress responses, transcript collections specific to stress response, dynamic changes in

protein and metabolic profiles, and protein interactions resulted from plant stress responses (Perez-Clemente et al. 2013).

Proteomic approaches are crucial given our improved understanding that changes in gene expression does not necessarily result in changes in corresponding protein levels. Proteomic studies provide a more direct correlation between protein abundance and stress responses. Ethylene-responsive element binding factors (ERFs) (Fujimoto et al. 2000; Onate-Sanchez and Singh 2002; Nakano et al. 2006), NAC proteins (Nakashima et al. 2011; de Oliveira et al. 2011), basic domain-leucine zipper (bZIP) (Satoh et al. 2004; Weltmeier et al. 2006), MYB proteins (Agarwal et al. 2006; Dubos et al. 2010; Zhang et al. 2011), and MYC proteins (Agarwal and Jha 2010) were identified by proteomic approaches based on their responses to stress tolerance. Metabolics approach on the other hand would be the ultimate components to improve understanding on many physiological processes in plants. Targeted analysis (Djoukeng et al. 2008), metabolic fingerprinting (Chatterjee et al. 2010), metabolic profiling (Seger and Strum 2007), and protein profile analysis (Wang et al. 2013) have been developed in the past decade to develop better understanding on metabolites on different physiological processes. Several metabolites such as abscisic acid, jasmonic acid, salicylic acid, polyamines, proline, glycine betaine, unsaturated fatty acids, ROS, and malondialdehyde are identified as metabolites associated with environmental stress (Perez-Clemente et al. 2013).

2.3 Seed Development and Abiotic Stress

In cereals that serve as a staple crop to growing parts of the world, it is obvious that grain number translates as a direct yield attribute. Increase in grain number contributes to increase in cereal grain yield. Though a simple concept, controlling the grain numbers to boost yield has not been easy, and researchers have had limited success. This is mainly because of the sensitive development phases, in particular the early reproductive development; early stages of pollen development are hindered by abiotic stress in most self-fertilizing cereals, including rice and wheat. Aborted pollen due to stress means fewer fertile spikes per plant and even fewer fertile spikelets per spike, which directly impact the number of grains per spikelet. It is essential to understand the physiological and molecular mechanisms of floral development and inflorescence architecture. Given that abiotic stress and climate change have been intertwined, it may be a bigger than envisaged problem to protect crops from duress.

3 Biogenetic Components Influencing Yield

Genetic variability for grain yield in early-maturing maize genotypes has been reported and could be used to improve yields (Pswarayi and Vivek 2008). Selection or high yield is associated with a high genetic complexity and a high environmental interaction. As such, selection of yield and secondary traits that are strongly correlated and

highly heritable is desirable. Secondary traits such as anthesis-silking interval (ASI), ears per plant, and senescence rate have been used to identify high grain-yielding genotypes under drought and low-nitrogen conditions (Banzinger et al. 2004; Derera et al. 2007). In other studies, the single or combined use of delayed senescence, kernel number, and cob size has been used to indirectly select for grain yield in maize (Lee and Tollenaar 2007; Zheng et al. 2009). In maize, grain-filling rate and grain-filling duration affect yield and find use as indirect selection tool for grain yield in both early and late maize genotypes. Grain filling in maize occurs in three stages: the lag phase, during which cell division and differentiation occurs; the linear phase, during which the rapid dry matter accumulation occurs; and the final phase, when the seed attains the physiological maturity (Lee and Tollenaar 2007). Over 90 % of the total dry matter in the grain is accumulated during linear phase and is known as the effective grain-filling duration (Lee and Tollenaar 2007). The effective grain-filling duration can be extended by selecting for shorter lag and final grain-filling phases. There is limited information on the inheritance of grain-filling rate and effective grain-filling duration and their use as indirect selection tools for high yield in early-maturing maize. Several maize genotypes with varying grain-filling attributes were identified (Gambin et al. 2007; Borras et al. 2009).

3.1 Kernel Numbers

Kernel (grain) numbers and weight are two important traits that influence yield in cereals. Both traits are influenced by genetic factors, physiological processes, and environmental conditions that occur during grain filling and development. Grain-filling-associated traits have been historically considered to be secondary traits for breeding high grain-yielding early-maturing maize genotypes.

Carvoca and Otegui (2007) reported that in maize the grain-filling rate is influenced by sink capacity, a trait that is dependent on the total number of kernels, which is in turn related to efficiency in kernel set and is influenced by the ASI. However, the existence of a negative correlation between grain-filling rate and effective grain-filling duration complicates the simultaneous exploitation of these traits. Yield decline of up to 90 % in maize has been reported as the ASI increases from −0.4 to 10 days (Bolanos and Edmeades 1993). A long ASI also reduces the number of grains per plant and grain yield in maize (Edmeades et al. 1993). From these observations, it appears that the grain yield is heavily influenced by ASI especially under stress conditions. In addition, resource utilization or sink capacity has also been described as key factors affecting grain yield in maize (Tollenaar and Wu 1999). Resource utilization can be improved by selecting for larger and heavier seed size as well as more kernels per plant (Zheng et al. 2009). These observations highlight the association between grain filling, flowering traits, and

grain yield. These traits have the potential to be used as indirect selection tools for high-yield early-maturing maize as they are predictive of the genotype's sink capacity and, hence, yield.

3.2 Gametophyte Development

Studies in cereal crops report the effect of post-anthesis stress on grain filling and grain size (Yang and Zhang 2006; Sinclair and Jamieson 2006) and fail to elaborate on pre-flowering stresses experienced by plants. As grain number is a direct yield component, it is logical to assume that plants disown vital organs such as tillers and florets so all the energy is directed to produce larger-sized seeds albeit in smaller numbers for dissemination and perpetuation. This logical assumption is actually unfavorable when it comes to crop plants where yield, or grain numbers, or grain size are important attributes. Grain numbers relate more to yield than grain size *per se*; however, grain size might influence market preferences. It may be ideal to have bigger grains and larger numbers to better reward growers. Fischer suggested a 10–14-day pre-anthesis window as a key determinant of grain numbers in wheat (Fischer 1973, 2011), although considering the reproductive phase is just beginning and the androecium is small and wrapped by leaf sheath at this stage. Similar influence of stress during pre-anthesis has been reported in rice. In rice, right from meiosis, the reproductive development becomes irreversibly committed to male and female gamete development. Meiosis, which reportedly occurs simultaneously in the male and female organs (Bennett et al. 1973), if impacted by a stress condition, can lead to drastic reduction in grain number. For instance, in self-pollinating cereals such as wheat and rice, where pollen viability is short, frost or drought stress during microspore development can lead to sterile pollen formation and, therefore, have detrimental effects on grain number (Oliver et al. 2005; Ji et al. 2010).

Flowering time modulation is an important adaption mechanism to overcome environmental adversaries. In self-fertilizing crops, such as rice and wheat, flowering is not synchronized and proceeds from the top to down, i.e., top of the panicle to the bottom in rice or from the middle of the ear towards the top and bottom in wheat, which could compound their susceptibility to stress conditions. Contrastingly, the maize ovary is sensitive to drought stress during the reproductive stage (Zinselmeier et al. 1995), also manifested by the fact that male and female flowers are separate and less dependent on self-pollination than rice and wheat. Environmental signals such as temperature and photoperiod control specific switches leading to transition from vegetative to reproductive stages (Amasino 2010; Trevaskis 2010). Short- and long-duration varieties basically aid plants to time their response to specific environments and thus avoid stress conditions, which may not necessarily improve yield. It may not be easy for condensed crop duration to match or produce higher yields than an extended duration crop. Despite these

strategies, abiotic stress can manifest itself anytime, and crops are not exempt in field conditions. Avoiding the stress is one thing, and tolerating the stress is another, which perhaps is the one worth searching.

3.3 Cytoplasmic Male Sterility

Cytoplasmic male sterility or CMS results from specific nuclear and mitochondrial interactions in the plant, while male sterility refers to the failure of plants to produce functional anthers, pollen, or male gametes. CMS is under extranuclear genetic control (under control of the mitochondrial or plastid genomes) and typically shows non-Mendelian inheritance, with male sterility being inherited maternally (Budar and Pelletier 2001). Such maternal inheritance plays an active role in the evolution of gynodioecious species. These mitochondrial dysfunctions affect only the anthers and not the whole plant. While male sterility can result from adverse growth conditions and from diseases, some of the CMS mutations are known to disturb mitochondrial metabolism in the tapetum of anthers (Mizelle et al. 1989). This may result in poor respiratory performance and an inability of the mitochondria to meet energy requirements for successful gametogenesis (Pelletier and Budar 2001; Chase 2006). The result is premature tapetal cell death and abortion of pollen development. The phenotypic manifestations of male sterility are very diverse from the complete absence of male organs, the failure to develop normal sporogenous tissues (no meiosis), the abortion of pollen at any step of its development, the absence of stamens dehiscence, or the inability of mature pollen to germinate on compatible stigma. Although there exists multiple causes for pollen abortion, cytoplasmic male sterility, which depends on mitochondrial genes, is the determinant of gynodioecy, most frequently met in natural populations. All these studies indicate the importance of mitochondria on plant gamete biology.

The mitochondria also influences programmed cell death, and CMS has been vital in demonstrating the role of plant mitochondria in CMS (Chase 2006). CMS and abiotic stress at the young microspore stage, in anthers, induce programmed cell death response in the tapetum; however, the cell death response could likely be a combination of two totally different upstream triggering pathways which is not fully resolved. As CMS has been exploited in plant heterosis breeding for hybrid seed production for a long time, we need to carefully consider the longer-term influence of mitochondria on gamete development. The use of gametocides or chemical hybridization agents (CHA) has been employed in reversible switching of gametes (Cross and Ladyman 1991) or for arresting male fertility. The mode of action and precise targets are subjects of investigation, though some of these components have been shown to interfere with pollen meiosis and early microspore development. It is very likely that some chemical agents, through interference with tapetal function, have a similar result to the effect of abiotic stress on pollen development. It is becoming clear that the tapetum is distorted via a bacterial ribonuclease gene (Mariani et al. 1990) or callose deposition

modulation (Worrall et al. 1992) and inhibition of respiratory enzymes (Yui et al. 2003). These studies demonstrate the susceptibility of male gametophyte development and the relationship to tapetal health influencing pollen development (Pelletier and Budar 2001).

3.4 ABA Hormone

Abscisic acid (ABA), an important phytohormone, is known to play a significant role in stress signaling by integrating numerous stress-related signals and scheming downstream stress responses. ABA plays important roles in several physiological processes including seed dormancy, germination, seed development, stomatal closure, embryo morphogenesis, storage proteins and lipid synthesis, leaf senescence, and defense against pathogens (Swamy and Smith 1999). As abundance of ABA often follows stress signaling, it is referred to as the plant stress hormone (Mahajan and Tuteja 2005).

Response of ABA to stomatal closure is considered as a major stress response conferring drought tolerance in plants. During day time, when there is active photosynthesis, CO_2 diffusion into the chloroplast occurs through stomata and the stomata remain opened for an extended period of time. During this process, most of the water (~90 %) absorbed by plants is lost through transpiration. Under drought conditions, ABA alters ion transport of guard cells so as to promote stomata closure thereby, slowing the transpiration process (Pillai et al. 2012). Several studies support the hypothesis that ABA-dependent stomatal closure is dosage dependent by either elevated levels of ABA in vivo or modulation of guard cell response to ABA (Iuchi et al. 2001; Wang et al. 2005, 2009). Mahajan and Tuteja (2005) report that ABA-dependent stress pathways are mostly related to osmotic stress gene expression. Loss of function mutants were used to study the effect of ABA under water stress in many plant species including maize (Swamy and Smith 1999). Many of these mutants were reported to have similar growth and development with respect to their wild types under normal growing conditions; however, ABA-deficient mutants readily wilt and die, if the stress persists. Similar results were obtained under salt stress, where the ABA-deficient mutants expressed poor growth (Swamy and Smith 1999; Xiong et al. 2001).

In addition to drought, many of the abiotic conditions such as cold and salinity are known to result in desiccation of the cell and osmotic imbalance. There could be a common signaling pathway shared by ABA and elements in various stress signals to maintain cellular homeostasis (Shinozaki and Yamaguchi-Shinozaki 2000; Tuteja 2007; Agarwal and Jha 2010). Research indicates that the interaction between various signaling pathways could be mediated by elements like calcium (Knight et al. 1997; Chinnusamy et al. 2004).

Transcription factors such as AREB1, MYC/MYB, RD22BP1, and DREB2A/2B are known to regulate the ABA-responsive gene expression in plants (Kim et al. 2004; Mahajan and Tuteja 2005; Reyes and Chua 2007). These regulatory

elements require interaction with their corresponding *cis*-acting elements such as ABRE, MYCRS/MYBRS, and DRE/CRT (Mahajan and Tuteja 2005; Tuteja 2007; Rahaie et al. 2013). For example, DREB2A and DREB2B are known to *trans*-activate the DRE *cis*-element of osmotic stress genes to retain the osmotic equilibrium of the cell (Mahajan and Tuteja 2005). MYC and MYB proteins are synthesized only after accumulation of native ABA, indicating their involvement at later stages of stress responses (Tuteja 2007). There are also reports indicating ABA-induced accumulation of miR159 and subsequent control of transcript levels of two MYB factors during *Arabidopsis* seed germination (Reyes and Chua 2007).

Nakashima et al. (1997) reported that an ABA-independent pathway also exists in the dehydration stress response of *Arabidopsis*; an example is *ERD1* (early responsive to dehydration 1—the Clp protease regulatory subunit encoding gene) that responds to dehydration and salinity stress before the accumulation of ABA. Drought stress-regulated ABA biosynthesis depends on a key enzyme, 9-*cis*-epoxycarotenoid dioxygenase (NCED) involved in ABA biosynthesis (Qin and Zeevaart 1999; Iuchi et al. 2001). NCED3 and/or its corresponding orthologs could be valuable to improving drought tolerance in cereal crops. Protein farnesylation, a post-translational modification resulting in attachment of C_{15}-farnesyl residues to the carboxyl termini of specific target proteins, has been implicated in negative regulation of ABA signaling pathway in guard cells (Pei et al. 1998). The utility of farnesyltransferases as biotechnological targets for enhancing drought tolerance was studied in several crops (Wang et al. 2005, 2009). Rapid adjustments of ABA in response to environmental changes are proposed to be due to the activation of inactive ABA pools by polymerized AtBG1, a β-glucosidase, that hydrolyzes glucose-conjugated molecules (Lee et al. 2006).

Many of the abiotic conditions are known to result in desiccation of the cell and osmotic imbalance. There could be a common signaling pathway shared by ABA and elements in various stress signals to maintain cellular homeostasis (Shinozaki and Yamaguchi-Shinozaki 2000; Tuteja 2007; Agarwal and Jha 2010). Research reports indicate that the interaction between various signaling pathways could be mediated by elements like calcium (Knight et al. 1997; Chinnusamy et al. 2004) and potassium (Hsiao and Läuchli 1986; Benlloch-González et al. 2008). These authors have noted that moderate starvation of potassium inhibits water stress-induced stomata closure and resistance to low-water conditions.

4 Genetic Approaches to Improving Cereal Yield Under Abiotic Stress

Numerous approaches to address the issue of abiotic stress have been highlighted in various publications. Starting from genetic engineering involving *cis*- and *trans-genes*, uses of engineered transcriptional regulators, supplemented by genomics and computational biology, are some of the popular approaches.

4.1 Plant Architecture

Leaf size and leaf angle affect plant architecture. For instance, it is believed that during the past century, yield increases in corn have been mostly due to adaptation of hybrid maize plants to higher plant densities, increasing from 30,000 plants/ha to over 80,000 plants/ha now (Duvick 2005). The leaves of these modern plants have become more upright; increasing the angle between the midrib and soil maintains light capture under high density (Pendleton et al. 1968; Lambert and Johnson 1978). Tian et al. (2011) found that the changes in leaf angles range from one maize variety to another by up to 80 degrees; the largest effect from a single gene was only 1.5 degrees. Similarly, rice plants with more upright leaves improved light capture and accumulation of leaf nitrogen for grain filling (Sinclair and Sheehy 1999). These indicate that leaf angle and leaf size influence leaf architecture and define canopy morphology and photosynthetic efficiency and yield. Using a genome-wide association study of the maize-nested association mapping panel, Tian et al. (2011) demonstrated that the genetic architecture of the leaf traits is dominated by small effects, with little epitasis, pleiotropy, or environmental interactions.

Studies using nearly 300 diverse maize lines and 1.6 million sites on the maize genome, where one individual may vary from another, to decipher leaf characteristics such as the genetic architecture of upper leaf angle, leaf length, and width using genome-wide association studies have revealed that variations at the *liguleless* genes (*lg1 and lg2*) contributed to more upright leaves (Pendleton et al. 1968; Lambert and Johnson 1978; Moreno et al. 1997; Walsh et al. 1998). The ligule and auricle are the regions separating the blade and sheath of a maize leaf, which allow the leaf blade to bend away from the stem. *lg1* and *lg2* mutants have no ligule or auricle, leading to considerably upright leaves than their normal wild-type counterparts (Moreno et al. 1997; Walsh et al. 1998). *lg2* mutant alleles lead to significant grain yield increase in maize hybrids (Pendleton et al. 1968; Lambert and Johnson 1978), whereas the effect of the *lg1* mutant alleles depends on genetic background (Lambert and Johnson 1978). The associations around *lg1* and *lg2* are critical to defining the upper leaf angle. Insightful findings into the genetic basis of maize leaf architecture can be used to develop plants with modified leaf architecture for high-density planting, and thus tolerate stress leading to improved yield.

4.2 Inflorescence Arrangement

Meristems are a pool of stem cells from which multiple plant organs develop, and meristems have vital role in developmental patterning and control of plant morphogenesis. Mutations affecting meristems have allowed us to understand meristem formation, maintenance, and enlargement (fasciation). Fasciation originates from the Latin word "*fascis*" meaning bundle and refers to variations in plant form

resulting from proliferative growth. Fasciation has been an interesting research area, because fasciated variants have contributed to yield increase as early as the 1940s (Luckwill 1943; Zielinski 1945). Taguchi-Shiobara et al. (2001) identified a novel *fasciated ear2* (*fea2*) mutant of maize. The *fea2* mutant plants developed larger meristems during inflorescence and floral shoot development, and ear inflorescence meristems showed severe fasciation, suggesting that the role of *fea2* could be to limit the growth of these meristems. Detailed analysis reveals that *fea2* encodes a leucine-rich repeat receptor-like protein that is targeted to the plasma membrane. It is interesting to note that mutants in *fea2* display severe fasciation of the ear and an increase in the number of vertical rows of seeds produced. Thus, *fea2* can increase the number of kernels, thereby the kernel number/ear. This appears to be a topic of great research interest for the Pioneer Hi-bred, a member of the plant biotechnology industry.

As plant growth and development depend on meristems that are reservoirs in plants, the equivalent of stem cells, when provided the right genetic signal, the cells in the meristem develop into plant organs such as stem leaves and flowers. Though it is logical to ask that if plants are big, do they make more grains, or does it make sense to divert the resources of the plants to grain-making and keep the plant itself small? In an attempt to answer several questions surrounding this topic, Bommert et al. (2013) hypothesized that increasing the size of inflorescence meristem would give rise to large number of flowers and thereby, after pollination a large number of seeds that would mature into large number of kernels (grains) in maize, called kernel row number. Using quantitative trait loci (QTL) mapping and TILLING resources, varying mutants of *fea2* were developed and their analysis shows that by producing a weaker-than-normal version of the *fea2* gene, whose protein is still functional, it is possible to increase meristem size and produce ears with more rows and more kernels in maize resulting in about ~13 % increase in kernel yield (Bommert et al. 2013). This advantage was possible without reducing the length of the ears or inducing fasciation to flatten the ears which can dramatically reduce yield when *fea2* is completely shut off.

4.3 Germplasm Tolerance to Stress

Nature harbors germplasm that are tolerant and sensitive to abiotic stress. Our ability to identify and characterize germplasm that are tolerant and sensitive to various stress should facilitate breeding and mapping of QTLs. In order to classify germplasm as tolerant and sensitive, adequate screening and scoring criteria need to be developed. Numerous reports mention that developing a screening approach and consistent scoring criteria is a major challenge. For example, drought is a multigenic trait and multiple interactions are reported. Mimicking drought alongside, the phases of plant development is a major challenge be it in controlled conditions or open field. Despite such bottlenecks, a few screens have been developed. Taking advantage of the high sensitivity of young microspore-stage pollen development, a screen was developed to select cold-tolerant rice germplasm in a collection of rice

varieties from the Yunan province in China, where rice is grown at high altitudes (>2,000 m) (Oliver et al. 2005). Similarly, a high-altitude sorghum line was found to have higher tolerance to stress at the young microspore stage (Brooking 1979). A wheat line derived from CIMMYT synthetic wheat was shown to have strong young microspore stage drought tolerance (Ji et al. 2010). These examples show that abiotic stress tolerance is present in cereals where the trait may have been maintained out of necessity, often imposed by topographic circumstances (e.g., high-altitude rice for cold tolerance). It may likely be possible that these tolerance mechanisms may have been lost from currently available commercial germplasm pool due to direct or indirect selection or due to negative selection drag when consistently selecting for other traits of interest. For example, in wheat germplasm from CIMMYT, which was selected under irrigated conditions, has been successfully adopted in semiarid countries such as Australia. This may have resulted in the loss of tolerance in current Australian wheat cultivars (Brennan 1989). Genes responsible for maintenance of grain number under drought may also have been lost due to selection pressure in breeding programs for grain size and weight as per the grain industry requirements. If there is a terminal drought, then genotypes that are conservative in number of grains they set may be selected, as these are also likely to have larger grains at maturity (Dolferus et al. 2011).

4.4 Association Mapping and Stress Biology

Genome-wide association mapping in cereal is a fairly recent approach. In a conventional biparental segregation-based mapping, allelic differences between the two parental lines are analyzed. Association mapping allows us to survey genetic variation across a wide spectrum of material/population. Historical field trial data or genetics of an existing breeding is useful in elucidating the genetic basis of some agronomic traits. Using this approach, marker-trait associations for a whole set of agronomically important traits including stress tolerance could be detected. With a mix of the kinship and the Q-matrix approach, it is evident that many loci are easily detected representing either many known major genes or a QTL.

Many traits in cereals that are agronomically critical tend to be quantitatively inherited. In doing so, often the genes responsible for the variation of these traits are hard to discover and detect. Classical mapping populations developed from a cross of two parents such as recombinant inbred lines and doubled haploid lines have been employed to identify and map many quantitative trait loci (QTL) in cereals (Börner et al. 2002; Quarrie et al. 2005; Huang et al. 2006; Kumar et al. 2007). Another approach to identify QTLs is association mapping, which in simple terms is based on correlating a genotype with the phenotype in germplasm collections or naturally occurring populations (Breseghello and Sorrells 2006a; Kraakman et al. 2006; Roy et al. 2006). The basis for this approach is that linkage disequilibrium (LD) tends to be maintained over many generations between loci which are genetically linked to one another. High LD is expected to be observed between loci in tight linkage as recombination events, since the mutation should have eliminated

LD between loci that are not in close distance (Breseghello and Sorrells 2006b). Using this approach, it is possible to screen a very large gene pool with adequate representation of all genes. This approach may also shorten the study duration by eliminating the bulk of mapping studies typically performed via multiple crossing cycles in a population. Furthermore, it facilitates the mapping of many traits in one set of genotypes, resulting in a finer mapping resolution often resulting in small confidence intervals of the detected loci compared to classical mapping, where the identified loci need to become finely mapped (Remington et al. 2001). A false-positive association between a trait and a marker(s) is a potential threat mostly due to the population structure and, therefore, mandates a vigorous statistical analysis (Falush et al. 2003).

Neumann et al. (2011) conducted a genome-wide association study in a winter wheat by employing a large number of diversity array technology markers for genotyping a winter wheat core collection of 96 accessions. The germplasm was structured into two subpopulations and 20 agronomic traits were measured in field trials conducted over up to 8 growing seasons. A general linear model incorporating only the Q-matrix and a second mixed linear model that included the kinship matrix were used to perform association analysis. Nearly four hundred marker-trait associations significant in both models were detected, of which the intra-chromosomal locations of some of those were known and many unknown. Such unknown marker-trait data are worth further exploration and are bound to assist in mapping stress-linked QTLs in cereals (Clarkson et al. 2005). This idea was previously reported by Crossa et al. (2007), when these researchers discovered many marker-trait associations by analyzing historical data from multilocation field trials specifically for traits related to cereal grain yield and resistance to leaf diseases in wheat.

5 Prospective Approaches to Tackle Abiotic Stress in Cereals

As considerable resources are being deployed to tackle the problem of abiotic stress in cereals, challenges remain in not only translating the functional efficacy achieved using molecular tools to field performance but also ensuring that the incremental yield realized is economically significant to justify the additional investments. Other challenges involve understanding the association of numerous molecular events and the whole plant physiological response to abiotic stress and capitalizing on the knowledge. Does this mean we are lacking the right model to enhance our understanding of abiotic stress or is it just getting too complex to comprehend?

5.1 Do We Know the Bottlenecks?

Often yield reduction or loss is a major observation in abiotic stress studies. Abiotic stress varies widely in form and occurrence, and so it is crucial that cereal crops are able to tolerate a wide variety of stresses so that the yield component is

stable. As the tolerance mechanism involves a multifaceted response involving modification of physiological responses and biochemical reactions in a complex manner, it is important to identify, monitor, and understand the morphological, physiological, and molecular components associated with these responses and to be able to correlate these complex responses to yield stability and the ultimate realized yield. This continues to be a challenge, especially determining associations between molecular responses and whole plant level morphological and physiological traits. It may be possible with the current technologies to understand the complex nature of abiotic stress tolerance using genome-wide transcript analyses to identify genes associated to a trait of interest (Zong et al. 2013). Association mapping as described earlier can be helpful in such scenarios. Likewise, forward genetics with phenotypic screening of tagged populations (e.g., T-DNA insertion mutants, transposon tagged) can help identify genome-wide targets for identifying connections between trait functionality and transcripts. While mimicking or replicating a stress condition in stress studies can be challenging for plant-based experiments, these approaches described may still provide us with a more simple approach to understand the connection and replicate these changes in a repeatable manner. So in essence, is yield the bottleneck to understand abiotic stress? Yield is one visual component that is the resultant of plant and its environment (stress). Yield loss may be an after-the-fact observation, i.e., observed after the plant has gone through fighting stress. So considering yield alone as an indicator of stress may not be ideal, although achieving yield stability that can be transferable across more than one growing environment and tolerance that can address stress occurring at more than one physiological growth phase during the cropping system remains a challenge.

Adoption of proper stress management protocols is critical to stress research. A rigorous, standard, and repeatable system of studying stress response is very much needed. Several inputs are required to enable plant growth, and adopting a different practice or the slightest modification in a complex interactive network can impact our inference.

5.2 Plant Phenomics

Genomics revolution has contributed tremendously to plant biology, especially in the field of biotechnology. Plant scientists are loaded with enormous amounts of information on genome structure and evolution, with the ability to dramatically change the way new crop varieties are developed in order to tackle the food security challenge. Data mining requires skill, and it is cumbersome to manage the steady flow of terabytes of data that is being generated daily and needs to be analyzed to search for functional meanings. Despite the high throughput in sequence data production, the association of genes with their function is lagging behind. The study of the relationship of specific genes with the measurable characteristics of a plant (phenotype) is crucial to understand the specific role and function of genes.

Phenotype is the end product of interactions that occur between genes and the environment (signals). To discover gene function, scientists usually play with these two factors, using mutants of target genes (or candidate genes), either naturally occurring or available within the genetic diversity of the species or experimentally induced, and growing plants under defined and preset environmental conditions. Changes in phenotypes typically offer clues for the role or function of the target gene in the plant's physiology or biometabolism. Target genes could be either knocked out from performing their function fullest (loss of function) or expressed (gain of function) and/or overexpressed. This contrasting scenario is likely to yield potentially opposite phenotypes although they often are invisible and hard to detect. The study of phenotype responses to the stressful environment requires the growth of plants under various environmental conditions. As such in plant breeding, selection schemes are applied in different fields, years, and seasons requiring intense human efforts and often subjective sampling. The measurement of phenotype is a time-consuming step characterized by laborious manual sampling and disruptive measurements. In plant abiotic stress response breeding, reliable phenotyping protocols are crucial, and their unavailability or limited development is a cause for worry.

High-throughput plant phenotyping or phenomics is becoming the bottleneck in plant biology to close the gap between plant genetics and physiology. This strong demand recently stimulated research interest in developing technologies and platforms that are able to augment the phenotyping process. Some of the notable entities in Europe include Crop design, Keygene, an international consortium of few public research institutions (http://www.plantphenomics.com), and several researchers in Australia, Canada, France, Germany, and Italy. Plant phenomics represent an ensemble of technologies that are based on nondestructive image analyses that exploit either the reflectance properties of the incident light on plant tissues or including reflectance and fluorescence imaging, thermal imaging, hyperspectral imaging, X-ray computed tomography, 3D-image analysis, and pattern recognition or structural and functional features obtained by MRI and X-ray CR scanning. In a typical automated setting, cameras can be moved to plants or vice versa to capture images, for example, in a greenhouse. The Plant Accelerator, Australia; PhenoPhab, Netherlands; Metapontum Agrobios, Italy; Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany; and organizations like Monsanto, USA, have achieved greater capabilities in 3D-image acquisition and analysis.

Association between genotype and phenotype must be better understood so that our ability to predict phenotype performance is enhanced. Genomics is considered a key to comprehend gene–phenotype associations at the level of candidate genes and sequences. This will be critically important for quantitative traits such as drought tolerance, where performance is regulated by many loci and influenced by multiple genotype × environment (GxE), gene × gene (GxG) interactions (epistasis), and gene × gene × environment (GxGxE) interactions. One way to delve into this aspect is by identifying and measuring all secondary traits associated with grain yield. These may serve as a guide to specific mechanisms that contribute to grain

yield under drought. Thus, water depletion patterns, leaf rolling, and canopy temperatures are indicative of root exploration and water extraction capacity, and chlorophyll concentration could be a reference to the plant's photosynthetic ability.

It must be noted that a phenome is different from a phenotype. Studying a phenome or phenomics aims to characterize phenotypes in a rigorous and formal manner and link these traits to the associated genes and/or alleles. While phenomics shares similarities to traditional gene screen approaches, it also differs by involving large-scale phenotypic data collection and analysis, whereas the phenome is a true catalog of measurements. Once a large population of genetic variants with the goal of sampling variation in many genes has been identified, each genotype needs to be assayed for a large number of traits using well-tested and high-throughput standard protocols to secure accurate and reproducible data. Growth conditions (viewed as a variable) need to be properly defined and continuously monitored. The data generated (both the phenotypic and metadata descriptors) from a specific experimental condition (e.g., a specific drought-simulated growth condition) is captured and formatted in a manner that can be subject to extensive data analytics. It is envisaged that such analysis would identify relationships between genotypes and phenotype as well as reveal correlations between seemingly unrelated phenotypes (Schauer et al. 2006; Lu et al. 2008) or genetic loci (Gerke et al. 2009). Phenotypes are defined by the interactions of GxE. Therefore, it is important to collect a large number of measures across multiple environments, at different developmental stages, and for multiple cell/tissue/organ types. As valuable it may be, it is unrealistic to sample all alleles for every measurable trait under every possible growth condition; the experimental design and methods of data analysis must be aligned to the preferred and expected outcome (Shasha et al. 2001).

5.3 Transgenic Versus Conventional?

Plant tissue culture methods have a wide scope for the creation, conservation, and utilization of genetic variability for the improvement of field crops. Micropropagation technology ensures true-to-type, rapid, and mass multiplication of plants for quick bulking up of new varieties and rejuvenation of old varieties. Cellular techniques such as anther or microspore culture, somaclonal variation, embryo culture, protoplast culture, and somatic hybridization are being exploited to generate useful genetic variability for incremental improvement of field crops. Using another culture or pollen culture, several cereal crops including rice, wheat, barley, and maize, amongst others, have been released. Double haploid approach is increasingly being used for the rapid development of populations for QTL mapping and construction of genetic linkage maps for traits of interest.

Abiotic stress tolerance is largely a quantitative trait, but several single gene-controlled secondary traits such as flowering time, ear type, plant height are known. Since abiotic stress tolerance trait is a multigenic response, it is safe to assume that

single genes have minor or negligible effect and it is difficult to visualize their effects. It may, therefore, be a wise idea to introgress multiple QTLs that contribute to an abiotic stress tolerance phenotype. Using a genome resynthesis approach, it is possible to introduce new genetic variation to expand the genetic pool and therefore enrich the QTL and breeding toolkit.

6 Conclusions and Future Perspectives

Yield increases are mostly the result of adaptations engineered by breeders such as high-density planting or modifications to the root system and/or nutrient uptake. Over time, it is possible that plants evolve to different adaptations and human needs. Plants are being planted closer (high-density planting), and they tend to evolve with the need and leaves of some cereal crops such as maize have become more upright to maintain access to sunlight in crowded plots. It is interesting to note a study by Tian et al. (2011) that suggests variation in traits is the sum of many small effects in genes, similar to an evolving concept in human genetics. In another study reported from Jackson Laboratory, Cold Spring Harbor Laboratory, researchers studying *fea3* mutant plants found that during reproductive stages, the *fea3* mutants developed enlargement of the inflorescence meristem causing fasciation in the mutant (Je et al. 2012). Fasciation leads to enlargement of the ear tip, extra kernel rows, and an overall irregular arrangement of rows, thus stressing the importance of discoveries in fundamental biology research in the modern—omics era. Knowledge of *fea* genes may be useful to crossbreed the weak *fea2* or *fea3* gene variant or allele associated with higher kernel yield with the elite maize lines used in food crops to explore potential higher-yield plants.

Though we discussed the advancements made via employing molecular biology or genetic engineering approaches in plant biotechnology to attack the stress issue, it is becoming increasingly clear that it is not a simple problem. Unlike relatively stable abiotic stress such as soil salinity or acidity, drought stress in most cereal growing regions is strongly dependent upon stochastic weather processes. Transient stresses contribute to a complete genotype × season, genotype × season × management interactions, since stress tolerance varies among genotypes and throughout the season of the cropping period. Yield monitors have enabled the farmer to understand yield variations within a field, and it usually relates to variation in soil texture and plant-available water. Such complex problems, i.e., abiotic stress caused mainly by heat and drought in cereals, may be well addressed using integrated approaches in plant biotechnology. Testament to this is the African Maize Program by CIMMYT that understood the complexities involved and have launched programs that try to address water efficiency in maize, drought tolerance in maize, and geography-specific maize suitable for African soil conditions.

This is also an example of public-private partnership trying to fight tooth-and-nail problem of abiotic stress in maize. Monsanto, BASF, and Pioneer Hi-bred are collective suppliers of proprietary germplasm, advanced breeding tools, subject matter expertise, and stress tolerance genes, while CIMMYT being the CGIAR institution is providing high-yielding maize cultivars adapted to the African climate and complementing with conventional breeding and testing for drought tolerance. There is increasing interest in the belief that stress tests conducted in the greenhouse differ from that performed in the field, given the resource competition and changing input factors that plants require for growth and development. The cultivars developed through such an initiative are intended to be distributed by the African Agricultural Technology Foundation to African seed companies and small farmers as part of their seed business. This integrated approach considers soil biology, local climate, conventional breeding, use of transgenes, modified chemical fertilizers, and use of high-yielding and stress-tolerant germplasm which is clearly a winning strategy than fighting it alone.

Such collective efforts are only possible when new information is utilized as it becomes available. Technology has enabled us to deploy custom imaging solutions from controlled environment screening and in-field analytics via remote sensing approaches. For example, taking advantage of the Next-Gen Sequencing (NGS) or transcriptomics and proteomics toolbox and combining them with conventional, *trans*-genic, and *cis*-genic breeding to excise markers (e.g., antibiotic genes) or use of TILLING to mutate genes would pave the way for a well-packaged product that can attack the abiotic stress problem nagging the plant biotechnology industry. "Seeds of Discovery" (SeeD) is a new and concerted initiative of CIMMYT (Prasanna 2012) aimed at discovering the extent of allelic variation in the genetic resources of maize and wheat through high-density genotyping, phenotyping for prioritized traits and novel bioinformatics tools. Newer model plants such as *Physcomitrella patens* and *Brachypodium distachyon* are the subject of extensive research with goal of enhancing our understanding of abiotic stress. *P. patens* is a haploid moss known to be tolerant to abiotic stresses (drought, salt, osmotic stress) and genetically amenable. *B. distachyon* is a wild grass relative of many cereal crops with a short life span and has a small genome that has been sequenced. Can we employ such an advanced toolkit to challenge ourselves against the traditional belief that crop plant genotypes that produce desirable traits (increased yield or higher nutritional quality) are better identified under field conditions of soil, climate, and biotic stress agents similar to where the final varieties will be grown commercially? It is encouraging to see that the traditional beliefs are being challenged. Nevertheless, we may have to accommodate some conventional wisdom such as encouraging and expanding the adoption of stress-tolerant crops to non-stress areas too otherwise the adoption could be limited to stress-prone areas. A right mix of conventional wisdom, germplasm availability combined with inference derived from the several advanced platforms, will enable us to address the abiotic stress challenge.

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Chapter 10
Improvement of Crop Production Under Saline Stress by a Biohydraulic Approach

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1 Introduction

Various researchers have dedicated efforts to relating genotype differences between salt-tolerant and salt-sensitive plants with some physiological and biochemical parameters for developing fast methods of screening for saline tolerance (Alian et al. 2000). In the majority of the agricultural crops, it is well established that the capacity of tolerating salinity depends on the genetic and biochemical characteristics of the species. The mechanisms of response to salt treatment have been studied in several species. The comprehension of saline stress, however, remains unclear because of the complexity of the process which has an ionic and an osmotic component, comprising morphological, physiological, and metabolic changes (Bray 1993). According to Munns and Termaat (1986) and Marschner (1995), there are three ways by which plants become stressed in saline soils: (1) the decrease in water potential of the root medium results in water deficit; (2) the toxic effects of ions, mainly Na^+ and Cl^-; and (3) nutritional imbalance by depression in the uptake and/or the transport to the shoots.

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The excessive accumulation of salts in cultivated soils is a frequent problem, particularly under irrigated conditions, becoming a permanent risk for food production (Bohnert and Jensen 1996). Generally, a method to reduce the salt accumulation in soils is the leaching from the rooting zone of the soil profile. However, this agricultural practice is not a sustainable strategy because it is a resource-consuming and expensive approach, while the availability of good quality water for irrigation and leaching is generally limited (Sohan et al. 1999). In addition, in recent years, the use of hydraulic methods to alleviate soil stress has received increased attention (Kahlaoui et al. 2011a, b, 2012). The improvement of the irrigation techniques has been employed to preserve water until the present. The aim of these improvements was to gain efficiency of water use while minimizing the cost. The subsurface drip irrigation (SDI) system is the best method to irrigate agricultural crops under certain conditions (Hachicha et al. 2006; Kahlaoui et al. 2011b, 2012).

Besides the environmental constraints and/or controlled application of various factors of stress, the understanding of the functional biology of plant response to various methods of cultivation is thus relevant to identify effective strategies to improve the efficiency of the production process and quality of products (Maggio et al. 2003). Currently, the increasing salinity in the irrigation water is compelling farmers of arid and semiarid regions to innovate their farming techniques for maintaining crop yields while they face the degradation of this main resource (Debaeke and Aboudrare 2004). During the last decades, various techniques used to improve the performance of plants subjected to salt stress have been advanced, besides to the breeding and biotechnological strategies (Maggio et al. 2003). Among these methods, foliar spray of stress metabolites that can be identified and assimilated by plants as components of the stress adaptive response has been included (Ashraf and Foolad 2007). The exogenous application of osmoprotective molecules has beneficial effects on plants subjected to salt stress (Ali et al. 2007). The actual mechanism by which these molecules exert their protective function is not clear at all. One the most common response in plants to stress is the overproduction and accumulation of various types of organic compatible solutes (Ashraf and Foolad 2007; Ahmad and Sharma 2008; Koyro et al. 2012; Rasool et al. 2013a, b; Kahlaoui et al. 2013; Hasanuzzaman et al. 2013). These compatible solutes are characterized by their low molecular weight and high solubility that are usually nontoxic at high cellular concentrations. They have many functions leading to the protection of plants against stresses. Among the functions included are the contribution to cellular osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilization of enzymes/proteins (Yancey et al. 1982; Bohnert and Jensen 1996; Ashraf and Foolad 2007). Because some of these compatible solutes also protect cellular components from dehydration injury, they are generally called as osmoprotectants. These solutes include proline, sucrose, polyols, trehalose, and quaternary ammonium compounds (QACs) such as glycine betaine, alaninebetaine, prolinebetaine, choline *O*-sulfate, hydroxyl prolinebetaine, and pipecolatebetaine (Rhodes and Hanson 1993; Ahmad and Sharma 2008; Koyro et al. 2012).

Despite much effort has been made to genetically modify plants for obtaining overproduction of various osmoprotectants, little success in reaching the desired

protective levels of these osmolytes in plants has been obtained. As an alternative, the exogenous application of various organic solutes improved the resistance of some plants to abiotic stresses (Ashraf and Foolad 2007; Hamdia and Shaddad 2010). This approach, which can contribute to increase the production of crops under environmental stress, however, has not received enough consideration. In this chapter, we postulate that the development of crop plants able to tolerate stress needs to integrate the study of hydraulic methods (SDI), physiological mechanisms, and genetic control at different plant developmental stages. Under this scope, the physiological role of proline (Pro), polyamines (PAs), and brassinosteroids (BRs), as well as their role in increasing the tolerance of plants to stresses, in particular, to salt stress, is reviewed and discussed.

2 Hydraulic Components (Subsurface Drip Irrigation)

The irrigation method used when irrigating with saline water may notably influence the accumulation and distribution of salts in the soil profile and consequently, the crop yield. Among irrigation methods, sprinkler irrigation involves a risk of injury to plants when the available water is saline due to foliar absorption of salts. During the daytime when the rate of evaporation is higher, the risk of damage is increased if the irrigation system is operated. Thus, trickle irrigation or drip irrigation is preferable as it maintains the soil humidity at high levels in the root zone, maintaining a low level of salt concentration and avoiding wetting the crop canopy. Common problems occurring in drip irrigation are the need to wash down the accumulation of salts from the wetting front and the avoidance of drippers clogging (Paranychianakis and Chartzoulakis 2005). Specifically, the SDI seems to be the most effective method for the irrigation with saline water and can avoid these problems. Indeed, the SDI leads to an increase in the water use efficiency and to overcome the decreasing trend on the quality of the irrigation water. As benefits for crop production, the SDI increases fruit yield, maintains a dry soil surface for improved weed control and crop health, and protects drip lines from damage due to cultivation and other practices (Hachicha et al. 2006; Enciso et al. 2007; Thompson et al. 2009). SDI systems are able to apply a little amount of water directly to the root zone of the plants where it can be frequently applied to maintain favorable moisture conditions. Compared to surface irrigation methods as furrow and drip irrigation, the SDI has shown to improve fruit yield and water use efficiency in several agricultural crops (Ayars et al. 1999; Camp 1998; Enciso et al. 2005) including tomato (Fig. 10.1a, b). Furthermore, the use of SDI can also decrease the blossom-end rot in tomato crop (Fig. 10.2a, b). The improvement of fruit yield and fruit quality by SDI was found also in several crops including tomato (Kahlaoui et al. 2011b), onion (Enciso et al. 2007), potato (Patel and Rajput 2007), and squash (Al-Omran et al. 2005). According to Ruskin (2000), the SDI could be employed to apply little amounts of water with high frequency. In comparison to the drip irrigation, the SDI led to save 46 % of water in a heavy texturized soil in which the movement of water occurred mainly

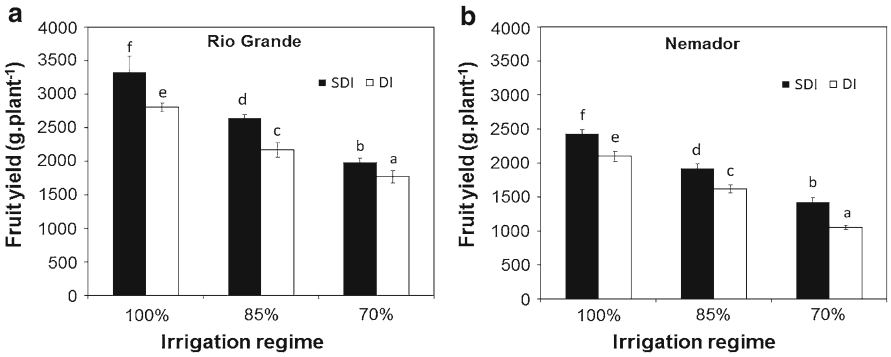

Fig. 10.1 Effect of irrigation systems on fruit yield of two tomato cultivars irrigated with saline water (6.57 dS m^{-1}). All values are the mean ± SE of three replications ($n = 3$), and *bars* with *different letters* are significantly different at ($P \leq 0.05$) according to LSD test

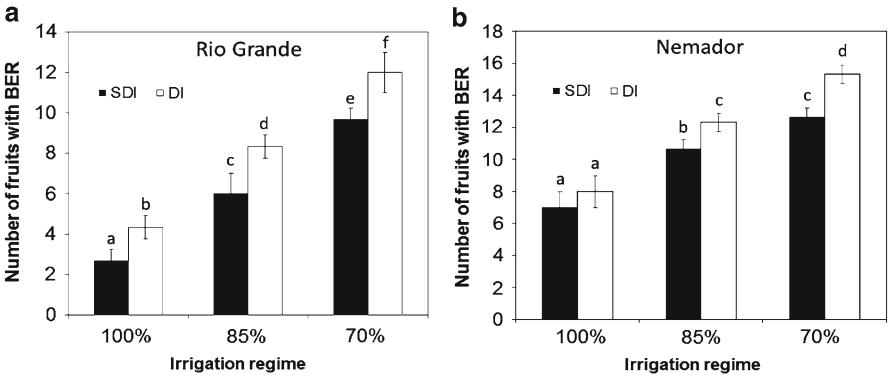

Fig. 10.2 Effect of irrigation systems on fruit BER number of two tomato cultivars irrigated with saline water (6.57 dS m^{-1}). All values are the mean ± SE of three replications ($n = 3$), and *bars* with *different letters* are significantly different at ($P \leq 0.05$) according to LSD test

due to capillary forces (Ruskin 2000). However, the study of Phene et al. (1992), on the impact of SDI in a clay loam soil when drip tubes were placed at 45.0 cm below the soil surface, showed that the soil water remained available at the root zone for the use by plants and no loss due to deep percolation was observed.

3 Biological Components

In areas subjected to salinity, the improvement of the productivity of crops is of main importance (Watanabe et al. 2000). In order to set saline soils into proper cultivation, salt-tolerant cultivars or species represent the best choice (Heuer et al. 2005). However, the accurate and successful selection or breeding of such cultivars and

species supposes an understanding of the mechanisms governing salt tolerance (Ghoulam et al. 2002). Other studies on the tolerance of plants to stress are biochemical mechanisms and the selective accumulation or exclusion of ions, control of ion uptake by roots and transport into leaves, compartmentalization of ions at the cellular and whole-plant levels, and accumulation and synthesis of compatible solutes (Parida and Das 2005).

3.1 Proline

The proline is an amino acid characterized by an exceptional conformational rigidity, and it is essential for the primary metabolism. The accumulation of proline was first discovered in wilting perennial rye grass (*Lolium perenne*) (Kemble and Mac Pherson 1954; Szabados and Savouré 2010). From that time, several studies showed that the proline is accumulated in higher plants when subjected to various environmental stresses as drought, high salinity, heavy metals, high light and UV irradiation, and oxidative stress (Szabados and Savouré 2010; Ahmad et al. 2012a, b; Katare et al. 2012; Rasool et al. 2013a, b). The proline has an osmoprotective function which was initially discovered in bacteria, after which it has been demonstrated a positive correlation between proline accumulation and salt tolerance (Sekhar et al. 2007; Ahmad et al. 2010, 2011). Such results have guided to the assumption that proline accumulates in stressed plants with a protective function, which has been reported by numerous reviews (Hare and Cress 1997; Verbruggen and Hermans 2008; Koyro et al. 2012). However, the accumulation of proline is not always evidently correlated with the tolerance to abiotic stresses. For example, salt and cold-hypersensitive mutants of Arabidopsis (*Arabidopsis thaliana*) have been found to contain high levels of proline (Lui and Zhu 1997). Nevertheless, several studies undertaken on transgenic plants or mutants have reported that the metabolism of proline has a complex effect on the developmental and stress response and that the accumulation of proline is important for the tolerance to certain environmental conditions (Ashraf and Foolad 2007).

The accumulation of proline has several functions in tissues subjected to abiotic stress as osmotic adjustment, C and N reserve for growth after stress relief, detoxification of excess ammonia, stabilization of proteins and/or membranes, and as scavenger of free radicals (Solomon et al. 1994). Moreover, the biosynthesis of proline may be related with the regulation of the cytosolic pH or the production of $NADP^+$ for stimulating the pentose phosphate pathway (Lutts et al. 1999). In spite of the beneficial effects of proline in plants, its accumulation and how it affects the salt tolerance remain under debate. For example, in wild tomato species (Shannon et al. 1987) and rice cultivars (Lutts et al. 1999), the increase in proline level proved to be inversely related to salt tolerance. Conversely, in other crop species, isolated cells, and transgenic plants, high levels of proline were measured in salt-tolerant samples (Winicov 1998; Hare et al. 1999). Notably, works carried out on *Nicotiana sylvestris* (Dix and Pearce 1981) and eggplant (Jain et al. 1987) did not report any appreciable increase in free proline content, while Moftah and Michel (1987) studying soybean

considered the enhanced proline level a mere stress effect, rather than a cause of stress tolerance. Consequently, the role of the accumulation of proline and it metabolism associated to the salt tolerance must be critically examined.

Proline in plants is synthesized from either glutamate or ornithine, predominating the glutamate pathway under osmotic stress conditions (Delauney and Verma 1993; Ashraf and Foolad 2007; Ahmad and Sharma 2008; Koyro et al. 2012). The first two steps of proline biosynthesis from glutamate are catalyzed by the key enzyme delta 1-pyrroline-5-carboxylate synthase (P5CS: γ-glutamyl kinase, EC 2.7.2.11; glutamate-5-semialdehyde dehydrogenase, EC 1.2.1.41). Then, the glutamic acid-5-semialdehyde (GSA) produced by these reactions is spontaneously converted into pyrroline-5-carboxylate (PSC), after which is reduced by P5C reductase (P5CR) to proline (Misra and Gupta 2005; Ashraf and Foolad 2007; Misra and Saxena 2009). A catabolism pathway controls the accumulation of proline thanks to the proline oxidase (PROX), which catalyzes the conversion of proline to glutamate. The activity of the enzymes involved in proline metabolism as affected by stress has been demonstrated in many plant species, including green gram (Misra and Gupta 2005), lentil (Misra and Saxena 2009), tomato (Fujita et al. 2003), peanut (Girija et al. 2002), and mulberry (Ahmad et al. 2010).

The exogenous application of proline plays a pivotal role in the improvement of the tolerance of plants subjected to stress (Ashraf and Foolad 2007; Nounjan et al. 2012). For example, the application of proline in salt-stressed tobacco reduced the effect of salt stress and increased the activities of antioxidant enzymes (Hoque et al. 2007). Moreover, the exogenous application of proline affects gas exchange enhancing the net CO_2 assimilation rate, the transpiration rate, and the stomatal conductance (Ali et al. 2007). It upregulates the levels of protective proteins of stress (Khedr et al. 2003) and reduces oxidation of lipid membranes (Okuma et al. 2004). The proline can be applied to plants by three different ways such as the application to roots, foliar pulverization, and pre-sowing seed treatment (Ashraf et al. 2008; Wani et al. 2012). Besides the beneficial effects of proline, high concentrations can lead to harmful or deleterious effects on growth and metabolism of plants (Ehsanpour and Fatahian 2003; Nanjo et al. 2003; Ashraf and Foolad 2007). For example, a concentration of 30 mM of proline in rice was the most effective for obtaining an improved germination and seedling growth under salt stress, whereas higher concentrations (40 or 50 mm) induced a reduction in growth and lower the ratio of K^+/Na^+ in leaves (Roy et al. 1993). Therefore it is still required to determine the optimum proline concentration in different species of plants.

3.2 Polyamines

Polyamine (PA) was first time reported in human spermatozoa more than 300 years ago (Van Leeuwenhoek 1978; Hussain et al. 2011). Compared to other compatible solutes, the success of PAs can be explained by their particular functions. PAs are small ubiquitous nitrogenous compounds, consisting in aliphatic hydrocarbons

substituted with two or more amino groups. Putrescine (Put), spermidine (Spd), and spermine (Spm) are the main PAs in plants. These amines participate in several processes of regulation such as cellular division and elongation, growth of roots, development of flowers and fruits, replication, transcription, translation, stabilization of membranes and cell wall, chromatin organization, ribosome biogenesis, and programmed cell death (Hussain et al. 2011). According to several studies, the accumulation of polyamine takes place under drought, salinity, extreme temperature, UV-B, heavy metals, mechanical wounding, and herbicide treatment (Pang et al. 2007; Hussain et al. 2011; Ahmad et al. 2012c).

The diamine putrescine is synthesized in plants via ornithine by arginase (EC 3.5.3.1) and ornithine decarboxylase (ODC, EC 4.1.1.17). Putrescine is also synthesized via agmatine by three sequential reactions catalyzed by arginine decarboxylase (EC 4.1.1.19), agmatine iminohydrolase (EC 3.5.3.12), and *N*-carbamoyl putrescine amidohydrolase (EC 3.5.1.53), respectively. Then putrescine can be converted to spermidine and spermine via two sequential amino propyl transferase reactions catalyzed by spermidine synthase (EC 2.5.1.16) and spermine synthase (SPMS, EC 2.5.1.22), respectively. In both reactions, an aminopropyl residue is transferred from the decarboxylated *S*-adenosyl methionine which is synthesized by *S*-adenosyl methionine decarboxylase (EC 4.1.1.50) (Naka et al. 2010).

The physiological implications of the accumulation of PAs and their regulatory role have attracted considerable attention (Bibi et al. 2010; Hamdani et al. 2011). The accumulation of PAs has been considered a mechanism which confers adaptive and protective functions under abiotic stress (Sfichi et al. 2004; Bibi et al. 2010; Hamdani et al. 2011). Consequently, the increase in the internal PAs content has been suggested to reverse the damaging effects and improves the tolerance of plants when exposed to various stresses (Unal et al. 2004; Hamdani et al. 2011). However, PAs occurring as polycations in the cytoplasm maintain the cation–anion equilibrium, while the reduction in the concentration of PAs in the cytoplasm is the consequence of the simultaneous displacement of the excessive cations from it (Janicka-Russak et al. 2010).

The impact of the PAs in resistance to the saline stress remains elusive and disputed, in spite of many experimental data. The role of PAs depends on plant species and plant organs, making the physiological interpretation difficult to be conclusive. Lin and Kao (2002) reported a reduction in Put, Spd, and Spm induced by salinity, whereas Chattopadhayay et al. (2002) showed that salt induced an increase in concentrations of PAs. In rice exposed to salts, Maiale et al. (2004) showed a decrease in Put and Spd while Spm increased. Even a foliar application of Put improved the behavior of rice cultivars exposed to NaCl (Krishnamurthy 1991), suggesting that the endogenous level of Put can be limiting for resistance to salts. In a salt-sensitive rice cultivar, salinity leads to excessive accumulation of Put, with minor changes of the content of Spd and Spm in shoots, while in salt-tolerant cultivars, the same stress caused a remarkable increase in the content of Spd and Spm and a reduction in Put (Krishnamurthy and Bhagwat 1989). The addition of Spd or Spm to a salinized nutritive solution mitigated considerably the damage induced by salinity in rice plants (Chattopadhayay et al. 2002). On the other hand, Ndayiragije and Lutts

(2006) showed that Put, Spd, and Spm did not reduce the suppression of growth induced by salinity in studies undertaken with rice plants. Still in rice, Yang et al. (2007) suggested that the salt-tolerant cultivars AU1, Co43, and CSC1 maintain a high concentration of Spd and Spm, whereas the Put content was not significantly changed at the studied growth stages of plants subjected to salt stress. When studying responses under water stress, endogenous levels of individual and total PAs in roots of chickpea increased in tolerant plants more than in sensitive plants of soybean. The damage by stress was more obvious as PAs levels decreased in sensitive species (Nayyar et al. 2005). The reduced levels of PAs in soybean, particularly Put and Spd, compared with chickpea, were correlated with higher damage caused by stress and lower water content.

The exogenous application of PAs has not only been employed as a convenient way to reveal their implication in the salinity response but also as an effective practice to increase tolerance and, eventually, productivity in crops under high salinity (Chattopadhayay et al. 2002; Hussain et al. 2011). Panicot et al. (2002) suggested that the excessive accumulation of free Put is considered toxic, unfavorable, and a negative factor in salt tolerance, even capable to cause cell death. The addition of Put to a nutritive solution increased endogenous Put, which was not enough to overcome the deleterious effects of salt stress and even reinforced the negative impact of NaCl in growth of shoot and root (Ndayiragije and Lutts 2006; Hussain et al. 2011). Nevertheless, the increase in endogenous levels of Spd and Spm in salt-tolerant rice plants or in drought-tolerant wheat cultivars (compared to sensitive cultivars) was correlated with a greater increase in the activities of antioxidant enzymes and consequently more closely associated with stress tolerance than Put (Krishnamurthy and Bhagwat 1989; Shen et al. 2000). Moreover, exogenous Spd has shown to play a main function in preventing the electrolyte and amino acid leakage or recovering the damage to the plasmalemma in rice cultivars exposed to salinity, in chilling tolerance, and in protection of cucumber leaves under water stress (Roy et al. 2005). Spm plays a protective role in Arabidopsis subjected to salt stress (Yamaguchi et al. 2006). Recently, studies on rice exposed to salt stress reported an increase in levels of Put and Spd and a reduction in Spm (Maiale et al. 2004). The foliar application of Put involved an improvement in the behavior of rice cultivars exposed to NaCl (Lutts et al. 1996). This suggested to authors that the endogenous level of Put can be a limiting factor for resistance to salt stress in rice. Ndayiragije and Lutts (2006) concluded that the addition of PAs to the nutrient solution led to a better discrimination of K^+/Na^+ in rice plants under salt stress, but this positive impact did not cause an improvement in growth.

3.3 Brassinosteroids

In addition to the auxins, gibberellins, cytokinins, abscisic acid, and ethylene, BRs represent a sixth class of hormones in plants with broad occurrence in the plant kingdom. Among BRs, there are three bioactive BRs including brassinolide, 24-epibrassinolide, and 28-homobrassinolide (HBL) which are most employed in

physiological studies (Hayat et al. 2012). They were identified for the first time in pollen of *Brassica napus* (Mitchell et al. 1970). Like other plant hormones, BRs are implicated in the regulation of several processes in the growth and development of plants (Mandava 1988). BRs play main roles in cell elongation and cell division in stem and inhibition of root growth and promote xylem differentiation and abscission (Mandava 1988; Nemhauser et al. 2004). Concurrently to these processes, BRs contribute to regulate several other processes such as induction of nucleic acid and protein synthesis (Khripach et al. 2003), photosynthesis (Hayat et al. 2007), increase fruit set (Ali et al. 2006), and the activation of several enzymatic activities (Hasan et al. 2008). The mechanism by which BRs control these processes is not completely elucidated yet (Clouse and Sasse 1998).

The BR biosynthetic pathway and the genes involved in BR biosynthesis have been identified primarily in Arabidopsis and also in rice and tomato. Brassinolide (BL) is the most active BR and it is synthesized from campesterol via several pathways. Stepwise metabolic experiments have found the presence of two parallel pathways from campestanol to castasterone termed as early and late C-6 oxidation pathways (Divi and Krishna 2009).

In plants exposed to various biotic and abiotic stresses, ameliorative roles of BRs were identified (Ozdemir et al. 2004). BRs exogenously applied involve an improvement in the tolerance to low and high temperature stress (Bajguz and Hayat 2009), heavy metal stress (Hayat et al. 2010), drought stress (Fariduddin et al. 2009), salinity (Ali et al. 2007), and water logging (Bajguz and Hayat 2009). For example, in maize seedlings (*Zea mays*) subjected to water stress, the application of BL increased the activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase, as well as the ascorbic acid and the carotenoid content (Van Staden and Jager 1998). Moreover, studies undertaken on rice plants exposed to salt stress and treated with total BRs showed a significant increase in the activities of CAT, SOD, and glutathione reductase (GR) and a light increase in APX (Nunez et al. 2003). Ozdemir et al. (2004) compared salt-tolerant and salt-sensitive varieties of rice and showed that the exogenous application of EBL improved the tolerance of the salt-sensitive plants in the short term. This significant improvement in the tolerance of the salt-sensitive rice variety was correlated to the increase in antioxidant enzyme activities induced by EBL. The exogenous application of total BRs increased the length of seedlings and the fresh and dry weight of three sorghum varieties (*Sorghum vulgare*), CSH-14 and ICSV-745 being susceptible to water stress and M-35-1 being resistant to water stress, when exposed to osmotic stress (Vardhini and Rao 2003). In a similar way, the application of EBL involved a substantial improvement of seed germination and growth of *Eucalyptus camaldulensis* seedlings under salt stress (Sasse et al. 1995).

4 Conclusions and Future Perspective

In this chapter, we reviewed and discussed various approaches directed toward the aim of increasing salt tolerance of food crops. Specifically we focused to the evidence of the two relevant components of the biohydraulic approach. In the hydraulic

component, the use of SDI in agriculture offers a very effective solution to water scarcity, and the research results encourage farmers to use this kind of irrigation system since it improves the water use efficiency while allows the irrigation with water of lower quality and increases the yield of agricultural crops.

In the biological component, our attention was focused on the exogenous application of specific osmoprotectants. The beneficial effects of the external supply of osmoprotectants vary according to several factors as the severity of stress, the plant species, the application method, and the concentration of the osmoprotectants as well. However, the roles and mechanisms of action of these osmoprotectants were found to be complex and remain debated. Indeed, besides the reports available about the use of Pro in crops, it is important to explore the effects of other osmoprotectants such as the PAs and BRs. Further, to understand the mechanisms of tolerance in plants to salt and other stresses by using exogenous osmoprotectants is promising and it needs additional research at the field level to generate techniques applicable to improve the crop production. As no agricultural practice alone can solve the stress problem, we address to the use of the integrative biohydraulic approach. SDI is an effective solution and should be subject of permanent research regarding the newer irrigation materials. Plant breeding using novel approaches that combine genetic, biochemical, and molecular techniques may provide results in the near future, leading to improve the tolerance of sensitive crops to abiotic stress. Complementarily, future research in compatible solutes (Pro, PAs, and BRs) can focus in a deeper understanding of their roles, as well as the effects of other plant hormones such as salicylic acid, used externally for increasing tolerance to salinity, drought, heavy metals, high and low temperature, water logging, and flooding in various crop plants.

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Chapter 11
Induced Mutagenesis for the Improvement of Pulse Crops with Special Reference to Mung Bean: A Review Update

Mohd Rafiq Wani, Mohammad Imran Kozgar, Samiullah Khan, M. Abass Ahanger, and Parvaiz Ahmad

1 Introduction

India grows a variety of pulse crops, also called as grain legumes, under a wide range of agroclimatic conditions and has a pride of being the world's largest producer of pulses. Unique characteristics like high protein content, nitrogen fixing ability, soil ameliorative properties, and ability to thrive better under harsh conditions make pulses an integral component of sustainable agriculture particularly in dry land areas (Ali and Kumar 2006). They are also rich source of energy, minerals, and certain vitamins of B complex group. Consequently, pulses help in checking the malnutrition among the children of our country. Indian population relies on pulses for meeting its protein requirement mainly because of its vegetarian food habit and high cost of animal-based protein. The country has witnessed a decreasing trend in the per capita availability of pulses from 61 g per day in 1951–1956 to less than 40 g in recent years (Satya Sundaram 2010). The problem of declining per capita availability can be addressed through rapid improvement in indigenous production levels. Although efforts have been expedited to bring additional area under the cultivation of pulses, it is important to increase the production by exploiting the yield

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Table 11.1 All-India area, production, and yield of total pulses

Periods	Area (million ha)	Production (million tonnes)	Yield (kg ha$^{-1)}$
1999–2000	21.12	13.42	635
2000–2001	20.35	11.08	544
2001–2002	22.01	13.37	607
2002–2003	20.50	11.13	543
2003–2004	23.46	14.91	635
2004–2005	22.76	13.13	577
2005–2006	22.39	13.39	598
2006–2007	23.19	14.20	612
2007–2008	23.63	14.76	625
2008–2009	22.09	14.57	659
2009–2010	23.28	14.66	630
2010–2011	26.28	18.09	689

Source: Directorate of Economics and Statistics, Department of Agriculture and cooperation, Ministry of Agriculture, Govt. of India

potential of existing varieties through genetic manipulation. The estimates for 2010–2011 indicate that the pulses occupy an area of 26.28 million hectares and produce 18.09 million tonnes with an average yield of 689 kg ha^{-1} (Table 11.1).

During the last decade, the average growth rate of pulses production was far below the required growth rate to meet the domestic requirement. The non-availability of high-yielding varieties is a major constraint in achieving higher productivity of pulses. Nonsynchronous maturity, long duration, flower drop, and susceptibility to diseases are the other problems associated with varieties of major pulses. The state-wise data on area, production, and yield of pulses in India is given in Table 11.2. The use of induced mutations over the past five decades has played a major role in the development of superior plant varieties all over the world. Among the mutant varieties, the majority belong to food crops (Fig. 11.1).

Mung bean (*Vigna radiata* (L.) Wilczek), which ranks third to chickpea and pigeon pea, is an important pulse crop in Southeast Asia and the Indian subcontinent (Fig. 11.2). Mung bean is grown in almost all the states of India and is cultivated as a kharif (monsoon) and summer season crop in different agroecological regions. Loam to sandy loam soils with good internal drainage are considered ideal for mung bean cultivation. In India, mung bean was grown over an area of 3.55 million hectares with the production of 1.82 million tonnes in 2010–2011 (Table 11.3). The average yield of 512 kg ha^{-1} is low and is not sufficient to meet the growing demand. For breaking yield plateau in mung bean, efforts are needed to develop high-yielding varieties with appropriate growth habit. In some cases, the progress obtained for productivity has exploited the variability to such a large extent that only further progress from classical methods of breeding becomes more and more difficult. The possibility offered by mutagenic agents to induce new genetic variation is, therefore, of extreme interest. Since mung bean is a self-pollinated crop, mutation breeding could be rewarding for broadening the genetic base of important traits such as yield attributes.

Table 11.2 State-wise area, production and yield of pulses in India

State	Area (million ha)	Production (million tonnes)	Yield (kg ha^{-1})	Area (million ha)	Production (million tonnes)	Yield (kg ha^{-1})
2008–2009			2009–2010			
Madhya Pradesh	4.56	3.68	808	4.94	4.30	871
Maharashtra	3.08	1.66	537	3.38	2.37	702
Uttar Pradesh	2.22	2.00	899	2.54	1.90	748
Andhra Pradesh	1.77	1.45	818	1.93	1.43	740
Karnataka	2.09	0.97	466	2.48	1.12	451
Rajasthan	3.67	1.83	497	3.50	0.71	204
Gujarat	0.78	0.61	777	0.73	0.52	705
Chhattisgarh	0.86	0.50	580	0.81	0.49	604
Bihar	0.59	0.47	801	0.56	0.47	836
Orissa	0.80	0.39	481	0.87	0.40	461
Jharkhand	0.39	0.28	724	0.32	0.22	709
Tamil Nadu	0.54	0.16	307	0.53	0.20	382
West Bengal	0.18	0.13	704	0.18	0.15	826
Haryana	0.18	0.18	980	0.13	0.10	758

Source: Directorate of Economics and Statistics, Department of Agriculture and cooperation, Ministry of Agriculture, Govt. of India

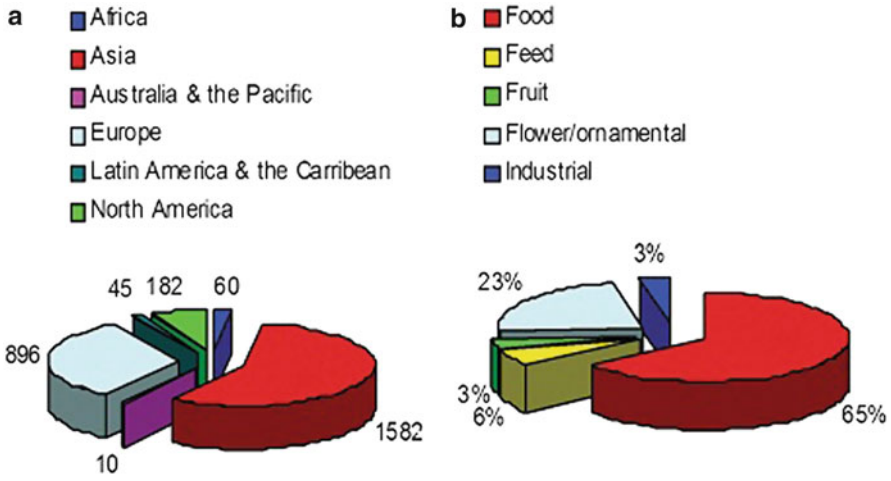

Fig. 11.1 Plant varieties derived from induced mutations: (**a**) the number of mutant varieties in different continents and (**b**) proportion of various plant types (*Source*: Adapted from Kharkwal and Shu 2009 with permission of Dr. Q. Y. Shu)

The Plant Breeding and Genetics Section of Joint FAO/IAEA Division, Vienna, helps plant breeders to develop improved cultivars through the use of induced mutations. Some improved varieties of mung bean developed through induced mutations are given in Table 11.4. Most of the varieties are found to be resistant against yellow

Fig. 11.2 Mung bean crop at its maturity (*Source*: http://www.aicrpmullarp.res.in)

Table 11.3 All-India area, production and yield of mung bean

Periods	Area (million ha)	Production (million tonnes)	Yield (kg ha$^{-1)}$
2000–2001	3.01	1.03	340
2001–2002	3.09	1.11	360
2002–2003	3.01	0.87	288
2003–2004	3.55	1.71	475
2004–2005	3.34	1.06	317
2005–2006	3.11	0.95	304
2006–2007	3.19	1.12	349
2007–2008	3.73	1.52	409
2008–2009	2.84	1.03	364
2009–2010	3.07	0.69	225
2010–2011	3.55	1.82	512

Source: MULLaRP information system (ICAR), India

mosaic virus (YMV). Two varieties, namely, Co 4 from TNAU, Tamil Nadu, and Pant Moong 2 from Pantnagar, were released in India in the year 1982. After that, the release of early maturing cultivars resistant to TMV and suitable for different cropping systems has resulted in an increased area and production in several states of India particularly Bihar, Gujarat, Maharashtra, Rajasthan, and Punjab (Solanki et al. 2011). Numerous mutant varieties have been released since then for commercial cultivation in various legume crops (Table 11.5). During the last 70 years, around 3,139 mutant varieties have been officially released either as direct mutants or from their progenies for commercial cultivation in the world. The major contribution is from cereals followed by ornamentals, legumes, and oilseeds (Fig. 11.3). Highest number of mutant varieties have been released in China (26.21 %) followed by Japan (15.35 %), India (10.57 %), Russia (7 %), the Netherlands (5.6 %), Germany (5.51 %), the USA (4.42 %), Pakistan (1.68 %), Bangladesh (1.43 %), and others (22.17 %) as depicted in Fig. 11.4. Many induced mutants were released

Table 11.4 Details of mung bean varieties developed through mutation breeding

Variety name	Country	Year of release	Remarks
Dhauli (TT9E)	India	1979	High yield, early maturity, resistance to YMV
Co 4	India	1982	High yield, early maturity, resistance to drought
Pant Moong 2	India	1982	Developed by gamma rays (100 Gy). Resistant to MYMV, more pods, high yield
NIAB Mung-28	Pakistan	1983	Developed by gamma rays (200 Gy). Early and uniform maturity, high yield
ML 26-10-3	India	1983	Developed by gamma rays. Resistant to yellow mosaic virus (YMV), high yield
TAP-7	India	1983	Developed by gamma rays. Early maturity (5–7 days), resistant to mildew and leaf spot, higher yield
NIAB Mung 19-19	Pakistan	1985	Developed by irradiation with gamma rays (400 Gy). Early maturity (60–65 days), high yield, high tolerance to mung bean yellow mosaic virus
NIAB Mung 121-25	Pakistan	1985	Developed through gamma rays (200 Gy). Early maturity (60–65 days), determinate type, high yield (44 %), recommended as spring and summer crop
NIAB Mung 13-1	Pakistan	1986	Developed by gamma rays (100 Gy). Early maturity, shortness, more pods, harvest index (28 %), TGW (40.5 g), higher yield (44 %)
NIAB Mung 20-21	Pakistan	1986	Developed by gamma rays (400 Gy). Early maturity, shortness, harvest index (31 %), high yield (65 %), tolerance to yellow mosaic virus, resistance to *Cercospora* leaf spot
Camar	Indonesia	1987	Developed by gamma rays (100 Gy). Resistant to *Cercospora* leaf spot and *Uromyces* species, high yield, tolerant to saline and acidic soils
NIAB Mung 51	Pakistan	1990	Developed by gamma rays (100 Gy). Early and synchronous maturity, non-shattering pods, tolerant to MYMV and CLS diseases, larger seed size, higher yield potential
NIAB Mung 54	Pakistan	1990	Developed by gamma rays (100 Gy). Early and synchronous maturity, non-shattering pods, tolerant to MYMV and CLS diseases, larger seed size, higher yield potential, crop vegetation: summer (71 days) and spring (73 days)
Binamoog-1[a]	Bangladesh	1992	Winter variety; 90–95 days needed for maturity, resistant to *Cercospora* leaf spot and tolerant to YMV disease
MUM-2	India	1992	Developed by the treatment of EMS. High yield, resistant to diseases
BM 4	India	1992	High yield, early maturity, resistant to YMV
NIAB Mung 92	Pakistan	1992	Developed by hybridization with mutant NIAB Mung 36. Resistant to MYMV, early maturity, large seed size, resistance to grain shattering

(continued)

Table 11.4 (continued)

Variety name	Country	Year of release	Remarks
LGG 450	India	1993	High yield, early maturity, resistant to YMV
LGG-407	India	1993	High yield, early maturity, resistance to YMV
TARM-2	India	1994	Developed by hybridization with a mutant RUM-5. High yield, medium to late maturity, resistant to powdery mildew diseases
Binamoog-2	Bangladesh	1994	Developed by hybridization with gamma ray-induced mutant MB-55(4). Larger seed size, early and synchronous maturity (7–10 days earlier), high yield, tolerant to leaf YMV and *Cercospora* leaf spot
TARM-18	India	1996	Developed by hybridization with a mutant variety TARM-2. High yield, resistant to powdery mildew disease
TARM-1	India	1997	Developed by hybridization with a mutant RUM 5. High yield, resistance to powdery mildew disease, medium maturity
Binamoog-3	Bangladesh	1997	Developed from cross (Mutant MB55-4 x AURDC line V1560D). Seed yield, synchronous pod maturity, tolerance to yellow mosaic virus and *Cercospora* leaf spot
Binamoog-4	Bangladesh	1997	Developed by irradiation of hybrid seeds from cross (Mutant MB55-4 x AURDC line V1560D). Mutant MB-55-4 was induced by gamma rays (200 Gy). Main improved attribute of mutant variety are high seed yield, synchronous pod maturity, early maturing, dwarf plant type, tolerant to yellow mosaic virus and *Cercospora* leaf spot
NIAB Mung 98	Pakistan	1998	Developed by hybridization with mutant variety NIAB Mung 20-21. Resistant to yellow mosaic virus and *Cercospora* leaf spot, high yield and medium seed size
AEM-96	Pakistan	1998	Developed through gamma irradiation (200 Gy)
Binamoog-5	Bangladesh	1998	Developed from cross between Mutant MB55-4 and AURDC line V1560D). Higher seed yield, synchronous pod maturity, tolerance to leaf YMV and *Cercospora* leaf spot
Chai Nat 72	Thailand	1999	Developed by gamma rays (600 Gy). High yield, larger grain size, resistant to fungal diseases
Binamoog-6	Bangladesh	2005	Developed by gamma irradiation by using advanced mutant line, VC-6173-10. Increased pods, reduced seed size, tolerant to leaf YMV and *Cercospora* leaf spot
Binamoog-7	Bangladesh	2005	Developed from Binamoog-2 with 0.75 % of EMS treatment. Higher seed yield, synchronous pod maturity, tolerance to leaf YMV and *Cercospora* leaf spot.

(continued)

Table 11.4 (continued)

Variety name	Country	Year of release	Remarks
TMB-37	India	2005	Early maturity (55–57 days), resistant to YMV, high yield, large seed size
NIAB MUNG 2006	Pakistan	2006	Developed by hybridization with mutant NIAB Mung 92. Purple hypocotyl and stem, high number of pods and clusters, resistance to diseases
TJM-3	India	2007	Developed by hybridization with mutant variety TARM-1 Resistant to YMV, powdery mildew and *Rhizoctonia* root-rot diseases, early maturity and large seeds
TM-96-2	India	2007	Developed by hybridization with mutant variety TARM-2. Resistant to powdery mildew and *Cercospora* leaf spot diseases, early maturity
TM 2000-2	India	2010	Resistant to powdery mildew diseases

Source: Joint FAO/IAEA, Vienna Mutant Variety Database (MVD); http://mvgs.iaea.org
[a]*Source*: http://www.bina.gov.bd/index.php?option=com_content&view=article&id=71&Itemid=85

Table 11.5 Pulse varieties released through mutation breeding in India

Crop/variety	Year of release	Mutagen
Mung bean (*Vigna radiata* (L.) Wilczek)		
BM 4	1992	[a]
Co 4	1982	Gamma rays
Dhauli (TT9E)	1979	[a]
LGG-450	1993	[a]
LGG-407	1993	[a]
ML 26-10-3	1983	Gamma rays
MUM-2	1992	EMS
Pant Moong 2	1982	Gamma rays
TAP-7	1983	Gamma rays
TARM-1	1997	Gamma rays
TARM-18	1996	Gamma rays
TARM-2	1994	Gamma rays
TJM-3	2007	Gamma rays
TM 2000-2	2010	[a]
TM-96-2	2007	Gamma rays
TMB-37	2005	[a]
Chickpea (*Cicer arietinum* L.)		
Kiran (RSG-2)	1984	Neutrons
Pusa 408 (Ajay)	1985	Gamma rays
Pusa 413 (Atul)	1985	Gamma rays
Pusa 417 (Girnar)	1985	Gamma rays
BGM 547	2006	Gamma rays
Pusa 547	2006	Gamma rays

(continued)

Table 11.5 (continued)

Crop/variety	Year of release	Mutagen
Pigeon pea (*Cajanus cajan* Millsp.)		
Co 3	1977	EMS
Co 5	1984	Gamma rays
TAT 10	1984	Fast neutrons
TAT 5	1984	Fast neutrons
TJT 501	2009	[a]
Trombay vishakha-1	1983	Fast neutrons
TT-401	2007	[a]
Pea (*Pisum sativum* L.)		
Hans	1979	EI
Lentil (*Lens culinaris* Medik)		
Rajendra Masoor 1	1996	Gamma rays
S-256 (Ranjan)	1981	Radiation
Urd bean (*Vigna mungo* (L.) Hepper)		
Co 4	1978	EMS
DU-1	2007	Gamma rays
Manikya	1988	[a]
TAU-1	1985	Gamma rays
TAU-2	1992	Gamma rays
TPU-4	1992	Gamma rays
TU-94-2	1999	Gamma rays
Vamban-2	1997	[a]
Cowpea (*Vigna unguiculata* Walp.)		
Co 5	1984	Gamma rays
COCP 702 [CoVu 702 & CO (CP) 7]	2002	Gamma rays
Cowpea-88	1990	Radiation
Gujarat cowpea-1	1984	[a]
TRC 77-4 (Kalleshwari)	2007	Gamma rays
V16 (Amba)	1981	DMS
V240	1984	DMS
V37 (Shreshtha)	1981	DMS
V38 (Swarna)	1981	DMS
Pusa Parvati	1970	X-rays
Soybean (*Glycine max* L.)		
Birsa Soybean-1	1983	Spontaneous mutation
MACS-450	2000	Crossing with mutant
NRC 12	[a]	[a]
NRC 2	[a]	[a]
TAMS 38	2007	[a]
TAMS 98-21	2005	Gamma rays
Common bean (*Phaseolus vulgaris* L.)		
Pusa Parvati	1970	X-rays
Moth bean (*Vigna aconitifolia* (Jack.) Marechal)		
RMO 40	1994	Gamma rays+EMS

(*Source*: Joint FAO/IAEA, Vienna Mutant Variety Database (MVD); http://mvgs.iaea.org)
[a]Not available

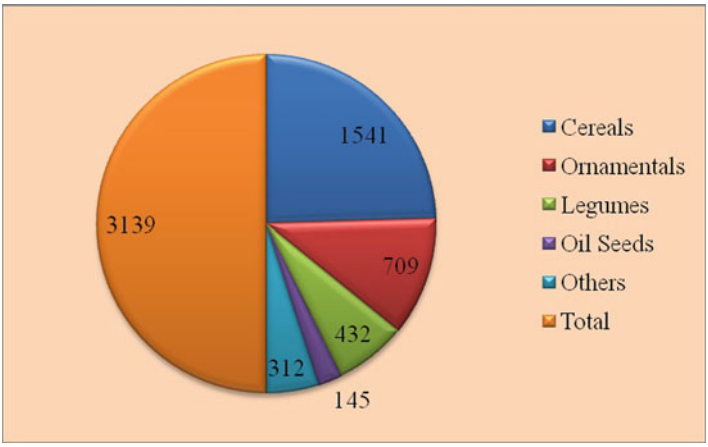

Fig. 11.3 Number of mutant varieties released in the world (*Source*: Joint FAO/IAEA, Vienna Mutant Variety Database (MVD); http://mvgs.iaea.org)

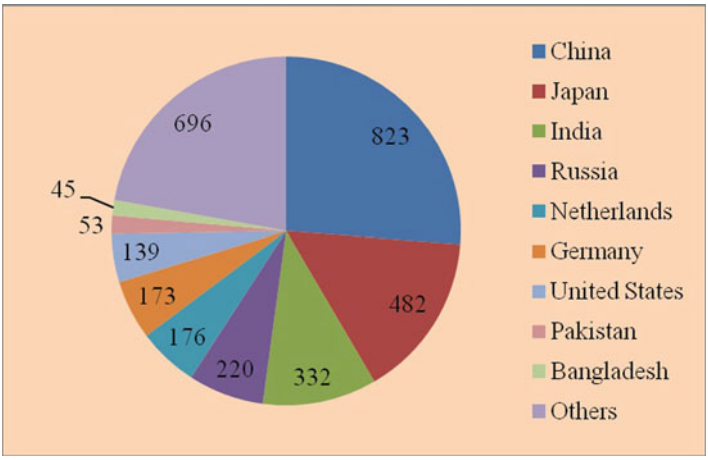

Fig. 11.4 Country-wise mutation varieties released in the world (*Source*: Joint FAO/IAEA, Vienna Mutant Variety Database (MVD); http://mvgs.iaea.org)

directly as new varieties, while others were used as parents in crossbreeding programs to derive new varieties. Mutation breeding has contributed in developing 432 mutant varieties in legumes. In India, so far about 84 legume varieties have been released and are in commercial cultivation. These are groundnut (26), mung bean (16), cowpea (9), urd bean (8), pigeon pea (7), soybean (6), chickpea (6), lentil (2), and others (4) as illustrated in Fig. 11.5. This shows the significant contribution of mutation breeding to pulse crop varietal improvement in India.

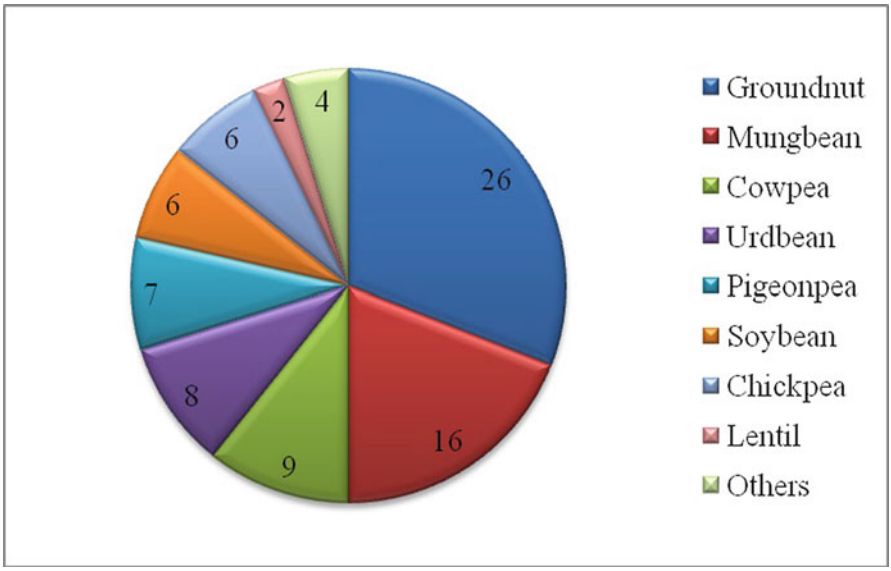

Fig. 11.5 Number of mutant varieties released in major pulse crops in India (*Source*: Joint FAO/IAEA, Vienna Mutant Variety Database (MVD); http://mvgs.iaea.org)

2 Economic Importance of Mung Bean

Seeds of mung bean are highly nutritious containing about 24 % protein, 1.15 % fat, 16.3 % fiber, 3.32 % ash, and 62.62 % carbohydrate on dry weight basis and provide approximately 347 kcal energy (Table 11.6). The mineral profile is primarily composed of potassium (1,246 mg/100 g), phosphorous (367 mg/100 g), calcium (132 mg/100 g), and iron (6.74 mg/100 g). Mung bean protein is considered to be easily digestible. Being rich in quality proteins, minerals, and vitamins, it is an inseparable ingredient in the diets of a vast majority of Indian population. The dried grains of mung bean can be split or eaten whole after cooking and made into a soup or dhal (porridge). Mung bean is also eaten as sprouts. Green pods and seeds can be cooked as vegetables. It is also recommended as a medicinal diet in case of flatulence and to the sick. Being rich in vitamin B complex, it is regarded as preeminent remedy for beriberi. In addition, the dried green stalk and leaves of mung bean are used as fodder.

3 Induced Mutagenesis

Mutation is a sudden heritable change in DNA, the hereditary material of life. It is now well established that ultimate source of new variation is mutation, which leads to creation of new genetic variation indispensable for the improvement as well as

Table 11.6 Nutrient contents of mature raw seeds of mung bean

Nutrient	Units	Value per 100 g
Water content	g	9.03
Calorie content	kcal	347
Protein content	g	23.86
Fat content (lipids)	g	1.15
Ash content	g	3.32
Carbohydrate content	g	62.62
Dietary fiber content	g	16.3
Sugar content	g	6.6
Calcium (Ca)	mg	132
Iron (Fe)	mg	6.74
Magnesium (Mg)	mg	189
Phosphorus (P)	mg	367
Potassium (K)	mg	1,246
Sodium (Na)	mg	15
Zinc (Zn)	mg	2.68
Copper (Cu)	mg	0.941
Manganese (Mn)	mg	1.035
Selenium (Se)	µ	8.2
Vitamin C (ascorbic acid)	mg	4.8
Thiamine content (vitamin B-1)	mg	0.621
Riboflavin content (vitamin B-2)	mg	0.233
Niacin content (vitamin B-3)	mg	2.251
Pantothenic acid content (vitamin B-5)	mg	1.91
Vitamin B-6 content	mg	0.382
Folic acid content	µg	0
Vitamin B-12 content	µg	0
Vitamin A content	µg	114
Vitamin E (alpha-tocopherol)	µg	0.51

Source: http://www.calorie-counter.net

evolution of crop plants. Mutations may occur spontaneously or can be induced artificially. Mutations provide an opportunity to create hitherto unknown alleles, so that the plant breeder does not remain handicapped due to limited allelic variation at one or more gene loci of interest. Use of induced mutants in the breeding programs for developing superior varieties is known as mutation breeding which has been used extensively for developing new crop cultivars and for changing the plant traits. Mutation in a gene, leading to the improvement of characteristics of well-adapted existing cultivars or to derive new cultivars using mutant traits, has been the basis for enhancing the germ plasm (Solanki et al. 2011; Gnanamurthy et al. 2013b). Besides, the developmental mutants can also be used for molecular analysis to study the morphological evolution and phylogeny.

Mutation breeding is a well-functioning branch of plant breeding supplementing to conventional methods in a favorable manner. The prime strategy in mutation-based

breeding has been to upgrade the well-adapted plant varieties by altering one or two major traits which limit their productivity or enhance their quality. In that sense, it provides a rapid method to improve local crop varieties, without going through extensive hybridization and backcrossing. One of the chief advantages of mutation breeding is that it can give rise to many diverse mutant alleles with a different degree of trait modification. In contrast, transposon or T-DNA insertional mutagenesis generally leads to complete disruption of gene function rather than in generating allelic series of mutants with partial loss of function and thus not producing the range of mutation strengths necessary for crop improvement (Chopra 2005). Consequently, conventional mutagenesis is still favored for crop improvement.

The concept of inducing mutations and utilizing them for improving cultivated plants is more than 85 years old. It was put to use for the first time by Muller in 1927, when he succeeded in inducing certain variations in *Drosophila*. Stadler (1928), while working on the effect of X-rays in barley, reported that it was possible to obtain high mutation rates through irradiation. Thus, in the beginning, mutation breeding was based primarily upon X-rays, gamma rays, and thermal neutrons. The constantly increasing level of knowledge in experimental mutation research was to a high extent due to the fact that not only physical but also chemical mutagens became available. The first elaborate report was presented by Auerbach and Robson (1942), who showed that mustard gas could induce mutations as well as chromosomal breaks in *Drosophila*. Some of these chemical mutagens, for instance, ethylmethane sulphonate (EMS), methylmethane sulphonate (MMS), ethylene imine (EI), diethyl sulphate (dES), *N*-nitro-*N*-methyl urea (NMU), *N*-nitroso-*N*-ethyl urea (NEU), sodium azide (Na N_3), and hydrazine hydrate (HZ) among others, were found to be more effective than X-rays and gamma rays (Sander et al. 1978; Gaikwad and Kothekar 2004).

Experiments on higher plants have shown that chemical mutagens have much greater advantage over ionizing radiations. This is due to their milder effect on genetic material of the cell as against the physical mutagens which break the chromosome. Rapoport (1966) discovered overwhelming majority of strong chemical mutagens which are being used widely in genetic and breeding research. Using chemical mutagenesis in his research, I.A. Rapoport has made a valuable contribution to the theory, having developed his own concept of "microgenetics," i.e., about gene structure, function of genetic material, mechanism of mutation induction, their origin and manifestation in the progeny, mechanism of mitosis, and stability of genetic state in living forms. The nature, essential properties, and mode of action of physical and chemical mutagens have been reviewed by Kaul (1989). Our knowledge on the fundamental aspects of mutational process and the possible mechanism of action of various physical and chemical mutagens has fairly widened with reports and reviews of Gottschalk and Wolf (1983) and Kodym and Afza (2003). Though there are several unanswered questions regarding the action of mutagens, a more comprehensive account of them has been given by Sharma (1985). The preferred mutagens belong to the class of alkylating agents (EMS, MMS, dES, NMU, NEU). Alkylation refers to the substitution of an alkyl group (e.g., C_2H_5 of EMS) for

hydrogen in the nitrogenous bases (Sharma and Chopra 1994). The alkylation of DNA leads to the following effects (Ashburner 1990; Sharma and Chopra 1994):

1. Alkylation of the phosphate groups of DNA: Alkylation leads to the formation of phosphate tri-esters which are unstable and release the alkyl group. However, if enough alkyl groups remain unreleased, then the attached alkyl groups interfere with DNA duplication. Sometimes the phosphate tri-ester is hydrolyzed between the sugar and the phosphate and results in the breakage of the DNA backbone.
2. Alkylation of bases: The seventh position in the guanine is a preferred site for alkylation, but it has been established that the major mutagenic effects arise from O^6 alkylation of guanine. O^6 alkyl-guanine can pair with thymine and leads to base-pair transition.
3. Depurination: The alkylated guanine can separate from the deoxyribose leaving it depurinated. The gap can be filled up by any base during DNA replication leading to transversion or transition type of mutation.

The mutagenic action of ethylmethane sulphonate (EMS) was studied earlier in *Drosophila* (Fahmy and Fahmy 1957), bacteriophage (Loveless 1959), *Escherichia coli* (Strauss 1964), and *Arabidopsis* (Greene et al. 2003). Because of its ability to induce a high frequency and wide spectrum of mutations, EMS is now being widely accepted as a powerful mutagen and is used commonly in the induction of mutations in various crop plants.

Sodium azide (SA) has been reported to be highly effective in black gram (Misra 1995), lentil (Gaikwad and Kothekar 2004), and mung bean (Wani et al. 2011b). Sodium azide mutagenicity was first observed by Wyss et al. (1948) in their studies on the role of peroxides in radiation-induced mutagenesis. Later Berger et al. (1953) observed that sodium azide increased the frequency of penicillin- and streptomycin-resistant mutants in *Staphylococcus aureus*. They interpreted the observed mutagenicity as an indirect effect due to the inhibition of catalase and peroxidase by sodium azide, resulting in the accumulation of hydrogen peroxide in bacterial cells. The hydrogen peroxide was presumed to be the actual mutagen. Sodium azide mutagenicity, in higher plants, was discovered inadvertently in experiments using this chemical as a respiratory inhibitor in *Hordeum vulgare* (Spence 1965). Again, the inhibition of catalase and peroxidase by azide was presumed to result in an increased peroxide concentration in the cell and, therefore, the mutagenic effect. Its pH dependency was reported by Nilan et al. (1973) in barley. Sodium azide induces mostly gene mutations with negligible frequency of chromosomal aberrations (Kleinhofs et al. 1974). Combination of sodium azide, with physical or chemical mutagens, was found to increase the mutation frequency (Singh and Olejniczak 1983). Sodium azide is reported to produce high mutagenic effects in several crops, depending upon the treatment conditions (Ando and Tulmann Neto 1979; Hasegawa and Inoue 1980).

There is a certain amount of evidence about the mutagenic action of hydrazine in both prokaryotes and eukaryotes. It was sometimes classified primarily as an inactivating agent with weak mutagenic activity (Fishbein et al. 1970), but studies with bacterial species suggest that it can fairly be a potent mutagen with little or no toxic

effect. A useful review of the earlier work with special emphasis on the chemical basis for mutagenesis of hydrazine was given by Brown et al. (1966). Hydrazine was reported to induce a variety of morphological, chlorophyll, yield, physiological, and color mutants in several crop plants such as chickpea (Atta et al. 2003; Khan et al. 2005) and mung bean (Wani et al. 2011b, c). In general, hydrazine in these studies appeared to be as successful as other potent mutagens. However, it appeared to differ in two ways:

1. It produced a number of mutations detectable in M_1 generation, whereas the other mutagens produced fewer or none.
2. The spectrum of mutational changes (phenotypic classes) for hydrazine was generally different from that of other mutagens. Hydrazine has been reported to react with the pyrimidines in DNA to saturate the 5, 6 double bond, especially of thymine, to form N^4-amino-cytosine and to open up the pyrimidine ring with consequent loss of pyrimidines from DNA or through intermediate radical reactions including the formation of hydrogen peroxide (Kimball 1977). The mutations produced by hydrazine seem to be mainly or entirely single-locus changes.

Apart from easy handling and better efficiency, chemical mutagens have greater specificity than radiations (Wani et al. 2011b; Kozgar et al. 2012). They have been proved to be more potent and efficient in inducing mutations than physical ones (Kharkwal 1998a). Therefore, chemical mutagenesis has become the method of choice for genetic studies, remaining popular even with the advent of new technologies. Mutation breeding technique may have a greater role in crops like pulses, where a large part of natural variability has been eliminated in the process of adaptation to the environmental stress. In recent years, a lot of work has been undertaken on induced mutagenesis through physical and chemical mutagens. It has been clearly shown in number of plant species that the effect induced varies with the varying mutagens and with variation in mutagen doses. Thus, selecting a mutagen and its optimum dose for a genotype in any plant species is an important step in mutation breeding program.

4 Mutagen Dose and Genotypic Sensitivity

The frequency and the spectrum of mutations differ depending on the mutagen used and the dose applied. An optimum dose is the one which produces maximum frequency of mutations and cause minimum killings (Solanki and Waldia 1997). Many workers feel that a dose close to lethal dose-50 (LD_{50}) should be optimum. It is that dose of the mutagen which would kill 50 % of the treated individuals. In general, an overdose is likely to kill too many treated individuals, while an underdose would produce too few mutations. The LD_{50} of a particular genotype varies greatly. This is due to the fact that genetic architecture of an organism is a potent factor in determining the genotypic difference towards the mutagens. Polyploid species have been found to be slightly resistant to the action of mutagens than their diploid ones, and therefore, the effective dose is likely to vary in an individual crop. Effective mutagen doses can be selected on the basis of analysis of M_1 parameters (Konzak et al. 1965).

A decline in survival of the mutated population of chickpea has been associated with an increase in the dose of the mutagen (Singh 1988) which may result from cytogenetic damage and/or physiological disturbances as suggested by Sato and Gaul (1967). Van Harten (1998) reported that it is better to perform a "seedling growth test" with a range of doses to determine the optimal treatment conditions for a specific cultivar. With a view to enhance the mutation rate and to alter the spectrum of mutations, many variations in treatment methodology have been used by different workers. Treatments with chemical and physical mutagens have been given to dry as well as soaked seeds and seedlings at different developmental stages and at variable temperature and ionic concentrations. According to Solanki and Waldia (1997), the dose of a chemical mutagen treatment is dependent on several parameters, of which the concentration, duration of treatment, and temperature during treatment are most essential.

It is well known that the same mutagen dose can cause different degrees of effects in different species. Varied mutagenic sensitivity of different genotypes was reported by many workers. Bykovets and Vasykiv (1971) conducted mutation studies in leguminous crops like soybean, peas, and *Lathyrus* with three chemical mutagens, viz., NEU, NMU, and dES. It was found that all crops were not mutable to the same extent and that the maximum mutagenic effect appeared in peas, followed by soybean and *Lathyrus*. Khan et al. (1998) studied the mutagenic effect of maleic hydrazide (MH) in two varieties of *Vigna radiata* and found the var. PS-16 to be more sensitive than the var. K-851. Akbar et al. (1976) concluded from their studies in rice that the differences in radiosensitivity among rice varieties may be due to the difference in their recovery process involving enzymatic activity. Geeta and Vaidyanthan (1997) observed different phenotypic responses of two soybean cultivars to ethidium bromide and gamma rays. Differences to radiosensitivity were also reported by Nerker (1976) in *Lathyrus sativus*. Ahmad and Godward (1981) reported radiosensitivity in nine cultivars of chickpea. Out of these nine, two cultivars CSIMF and F10 were identified as the most radioresistant and radiosensitive, respectively. Kharkwal (1998a) reported mutagenic sensitivity in four varieties of chickpea on the basis of total germination rate, seedling damage, pollen sterility, and plant survival. Blixt (1970) reported that the varieties with large assortment of recessive alleles governing trait(s) show greater sensitivity and frequency of mutants than the varieties having more dominant alleles governing a trait. Findings of Khamankar (1984) in tomato with regard to the differential sensitivity of tomato genes to different physical and chemical mutagens are of considerable interest. He showed that the rate of mutations was different with different mutagens at certain loci. Some of the gene loci were affected by one mutagen but not by the other.

5 Biological Damage

Induced biological damage has been used as a criterion in determining the effect of the mutagen in question and also the sensitivity of the biological material. Seed treatment with physical as well as chemical mutagens is known to produce adverse

effects on seed germination, seedling growth, survival, and plant growth in general (Khan et al. 2004; Barshile et al. 2006). A linear relationship between increasing doses of EMS and reduction in biological traits, viz., germination, height, survival, and pollen fertility, was also observed by Rakshit et al. (2001) in mung bean and urd bean and Jain et al. (2013) in moth bean. Earlier studies of Gaikwad and Kothekar (2004) in *Lens culinaris* have shown a linear relationship between the mutagen dose applied and the parameters mentioned above. The reduction in survival is attributed to cytogenetic damage and/or physiological disturbances (Sato and Gaul 1967). Reduction in germination in mutagenic treatments has been explained due to delay or inhibition of physiological and biological processes necessary for seed germination, which include enzyme activity (Kurobane et al. 1979), hormonal imbalances (Chrispeels and Varner 1967), and inhibition of mitotic processes (Ananthaswamy et al. 1971). The greater sensitivity at higher doses of mutagens has been attributed to various factors, such as changes in the metabolic activity of the cells (Natarajan and Shiva Shankar 1965), inhibitory effects of the mutagen (Sree Ramulu 1972), and disturbances of the balance between promoters and inhibitors of growth regulators (Meherchandani 1975).

The reduction in seedling height, following mutagenic treatments, is mainly due to the uneven damage to the meristematic cells as a consequence of genetic injury. Variation in auxin level (Goud and Nayar 1968) and changes in the specific activity of several enzymes (Cherry et al. 1962) were correlated with reduction in seedling height after mutagenic treatments. However, there are a few reports of promoting effect of mutagens, when applied at lower doses. Kodym and Afza (2003) observed that germination is not a good indicator for an effective mutagen dose. Konzak et al. (1972) proposed that seedling height is used as an index in determining the biological effects of various mutagens in M_1 generation. Solanki and Sharma (1999) in lentil found seedling damage (leaf aberrations) to be the most effective index among all M_1 parameters. A positive relationship was observed by Singh (1988) between the degree of leaf aberrations in M_1 and morphological mutation rate in M_2 generation of chickpea. Pollen fertility is an index of meiotic behavior of chromosomes. The greater the abnormality in the behavior of chromosomes, the greater will be the sterility of pollen grains. High degree of pollen sterility was observed after mutagenic treatments in various crop plants (Khan et al. 1998; Mathur and Lal 1999; Wani et al. 2012b).

6 Cytological Aberrations

Cytological analysis with respect to either mitotic or meiotic behavior is considered one of the most dependable indexes to estimate the potency of mutagens. They also provide a considerable clue to assess the sensitivity of plants for different mutagens. Cytological and cytogenetic studies with mung bean have been relatively few due to its small size of chromosomes. Karpechenko (1925) reported the diploid chromosome number of mung bean as $2n=2x=22$, but Rao (1929) found it to be 24.

Most of the species are diploid with $2n=22$, showing perfect uniformity in chromosome number of this genus. The gamma rays, maleic hydrazide (MH), and their combination treatments showed disturbed mitotic behavior as noticed by Grover and Tejpaul (1982) in *Vigna radiata*. The sticky chromosomes, fragments and ring chromosomes at metaphase, and laggards and bridges at anaphase were found to be dose dependent. The combined treatments enhanced the chromosomal aberrations. Correspondingly, the meiotic process was also affected. A comparative study on the induction of chromosomal aberrations by gamma rays, *N*-methyl–*N*′-nitro–*N-nitrosoguanidine* (MNNG), ethylmethane sulphonate (EMS), and hydroxylamine (HA) was carried out by Grover and Virk (1986) in two varieties of mung bean. All the chemical mutagens and gamma rays were successful in inducing the chromosomal aberrations. The maximum frequency was noticed with gamma rays, followed by MNNG, EMS, and HA. The quadrivalents, trivalents, and univalents were encountered at metaphase-I in pollen mother cells accompanied by laggards, chromatin bridges, and irregular distribution of chromosomes at anaphase-I. In most of the mung bean varieties, pollen fertility showed a close relationship with meiotic abnormalities (Khan 1990). The least mutation frequency, at higher doses, may be attributed to chromosomal aberrations or saturation in the mutational events, resulting in the elimination of mutant cells during growth (Blixt and Gottschalk 1975).

The occurrences of univalents and multivalents at metaphase have been also reported in broad bean (Bhat et al. 2005a; Husain et al. 2013). Multivalent formation can be attributed to irregular pairing and breakage, followed by translocation and inversions. The major abnormalities at anaphase-I/II are bridges and laggards. The occurrence of laggards might be the result of delayed terminalization, stickiness of chromosomal ends, or failure of chromosomal movements (Jayabalan and Rao 1987; Bhat et al. 2006a). Bridge formation at anaphase may be attributed to interlocking of bivalents (Bhattacharjee 1953) and failure of chiasmata to undergo terminalization (Saylor and Smith 1996). In addition, the transmigration of chromatin material with cytomictic connections might have resulted in altered number of chromosomes. Variation in chromosome number in few pollen mother cells may be due to cytomixis which is considered as source of production of aneuploid and polyploid gametes (Yen et al. 1993; Bhat et al. 2006b). Chiasma frequency was variable in populations treated with different mutagens. Mitotic abnormalities like disorientation at metaphase, bridges at anaphase, fragmentation, and multinucleate condition were also observed by Shah et al. (1992) in gamma ray-treated *Vigna mungo*. Vandana and Dubey (1996) reported meiotic anomalies induced by EMS and DES in *Vicia faba*. These anomalies were found to increase with increasing concentrations of the mutagens applied. Overall frequency of meiotic anomalies induced by various concentrations of diethyl sulphate (DES) was higher than those of EMS.

A relative account of cytological and developmental effects of gamma rays, EMS, and MMS on meiotic features and pollen fertility in *Vicia faba* was provided by Bhat et al. (2005b). The induction of meiotic abnormalities was reported to be higher in MMS treatments, followed by gamma rays and EMS treatments. Precocious migration of univalents to the poles is a very common abnormality

among plants (Pagliarini and Pereira 1992). The other segregational abnormality (non-oriented bivalents) is rare but known to occur in *Chlorophytum comosum* (Pagliarini et al. 1993). The behavior of these and of laggard chromosomes is characteristic in that they generally lead to micronucleus formation (Koduru and Rao 1981). Khan and Tyagi (2009) reported bridges and laggards in soybean, when treated with EMS, gamma rays, and their combination.

In maize, sticky chromosomes were first reported by Beadle (1932) and are seen as intense chromatin clustering in the pachytene stage. The phenotypic manifestation of stickiness may vary from mild, when only a few chromosomes of the genome are involved, to intense, with the formation of pycnotic nuclei that may involve the entire genome, culminating in chromatin degeneration. Chromosome stickiness may be caused by genetic or environmental factors. Several agents have been reported to cause chromosome stickiness, including X-rays (Steffensen 1956), gamma rays (Al-Achkar et al. 1989), and some chemicals present in soil (Caetano-Pereira et al. 1995). However, the primary cause and biochemical basis of chromosome stickiness are still unknown. Gaulden (1987) postulated that sticky chromosomes may result from defective functioning of one or two types of specific nonhistone proteins involved in chromosome organization, which are needed for chromatid segregation. The altered functioning of these proteins leading to stickiness is caused by mutations in the structural genes coding for them (hereditary stickiness) or by the action of mutagens on the proteins (induced stickiness). The stickiness could also be due to depolymerization of nucleic acids caused by mutagens or due to partial dissociation of nucleoproteins and alterations in their pattern of organization (Evans 1962). Jayabalan and Rao (1987) suggested that stickiness of chromosomes might be due to the disturbances in cytochemically balanced reactions in nucleic acids. However, it seems most probable that some kinds of gene mutations lead to incorrect coding of some nonhistone proteins involved in chromosome organization. These proteins lead to chromosome clumping.

In angiosperms, cytoplasmic connection is a phenomenon widely described by Risueno et al. (1969). The first description was made by Gates (1908), who observed delicate threads of cytoplasm connecting adjacent pollen mother cells in *Oenothera*. According to Risueno et al. (1969), the role of cytoplasmic channels is related to the transport of nutrients between meiocytes. Although, cytoplasmic connections are very common in angiosperms, the movement of nuclear material through them is rare. In general, cytomixis has been detected at higher frequency in genetically imbalanced species such as hybrids, as well as in apomictic, haploid, and polyploid species (Yen et al. 1993). Among the factors proposed to cause cytomixis, the influence of genes, fixation effects, pathological conditions, herbicides, and temperature have an ample impact (Caetano-Pereira and Pagliarini 1997).

Cytomixis may have serious genetic consequences by causing deviation in chromosome number and may represent an additional mechanism for the origin of aneuploidy and polyploidy. In various crops, the abnormal spindles have been reported (Harlan and De-Wet 1975). The spindle apparatus is normally bipolar and acts as a single unit, playing a crucial role in the alignment of metaphase chromosomes and their movement during anaphase. Distortion in meiotic spindles may be responsible

for unreduced gamete formation. The formation of unreduced gametes has been investigated in studies of evolution (Harlan and De-Wet 1975) and in breeding programs (Veilleux 1985). It was reported that meiotic abnormalities cause male sterility (Goyal and Khan 2009). Chromatin bridges and micronuclei were described for the first time in interspecific hybrids of *Glycine max* and *Glycine soja* by Ahmad et al. (1977), who found that the extent of abnormalities was influenced by environmental conditions. The same abnormalities were reported by Ahmad et al. (1984), who concluded that chromosome behavior and fertility depends on the percentage of hybrids and on environmental temperature. Studies on different plant species have shown that the decline in seed production is correlated with meiotic irregularities (Khazanehdari and Jones 1997).

7 Mutagenic Effectiveness and Efficiency

Before the initiation of any sound breeding program, knowledge of relative biological effectiveness and efficiency of mutagens and their selection is essential to recover high frequency of desirable mutations. Kodym and Afza (2003) suggested that if no relevant data on appropriate doses of mutagens are available, a preliminary experiment with different doses must be conducted to determine the mutagenic effectiveness and efficiency. It is not necessary that an effective mutagen shall be an efficient one also (Koli and Ramkrishna 2002; Gaikwad and Kothekar 2004; Goyal and Khan 2010). Both of these though are two different properties, but the usefulness of any mutagen in plant breeding program depends on both of them.

Mutagenic effectiveness is a measure of the mutations induced per unit dose of a mutagen, while mutagenic efficiency gives an idea of genetic damage (mutation) in relation to total biological damage caused in M_1 generation (Konzak et al. 1965). According to Kaul (1989), the most desirable mutagen is the one that is least damaging and highly useful mutation yielder. The response of biological system, to physical and chemical mutagens, is influenced to a varying degree by numerous biological, environmental, and chemical factors. These factors modify the effectiveness and efficiency of different mutagens greatly, and the same is true for the mutation rate (Nilan et al. 1973). The effectiveness and efficiency of mutagens has thus been reported to vary to a greater extent in various pulse crops such as lentil (Reddy and Annadurai 1992), mung bean (Wani et al. 2011b), urd bean (Kousar and Babu 2010), horse gram (Bolbhat et al. 2012), and soybean (Satpute and Fultambkar 2012). Kharkwal (1998a) studied the comparative mutagenic effectiveness and efficiency of physical (gamma rays and fast neutrons) and chemical (NMU and EMS) mutagens on three varieties of chickpea. Both the effectiveness and efficiency were found to be higher at lower concentrations of the mutagens. According to Mahapatra (1983), sodium azide at low pH was more effective and efficient than gamma rays, EMS, and NMU in inducing mutations. Among monofunctional mutagens, while methylating agents are more toxic and have to be used only at lower concentrations (Fujimoto and Yamagata 1982), ethylating agents, being less toxic, can be applied

at relatively higher concentrations to yield more favorable mutations. Intervarietal differences with respect to effectiveness and efficiency of the mutagens have been reported by Sharma (2001). It was found that a treatment which induces least biological damage by and large shows a high degree of mutagenic effectiveness and efficiency (Khan et al. 2005). Wani et al. (2011b) worked out the effectiveness and efficiency of EMS, HZ, and SA in mung bean and concluded that the moderate concentrations of mutagens were found to be the most effective and efficient in inducing the mutations.

8 Chlorophyll Mutations

Enhancement of mutation frequency and alteration of mutation spectrum in a predictable manner are the two important goals of mutation research. In the past, varied approaches have been tried to achieve these goals (Swaminathan and Sharma 1968). Chlorophyll mutation frequency is useful in assessing the potency of a mutagen. Hence, scoring of chlorophyll mutations has proved to be a much dependable index for evaluating the genetic effects of mutagenic treatments. The chlorophyll-deficient mutants have been successfully used as genetic markers in plant breeding programs for obtaining the preliminary information vis-à-vis the role/effect of different mutagens, in addition to find out the response of a particular genotype to a particular mutagen. Several authors have so far reported the occurrence of different types of chlorophyll mutations such as albina, xantha, chlorina, viridis, virescent, and tigrina in M_2 generation following treatments with various mutagenic agents (Gaibriyal et al. 2009; Wani et al. 2011b; Bhat et al. 2012; Mahamune and Kothekar 2012; Girija and Dhanavel 2013a). Ionizing radiations generally produce a higher proportion of albina mutations than chemical mutagens (Swaminathan et al. 1962; Cheema and Atta 2003). Kaul and Bhan (1977) observed high frequency of albina types in EMS-treated population of rice as compared with gamma ray-treated ones. Kleinhofs et al. (1978) observed the azide spectra for albina, viridis, and xantha to be somewhat similar to dES spectra. They further reported that the higher proportion of viridis in relation to albina, induced by azide as compared to gamma rays, may be due to the vast difference between the two mutagens for inducing the chromosome breaks.

Combined treatments of different physical and chemical mutagens alter the mutation frequency and spectrum to a substantial extent. Singh et al. (1999) reported that combined treatments of gamma rays and EMS were most effective in producing the greater chlorophyll mutation frequency than their individual treatments in *Vigna mungo*. Synergistic effects of combined treatments of gamma rays and EMS for inducing chlorophyll mutations in barley were reported by Khalatkar and Bhatia (1975). Similar synergistic effects were also reported in black gram (Gautam et al. 1992) in the combination treatments of gamma rays and EMS. In cereals and legumes, most of the mutagens, given in combination, exhibit synergism, but in *Pisum* (Arora and Kaul 1989), the combined treatment (gamma rays + EMS)

revealed antagonism. The synergism among two mutagens may be firstly because of the first mutagen treatment making accessible otherwise non-available sites for reaction to the second mutagen and secondly, premutational lesions induced by the first mutagen become fixed due to an inhibitory effect of the second mutagen on repair enzymes (Payez and Deering 1972). Both these pathways should yield a frequency of mutations higher than the total of the two mutagens applied individually. EMS was reported to induce a wider spectrum of chlorophyll mutations than the ionizing radiations. The superiority of EMS in inducing chlorophyll mutations at a higher frequency than the other mutagens was also advocated by Reddy et al. (1993) in lentil, Khan et al. (2005) in chickpea, and Wani et al. (2011b) in mung bean. This confirms that EMS is a more potent chemical mutagen in inducing chlorophyll mutants and supports the earlier report of Khan et al. (2005) in *Cicer arietinum*. EMS is supposed to be specific to certain chromosomal regions (Goud 1967) containing genes for chlorophyll development and has been reported to induce a high frequency of chlorophyll mutations (Swaminathan et al. 1962). Thakur and Sethi (1995) reported that the frequency of chlorophyll mutations was 2–3 times higher in sodium azide than in EMS and gamma rays treatments in barley. Khan and Siddiqui (1992), while using EMS, MMS, and SA, reported the dose-dependent induction of chlorophyll mutations in *Vigna radiata*. Higher concentration/dose of mutagens has been reported to be more effective in inducing greater frequency of chlorophyll mutations (Amarnath and Prasad 2000; Das and Kundagrami 2000). There are strong indications that total mutation frequency (chlorophyll and morphological) and spectrum are associated with the dose of the mutagen. Nevertheless, according to Gaul (1964), the highest dose is not always the most effective in inducing the preferred mutations.

9 Morphological Mutations

Mutations have been variously classified and described by Swaminathan (1964). Kaul (1989) classified mutations into macromutations or megamutations (morphological mutations) and micromutations. Macromutations, whether resulting from single-gene changes or chromosomal aberrations, behave as monogenic traits and follow the Mendelian pattern of inheritance. On the other hand, micromutations are governed by the principles of quantitative genetics. The developmental mutations affecting more than one trait can help in understanding plant's developmental plan and to identify genes that play an important role in this progress. The morphological mutants represent not only a genetic tool in understanding the physiology of crop plants but a source of genes which can be introduced into different pulse breeding programs to broaden the genetic base. Presently, a number of mutants induced with chemicals and radiations are being used to produce saturated genetic maps and explore genomics, gene expression, and gene regulation (Ahloowalia and Maluszynski 2001). Induction of morphological mutations by physical or chemical mutagens has been reported in *Lens culinaris* (Solanki and Sharma 1999), *Cajanus*

cajan (Ravikesavan et al. 2001), *Cicer arietinum* (Shah et al. 2011; Khan et al. 2011), cowpea (Girija et al. 2013; Gnanamurthy et al. 2013a), and moth bean (Jain et al. 2013).

Mutations affecting growth habit, foliage, and maturity have been reported in mung bean (Sangsiri et al. 2005; Wani et al. 2011c). By using EMS, HZ, and SA, a wide range of morphological mutants were identified by Wani et al. (2011c) in two varieties of mung bean. These mutants involved traits affecting plant height, growth habit, seed, and pod. EMS induced comparatively more mutations affecting growth habit (bushy and prostrate) than HZ and SA. The more frequent induction of certain mutation types by a particular mutagen may be attributed to the fact that the genes for these traits are probably more responsive to different mutagens with different modes of action. Nilan (1967) reported that different mutagens and treatment procedures might also change the relative proportion of different mutation types. Though these mutants may not be useful for their direct use because of reduced yield, however, they may be used in hybridization programs to transfer useful traits for the development of high-yielding varieties of mung bean. Although it is not easy to eliminate the negative effects of the pleiotropy, the pleiotropic pattern of mutant genes can be still altered to some extent by transferring it into a specific genotypic background (Sidorova 1981). Tyagi and Gupta (1991) reported that each gene which is of agronomic interest can mutate; hence a wide spectrum of viable mutants can be expected in mutation experiments. The "advance stigma mutant" of mung bean was reported by Raghuvanshi et al. (1978). The calyx of the flowers was enlarged and the style protruded out of the bud which is a favorable peculiarity in hybridization programs. A dwarf mutant was obtained by Rehman et al. (2001) in black gram which was vigorous in growth and had bold seeds. Unbranched mutants were earlier reported by Mouli and Patil (1976). Early flowering mutants have been reported by Kumar and Dubey (1998). According to George and Nayar (1973), earliness in flowering may be due to the physiological changes caused by irradiation. Flower color mutations have been reported in various plants (Atta et al. 2003; Datta and Goel 2005). There are several reports on the effect of physical and chemical mutagens on the size, shape, and color of the seeds (Mohanasundaram et al. 2001; Joshi and Verma 2004). Dwarf, short culm, and early flowering mutants were induced in rice by azide treatment (Awan et al. 1980).

Frequency of morphological mutations has been found to increase with increase in the dose of the mutagen. Datta and Sengupta (2002) reported that spectrum of morphological mutations was wider in lower doses of the mutagens, while Vanniarajan et al. (1993) observed the higher frequency of morphological mutations at medium doses of gamma rays and EMS treatments. Kumar and Mani (1997) are of the opinion that spectrum and frequency of morphological mutations vary with mutagen and duration of treatment. Genetic differences of the experimental organism also have a role in the recoverable frequency and spectrum of morphological mutations (Sharma 2001). The morphological mutants can also help to identify linkage groups. A tight linkage between morphological mutants and economically important traits, such as disease resistance, yield QTLs, and quality traits, will help greatly to transfer economically important traits into good agronomic background.

10 Quantitative Traits

Improvement of cultivated plants largely depends on the extent of genetic variability available within the species. Mutagenesis has provided a handy tool to enhance the natural mutational rate and thereby enlarge the genetic variability and increase the scope for obtaining the desired selections. The significance of small mutations in evolution was first recognized and emphasized by Baur (1924). Most of the plant attributes of interest to plant breeders are quantitative traits which are controlled by polygenic interactions. Gaul (1965) emphasized the significance of micromutations in plant breeding by stating that "there appears to be no doubt that micromutations may affect virtually all morphological and physiological characters as do large mutations and they might have higher mutation rate than the macromutations." There are much differences of opinion among the breeders on the relative incidence of induced polygenic variation in negative or positive direction and shift of the mean in M_2 and later generations (Gaul and Aestveit 1966; Rao and Siddiq 1976). Many workers hold the view that induced mutations can be used to generate useful variations in quantitatively inherited traits, where appropriate selection has been applied for improvement (Lawrence 1965; Tickoo and Chandra 1999). It has been observed that induced mutations occur more or less randomly in the genome and inheritance is almost ever recessive; therefore, homozygosity is required for expression (Micke 1999). Several workers have so for reported encouraging results about the induction of useful quantitative variability in different pulse crops like pigeon pea (Srivastava and Singh 1993), cowpea (Pandey 2002; Girija and Dhanavel 2013b), urd bean (Singh and Singh 2001), lentil (Khan et al. 2006), faba bean (Mejri et al. 2012), chickpea (Khan and Wani 2005; Kozgar et al. 2012), and mung bean (Wani et al. 2011a, 2012a, b).

After the studies of Brock (1965, 1967), it became a common practice to advance only normal-looking M_2 plants to M_3 generation and apply the first dose of selection not earlier than M_3. Comparative studies of selection in M_2 and M_3 generations revealed in many cases that the two generations may not differ in respect of selection response (Scossiroli 1968). Tickoo and Jain (1979) concluded that promising progenies can be identified with high degree of confidence in M_2 on the basis of mean and variance. Jana and Roy (1973) selected M_2 families on the basis of significantly changed mean only. Bhadra (1982) employed inter- and intra-family selection in black gram on the basis of mean and variance in M_2 generation. Selection for quantitative traits, such as yield, should preferably be carried out in early generation because most of the desired combinations of favorable alleles are likely to be lost in advanced generations due to intensive or even no selection for other traits. Kharkwal (1983) also emphasized the effectiveness of such early generation selection for identifying superior lines for polygenetically inherited traits. The efficiency of M_2 generation selection has been reported in lentil (Sarker and Sharma 1988) and mung bean (Tickoo and Chandra 1999).

Heritability and genetic advance are important genetic parameters from the breeding viewpoint. They give an idea of effectiveness of selection in the material

to be carried forward. Frey (1969) reported that mutagen-derived variability for quantitative traits in crop plants is heritable and the response of selection is good. Trivedi et al. (2006) clearly brought out that in the treated population, the estimates of heritability were larger and varied from trait to trait. Johnson et al. (1955) suggested that heritability in combination with genetic advance was more helpful in predicting the effect of selection. Sharma (1977) studied induced variability in lentil and observed higher coefficient of genotypic variability for all the characters except seed size in M_2, suggesting that a part of the variability recorded is genetic which increased the heritability and genetic advance. Ravi et al. (1979) also reported higher heritability and genetic advance in lentil. In urd bean, Singh and Singh (2001) observed high estimates of heritability for pods per plant, pod-bearing branches, 100-seed weight, and total plant yield. High estimates of heritability and genetic advance showed that the number of pods per plant should be the main criterion for selection. Kaul and Kumar (1983) in rice studied the genetic variability for quantitative traits and concluded that heritability values for different quantitative traits were several times higher in the treated population than in the control.

In mung bean, different workers have reported increased variability for various quantitative characters in mutagen-treated population as observed by significant changes in the mean values and coefficient of variability as compared to the control. Positive or negative mean shifts were reported for various quantitative traits after mutagenic treatments (Tickoo and Jain 1979; Singh et al. 2001). Chaturvedi and Singh (1980) obtained some synchronously maturing mutants in M_2 generation from treated seeds of mung bean with NMU. Yadav and Singh (1988) also observed mutation for synchronous maturity by exposing mung bean var. PS-16 with gamma rays. Khan (1986), using EMS and gamma rays singly or in combination, observed an increase in the genetic variability for various quantitative traits in the var. PS-16 of mung bean. Rajput (1974) reported increased variability in M_2 population after irradiation of mung bean with gamma rays. Krishnaswami et al. (1977) studied the phenotypic responses of mung bean to X-rays and gamma rays and reported an increase in variance for quantitative traits in M_2 generation. Khan et al. (1998) studied the mutagenic effects of maleic hydrazide (MH) on plant height, days to flowering, and days to maturity in M_2 and M_3 generations of mung bean varieties K-851 and PS-16. The shift in mean values after the MH treatments was noticed in negative direction, except days to flowering in the var. K-851, for all the characters in comparison with their respective controls.

As stressed by Aastveit and Gaul (1967), in mutation breeding programs, there is imperative need to undertake studies on correlation coefficients in a routine manner, in addition to the estimation of genetic variability in characters such as yield and yield contributing traits. Bahl (1988) studied the change in correlations between various character pairs after mutagenic treatment. Kumar and Arora (1991) studied the relationships among various plant characteristics and yield in chickpea. Several highly significant changes towards desirable side were induced in correlation coefficients of character pairs in the mutagen-treated population (Kharkwal 2003; Khan and Wani 2005; Mubeen et al. 2007).

Contrary reports are available in literature on the extent of success of induced mutations for high grain yield coupled with high protein content of the mutants. Some workers are of the opinion that high protein content is difficult to combine with high yield as these two traits reveal almost negative correlation (Abo-Hegazi 1980; Gottschalk and Muller 1982). Gottschalk (1990) explained that there is no doubt that these traits are controlled by genes and mutations in these genes can alter the protein makeup of the genotypes. It is, however, very difficult to discern their action reliably because these traits are highly influenced by environmental factors. That the protein production in plants is influenced by the interaction of gene(s) and environmental factor(s) has also been reported by Sengupta et al. (1986). However, high-yielding mutants coupled with high protein content were reported by Kharkwal (1998b) in chickpea and Naik et al. (2002) in mung bean.

11 Nutritional Aspects of Mutagenesis in Pulses

The yield and quality are the two important traits to be considered as important goals for improvement of pulses. Very little work has been done for improvement of nutritional values of these crops, especially in mung bean (Singh et al. 2011). Although the concept of nutritional value is very complex (Munk 1964) it seems to be gained if the content of protein and the amount of some essential amino acids, viz., methionine and cystine, could be increased. The protein content is influenced by many factors such as nitrogen manuring, irrigation, and time of sowing. The improvement of protein content in pulses through mutation has been reported by a number of workers. Dahiya (1978) observed a significant variation for protein content in mung bean when irradiated by gamma rays. He also reported that more intensive treatment had the greater impact in increasing the protein content. Wani et al. (2011a) reported that seed protein showed a nonsignificant negative correlation with yield in different mutant lines of green gram. In different mutants, the coefficient of variation for total seed protein content had not greatly altered over the control, indicating that further improvement is difficult to achieve. A similar negative correlation between yield and seed protein content has been reported earlier (Gottschalk and Muller 1982; Khan and Wani 2005). Protein content is influenced by the interactions of gene(s) and environment factor(s) as has been reported in chickpea (Singh et al. 1990). Farooq and Nizam (1979) reported a significant difference in protein with high yield in red gram after treatment with base-specific chemicals. Shaikh et al. (1982) observed variation in protein content in M_1, M_2, and later generations of chickpea following gamma irradiation.

Malik (1988) isolated early mung bean mutant having high yield and protein content after treatment with gamma irradiation in M_4 generation. Mutation with gamma rays and EMS induced early mutants in urd bean with increased pod numbers and protein content (Sharma et al. 2007). Gamma irradiations were successful in increasing the protein content and yield in mung bean (Chakraborty et al. 1998). Malik et al. (1999) and Alexiava (1991) reported early mutants in mung bean and

soybean, respectively, having high yield and protein content from gamma ray-irradiated population. Khan and Claydon (1975) determined the role of induced mutations in the improvement of winged bean. Their studies mainly confined to isolate mutants with a potential new source of protein. Dadke and Kothekar (2005) made an extensive effort to accomplish the qualitative improvement of winged bean pertaining to its crucial biochemical features like seed proteins and seed trypsin inhibitors. Excellent variability has been obtained with regard to such parameters, and significant success has been gained in developing and characterizing the low trypsin inhibitor containing the lines of winged bean through mutation breeding. Such lines can be released as new varieties for the large-scale commercialization of winged bean. They concluded that the development of mutants carrying lowered level of trypsin inhibitors would be quite relevant in winged bean improvement as several parts of the plant are traditionally eaten in the green and uncooked form. Odeigah et al. (1998) reported that total seed protein, globulin, and albumin fractions show differences in number and intensity of subunit bands, but the least differences were found in the albumin fraction, when treated with sodium azide, EMS, and gamma rays.

12 Mutations Affecting Nodulation in Pulses

Nodulation and its constituent traits have significant effect on yield and quality. Nodulation influences the availability of nitrogen through photosynthetic activity which consequently determines the yield of pulses. Plants having higher number of nodules per plant may be expected to be capable of fixing greater amount of nitrogen and could be better adopted for higher photosynthetic activity leading to increase the yield potential (Singh et al. 2011). Many spontaneous and large numbers of induced mutants that show altered nodulation pattern have been isolated in pea, soybean, common bean, faba bean, chickpea, groundnut, and pigeon pea. Yadav and Singh (1985) have reported induced increased nodulation in mung bean through mutagenic treatments. Lee et al. (1991) reported a significant difference in shoot dry weight, root dry weight, nodule number, and nodule dry weight in mutant population of soybean. Singh et al. (1997) reported an induced mutant which had a greater number of dry weights of nodules with higher yield, when compared with the parent in black gram. Sagan and Gresshoff (1996) reported a super-nodulating mutant of soybean which was characterized by a faster appearance of nodules and a more extensive nodule distribution over the root system.

A single super-nodulating mutant, "En6500," was isolated from 2,800 families of soybean (Akao and Kouchi 1992). The "En6500" produced an increased number of nodules, which was later observed to be inherited as a Mendelian recessive trait (Kokubun and Akao 1994). However, the growth and the yield were inferior to those of the original cv. Enrei, probably because of high consumption of carbon hydrates by the nodules and a limited root surface for absorption (Takahashi et al. 1995). Treatment with nitrosoguanidine increased nodule dry weight and

nitrogenase activity of root nodules in lentil (Rai 1985). Better N_2-fixing symbiosis may be brought by manipulating both *Rhizobia* and plant hosts and by eventually creating an artificial rhizosphere. Specificity of the *Rhizobium*-legume symbiosis is also governed by specific genes on the bacterial chromosome that code for proteins involved in recognition and uptake of specific signal molecules present in root exudates. The genotypic specificity of the nodulation process depends on gene interaction between *Rhizobia* and the host. Symbiotic interaction between *Rhizobium* and leguminous plants is very specific. The importance of nodulation mutants in basic studies on plant–microbe symbiotic interaction, nitrogen fixation, and breeding of cultivars with higher yield and nitrogen fixation rate is observed by Bhatia et al. (2001), and they reported that the nodulation mutants, after inoculation with specific bacterial strains, show either no nodulation (nod^-), few nodules ($nod^{+/-}$), ineffective nodulation (fix^-), hyper-nodulation (nod^{++}), or hyper-nodulation even in the presence of otherwise inhibitory nitrate levels (nts). Nodulation mutants have contributed to understanding of the genetic regulation of host-symbiont interactions, nodule development, and nitrogen fixation. Using the induced hyper nodulating mutant, a new soybean cultivar "Nitrobean60" has been released in Australia. This cultivar is reported to have given 15 % higher yield over cv. "Bragg" and contributed a higher amount of fixed nitrogen to the subsequent cereal crop in rotation (Singh et al. 2011).

13 Uses of Molecular Techniques

The uses of molecular markers in plant breeding programs have a striking area of interest and are being used in a unique approach to analyze and survey the induction of mutations elicited in a number of crop plant species of agronomic importance. Besides qualitative traits, to understand the inheritance of quantitative traits, molecular markers give breeders relatively more precise and reliable results. The use of molecular markers in pulses as well as other crop plants offers new opportunities by providing selection on the genotypic instead of phenotypic level (Toker et al. 2011). In order to make this process easier, morphological and ecological information, pedigree logs, and several mating designs have been used by pulse breeders. However, the efficiency decreases if the trait of interest cannot be measured or shows low heritability. The use of molecular techniques could fill the gap among the difficulties encountered during the crop improvement. Till date, many molecular markers in pulses have been used for different aims. The most widely used markers in pulse crops are RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), SSR (simple sequence repeat), ISSR (inter-simple sequence repeat), and STMS (sequence-tagged microsatellite sites).

Important prerequisites for undertaking molecular breeding are molecular markers, genetic maps, and markers associated with traits (Varshney et al. 2007). Isozyme markers were used for map development during early days of genomic studies.

But, expression of these markers was influenced by the environment, and their number was small coupled with a low level of polymorphism in cultivated genotypes (Gaur et al. 2012). In some earlier studies, RFLP and RAPD markers were also used for genetic mapping and diversity studies (Simon and Muehlbauer 1997). RAPD markers are based on amplified arbitrary sequences which scan a wider part of genome leading to the resolution of complex taxonomic relationships (Casiva et al. 2002). Using RAPD markers, not only *Fusarium* wilts but also ascochyta blight pathogens have been well studied in various pulse crops. However, the extensive use of molecular markers in genetics and breeding started only after the development of simple sequence repeat (SSR) or microsatellite markers. SSR markers have become the marker of choice in plant breeding due to their multi-allelic and codominant nature (Gupta and Varshney 2000). Another PCR-based fingerprinting technique widely used in pulses genomics and breeding studies is ISSR. The technique does not need any prior information about DNA sequence and overcomes many of the technical limitations of RAPD and AFLP because of its high reproducibility and simplicity (Uzan and Cagirgan 2009). Ratnaparkhe et al. (1998) reported that ISSR polymorphisms are useful for finding markers associated with disease resistance gene clusters in chickpea as well as other plants. ISSR combination with bulked segregant analysis has a wide application and can be useful for identifying the molecular markers linked to gene of interest (Ratnaparkhe et al. 1998; Uzan and Cagirgan 2009) and for developing the sequence-tagged microsatellite sites (STMS). The STM markers have wide application in chickpea genetic maps (Toker et al. 2011). Both genomic and transcript datasets have been utilized to develop SSR markers.

Single-nucleotide polymorphism (SNP) markers have drawn greater attention in recent years due to their higher abundance and amenability to high-throughput approaches. Diversity arrays technology (DArT) is a high-throughput genome analysis method enabling a rapid and economical approach for screening a large number of marker loci in parallel (Jaccoud et al. 2001). DArT technology utilizes the microarray platform to analyze DNA polymorphism. DArT markers have been used for different purposes: (1) developing high-density genetic maps and (2) studying genetic diversity in various crops. Considerable progress has been made in the development of genetic and genomic resources for pulse crops during the past decade. The availability of a large number of molecular markers, dense genetic maps, and markers associated with traits and transcriptomics resources has made it possible to integrate genomics technologies for pulse improvement.

In the present scenario, TILLING has emerged as a robust, high-throughput, non-transgenic method that can be applied to most species (Tisser and Bourgeois 2001). TILLING is a technique combining chemical mutagenesis with a sensitive DNA screening technique that enables the recovery of individuals carrying allelic variants at candidate genes. Here, large numbers of small changes, either DNA base-pair substitutions or small deletions spanning no more than a few base pairs, are induced in a series of lines. In these lines gene function can be ascertained by associating a phenotype with changes in a particular gene and novel alleles of known genes. This new reverse-genetic approach combining high frequency of

point mutations induced by special mutation techniques can detect heteroduplexes between wild-type and mutant DNA fragments using "denaturing high-performance liquid chromatography" or "DPHLC." In this approach, point mutations of high density are required for which highly efficient chemical mutagens and ionizing radiations are generally used to develop mutated generations. Reverse-genetic strategies that target lesions to specific genes hold great promise in speeding up the process of gene-function analysis and enhancing the efficiency of mutation breeding. The knowledge on functional genomics and basic genetics of the model legumes will provide much benefit to mutation breeders in the frame of marker-assisted selection. The marker-assisted selection has a great potential to develop cultivars with resistance to biotic and abiotic stresses (Toker et al. 2011). In less than a decade, TILLING has moved from a proof of concept to a well-accepted reverse-genetic method that has been applied to over 20 different species. Over the coming years, new technologies such as these will have increasing impact in practical plant breeding. However, they will require different types of mutations induced at specific frequencies.

14 Biotechnological Approaches

The global warming and major environmental pollutions drastically reduce the production and productivity of pulses especially mung bean in the last two decades. Reduction in yield has enforced to adopt novel strategies of biotechnology in order to develop pulse crops which are tolerable to drought and cold and resistant to various types of diseases like *Fusarium* wilt, ascochyta blight, etc. Conventional mutations have well-defined limitations, particularly in crop breeding; however, the use of in vitro techniques coupled with conventional mutagenesis has conquered this barrier. The major bottlenecks in mutation breeding of vegetatively propagated plants are formation of chimeras. Therefore, attempts are made to find out the ways to prevail over this situation. Management of chimera and in vitro technique has opened a new vista for isolating new cultivars through recovery of mutated cells. In vitro mutagenesis can induce stress tolerance among the crop plants to improve their yield and quality. Understanding the mechanism that regulates the expression of stress-related genes is a fundamental issue in plant biology and is of utmost necessity for the genetic improvement of plants. The plant breeder has on hand several in vitro techniques such as micro propagation, protoplasts, embryo rescue, and somatic embryogenesis, which increases the efficiency in obtaining variation, selection, and multiplication of the desired genotypes (Ahloowalia 1998). The benefits of in vitro mutagenesis are (a) mutagen treatment can be given to large cell or protoplast or somatic embryos, (b) fast multiplication of mutant plant material, (c) in vitro selection of mutation, and (d) less space required for shoot multiplication under the controlled conditions.

Tissue culture of periclinal chimeras often results in segregation of component genotypes, depending on the pattern of differentiation and proliferation of the shoots

formed (Lineberger et al. 1993). One positive aspect of such association is that depending on the type of explants to be mutagenized, larger populations of cell can be grown homogeneously and submitted to selection pressure within a small in vitro space. As it is well known, in order to increase the possibility to select useful mutants, larger populations of cells have to be screened. This is of special importance when researchers are looking for dominant mutations that occur in lower frequency as compared with recessive ones. Certain in vitro explants responded better to chemical mutagen treatment due to higher permeability than in vivo treatments (Tulmann Neto et al. 1997). The association of in vitro and mutagen treatment is also unique as for the use of haploid cells, protoplast, and cell suspension is concerned. Treatment of cell suspensions or protoplasts might avoid chimera formation, thus enable the selection of stable mutants. Even in situations where multicellular explants are mutagenized, resulting in chimerism, in vitro technique can be used to decrease the time needed for the advance of generations before selection is applied.

The techniques of biotechnology can be profitably utilized for the improvement of crop plants (Kozgar et al. 2013). However, it has been more difficult to regenerate functional plants from grain legumes. Though mung bean is an important pulse crop, very little attention has been paid to tissue culture research. Bajaj and Dhanju (1979) have reported in vitro regeneration of plants from apical meristem tips of mung bean. Singh et al. (1985) obtained calli from root hypocotyls and shoot explants of three-day old mung bean seedlings and the genotypes varied in their potential for root and shoot regeneration. Mathew and Bhatia (1983) regenerated functional plants from de-embryonated cotyledons of mung bean. The cotyledon explants were cultured on MS basal medium without addition of exogenous hormones. Plants with roots and shoots arose from the proximal end of the cotyledon within 25–30 days after transplanting into the sterile soil. These plants matured and produced viable seeds. Mathew et al. (1986) reported that when these seeds were grown, 14 % of the progenies showed a wide spectrum of morphological mutations which are similar to those obtained following seed treatment with radiation or chemical mutagens. The production of double-haploid chickpea embryos and regenerated plants through anther culture using Canadian cultivar CDC Xena (kabuli) and Australian cultivar Sonali (desi) were reported by Grewal et al. (2009). Multiple shoots were induced directly from seedling plant cultured on different types of media with different hormonal concentrations. Multiple shoot formation from hypocotyls was significantly higher as compared to other explants. In contrast, all the wild species of *Cicer* gave higher shoot formation as compared to cultivated species.

Micropropagation and production of somaclonal variants in chickpea and its wild relatives were found to be very useful for chickpea improvement. Efficient multiple shoot formation which could be induced in a number of explants is visualized to be useful for genetic transformation using Ti plasmid (Rao and Reddy 1992). Somatic embryogenesis and subsequent shoot development was achieved in cotyledon-derived cultures of *C. arietinum*. Callus was induced on Gamborg's basal medium supplemented with 3 % sucrose and different concentrations of 2, 4-D, but

2 mg l^{-1} was found to be optimum for the production of embryogenic callus (Rao and Reddy 1992). Poehlman (1991) has stated that biotechnological research has been largely directed towards forage legumes or soybean and relatively little to mung bean. This field of research is inordinately expensive and long term in attainment of practical benefits. Considering the economic importance of mung bean, it does appear likely that mung bean will be the focus of a major biotechnology research effort per se.

15 Conclusions and Future Perspective

Food insecurity problem has seen a major deterioration in the last few years; food prices are rising sharply, and the poor people of the world are threatened with serious malnutrition (FAO 2009). In recent years, the crops have been subjected to various types of abiotic and biotic stresses in nature by growing human population, global warming, pollution, urbanization, etc., which have lowered the production of most essential crops especially pulses. With the consistent increase in the world's population, per capita availability of pulses is getting reduced. Food insecurity will even get worse since population is still growing at an alarming rate while no significant expansion of arable lands is foreseen. Food and Agriculture Organization (FAO) estimates that world food production should increase by more than 75 % in the next 30 years to feed about eight billion people by 2025. Therefore, a new "Green Revolution," rather "Evergreen Revolution," is desperately needed to solve the food security issue at present and for future generations and years to come. Traditional mutagenesis has been widely used in forward genetic strategies and has led to the release of over 3,000 mutant plant varieties. Heritable genotypic variation is a major contributor to phenotypic diversification and thus a fundamental driver of evolution. Naturally occurring alleles and induced mutations can be used as essential tools to study the plant gene function and to develop crops with agronomically important traits.

Mutations may induce both qualitative and quantitative variation comparatively in a shorter period of time by altering alleles at known loci as well as at previously unknown loci, besides altering linkage groups. In the age of biotechnology, pulse genomic research is taking up substantial pace. The mutants having no breeding value generally thrown away by the researchers are now becoming an important tool in genomic research. Damage to the DNA of particular gene sequence is now possible in pooled samples taken from large mutated populations using novel mutation detection technique known as "targeting induced local lesions in genomes (TILLING)" (Mc Callum et al. 2000a) which is gaining enormous popularity in these days. Mc Callum et al. (2000b) though demonstrated this technique in *Arabidopsis* for the first time, it is adopted for other plant species as well. If the sequence of the targeted gene is known and the methodology for detection of single-nucleotide substitutions is available, TILLING can be applied successfully (Solanki et al. 2011). In order to tailor the mutation process, there will be an immense need

to understand how specific classes of mutations are generated and distributed over genomes. In the past, this has not been possible because of lack of analytical tools and an inadequate knowledge of both the processes of DNA damage and the architecture of plant genomes. In addition, only a restricted number of plant genes were sequenced. Today, high-throughput DNA sequencing methods coupled with bioinformatics and functional genomic approaches provide extensive knowledge on genome architecture. All in all, the stage is set to transfer the science of DNA damage induced by physical and chemical mutagens from human genetics to plant systems (Lagoda 2004). The integration of genomic technologies in breeding will greatly improve the efficiency of breeding programs in the development of better cultivars and reduce the time required for cultivar development.

Conclusively, this review points out that there are still ample sources of improving the varieties of mung bean as well as other pulses through conventional and non conventional methods. These breeding tools need to be manipulated in various combinations in order to achieve better success in the breeding program. The technology of recombinant DNA and gene cloning are the powerful tools to achieve desired goals. In future, sequencing of the mutated genes and development of molecular markers will receive more attention for pulse improvement and to lead success from lab to field for better future. Cultivars with improved efficiency with respect to yield; early maturity; uptake of micronutrients; tolerance to abiotic stresses like drought, cold, and salinity; and resistance to biotic stresses like disease and insect pests can be easily developed using mutation breeding and marker-assisted selection.

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Chapter 12
Crop Improvement Through Tissue Culture

L.F. De Filippis

1 Introduction

Population growth and environmental degradation are two major problems on the planet. Fulfilling the needs of this growing population is going to be difficult from the limited arable land available without avoiding famine, malnutrition and shortages of food (i.e. food security). Although there are legal, social and political barriers to the widespread use of any branch of plant biotechnology, advances in this field have substantially improved agriculture and human life to a considerable extent. One of the vital tools of plant biotechnology is the in vitro cultivation of plants, commonly called plant tissue culture (PTC) or micropropagation (MP), which is most useful as an experimental aid and enabling technology to agricultural production, plant breeding, plant modification, plant metabolite production, genetic manipulation, cloning of cultivars and the quick and clonal propagation of limited and rare stocks of plants according to desired needs.

The world population is increasing at an alarming rate and is projected to reach about 8.5 billion by 2025, and this factor alone has resulted in current food deficiencies causing malnutrition, which can be a serious health problem. Producing crops with improved quality and quantity is imperative for food demand through sustainable agriculture and could be achieved either by using conventional selection and breeding or through genetic engineering and tissue culture (Ashraf et al. 2012; De Filippis 2012). Biotechnology for crop improvement has now become a viable strategy to combat deficiencies in foods by enhancing metabolites, proteins, carbohydrates, lipids, vitamins and micronutrient composition (Jain and Brar 2010; Hakeem et al. 2012). With the increase in crude oil prices, climate change issues and limited reserves of fossil fuel, some crop plants have been diverted to alternate energy

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sources for biofuel and biomass. Among the potential biofuel plants are crops like corn, edible oilseeds and nonedible oil-producing plants like date palm and *Jatropha*. These crops can yield promising biomass for biofuel conversion (i.e. 3.5–4.0 kg seeds/tree; enough for about 1 L biodiesel or 2.2–2.7 tonnes of oil/hectare). However, the use of substantial amounts of corn and edible oils for biofuel purposes will lead to even greater shortage of food and escalating food prices. We cannot afford to use good agricultural arable land, which is most valuable for food production and biodiversity to be diverted to the cultivation of biofuel crops creating severe competition with their use as food crops (Sun 2008; Sudhakar Johnson et al. 2011).

The term 'tissue culture' is most often used to describe all types of aseptic plant culture procedures leading to plant growth and falls into various categories depending on the type of growth achieved and the initial explant source for the culturing process (Chawla 2009; Neuman and Kumar 2009). Therefore tissue culture techniques can involve any of:

(a) Plant protoplasts (cells without the cell walls)
(b) Callus (loose or compact cell arrangement)
(c) Tissues (apical or root meristems or pith segments)
(d) Organs (including buds or roots)
(e) Embryos (usually used for more specialised purpose)
(f) Plantlets (having both roots and stem/leaves) (Murphy 2007; Jain and Saxena 2009)

Presently, ten major areas of plant and crop production are recognised as part of the tissue culture process and are primarily based on end points or objectives of the culturing process:

(a) Study systems for fundamental plant cell physiology and biochemistry
(b) Propagation of elite plant materials (for breeding or conservation purposes)
(c) Generation of genetically modified fertile individuals from infertile lines (for breeding)
(d) Increased speed of production for use in breeding and/or propagation programmes
(e) Genetic modifications that is difficult or impossible to achieve in vivo
(f) Preservation of endangered species and hard to propagate plants by conventional means
(g) Storage and/or preservation of germplasm and whole genotypes
(h) Metabolic engineering for plant chemicals and/or pharmaceuticals
(i) Increased production of desired plant metabolites and/or biochemicals
(j) Production of disease-free stock plant material (Vargas and Flota 2006; De Filippis 2012)

Plant tissue culture growth takes place in a sterile, artificial and controlled environment and is often referred to as 'in vitro culture'. It must be emphasised that the eventual outcome of any form of tissue culture needs to have a final objective to try to acclimatise tissue culture plantlets to greenhouse and eventually open field conditions; otherwise the potential time, effort and resources put into tissue culture may

be wasted. Despite the progress made so far, tissue culture involving some crops, especially woody plants, has presented problems and is considered to be *recalcitrant* under in vitro conditions (Aftab and Preece 2007). Lack of suitable totipotent explant sources and reproducible regeneration protocols, release of toxic exudate polyphenols, slow growth and high rates of contamination have limited the success in micropropagation of many woody plants of economic importance. However, several nursery/greenhouse techniques may now be combined in tissue culture to obtain improved results and have increased the success in micropropagation of woody plants (Preece and Read 2007; Aftab 2012). In understanding growth in tissue culture, three concepts must be understood:

(a) *Plasticity*—ability of a plant to endure extreme growth conditions by quickly changing and adapting the growth and development of its plant cells, tissues and organs without dying.
(b) *Totipotency*—concept that any part of the plant can give rise to an entire new plant given the right conditions. In short, totipotency is the ability of a plant cell or protoplast to undergo a series of complex metabolic and morphological coordinated steps to produce a complete and normal plant without the participation and need for sexual (i.e. seed) reproduction.
(c) *Phytotoxicity*—compounds and processes that are toxic or inhibit plant growth.

The technology of plant tissue culture and commercial laboratories using tissue culture propagation had a rapid period of expansion, but over the last 25 years, there have been signs of amalgamation and a slowdown in growth. In the Netherlands alone where there are reliable figures, there were 67 companies producing 62 million plants a year. The number of companies today has decreased due to financial constraints, consolidation and an increase in size of operations, but the number of tissue culture plants produced has in fact increased (de Fossard 2000). Similar reduction of tissue culture companies is evident in many other countries, and this technology is less important now as a major source of technological export to developing countries in agriculture and horticulture. Nevertheless, it is the emerging developing countries that are quickly adopting and using plant tissue culture technology. The most important developed countries in plant tissue culture production/technology are the USA, Japan, Israel, Italy, Holland, France, Spain, Belgium, the UK, India and South Africa. The main developing countries for tissue culture adoption include China, Columbia, Brazil, Kenya and Vietnam (De Filippis 1999). The expansion and use of tissue culture has become important for some food crops where high quality is important as one of the most costly aspects of crop production, especially in planting material used, and when the product is to be exported to international markets. A good example of this is potato where the traditional mode of propagation is by planting tubers or sliced tubers, and this is prone to diseases such as *Erwinia*, *Rhizoctonia*, leaf roll virus and spindle tuber viroids. Accumulation of any of these pathogens causes planting stock degeneration and eventually inferior diseased stocks; and more importantly the product is not accepted for export. The use of tissue culture for pathogen elimination and superior planting stocks and the

rapid multiplication of these potato stocks have lead to its widespread use in developed and developing countries.

Details of the techniques involved in the broad technological area of plant tissue culture are beyond the scope of this chapter and will not be dealt with here, but I will refer you to recent books that address this (IAEA-TECDOC 2004; Razdan 2003; de Fossard 2007; Chawla 2009; Jain and Brar 2010). However, during the course of this chapter, some aspects of these techniques must be covered. Plant tissue culture is not a science in itself. Tissue culture is merely a range of enabling techniques in which the knowledge of many different scientific disciplines is used when a particular type of conventional propagation and breeding method cannot be used on that plant for improved traits, or a type of end-user plant and/or plant product is required. In vitro techniques can be used to aid conventional and molecular breeding programmes, because tissue culture has a number of advantages over traditional use of greenhouses and field conditions, including disease elimination and selection of elite breeding lines, and the rapid multiplication of these stocks. The practical use of 'somaclonal variation' (Sect. 2.6) and induced mutation for new genotypes, and screening of breeding stocks, is quick and exact using tissue culture, especially with marker assisted selection (MAS). But there are some disadvantages in that disease could quickly infect all material, can be expensive, requires more skills and material must be conditioned to grow in the field because the plantlets out of tissue culture tend to have low metabolic activity and are 'weak' if not 'hardened off' (Herman 2009; Davey and Anthony 2010).

The use of tissue culture in transformation of plants is considered essential with all the major plant transformation systems used, and genetically modified (GM) crops will be dealt with later. Transgenic plant biotechnology very often starts with tissue culture (Murphy 2007; Chawla 2009), simply because plant cell and tissue culture are sets of defined techniques that were always designed for quick growing and cloning any plant part in a defined culture media in aseptic and controlled conditions (Vargas and Flota 2006). These are exactly the conditions required (sometimes by legislation or bioethical approval) in genetically modified (GM) crops or transgenic crop development (Stirn and Lorz 2006; Rashid et al. 2012). Therefore, tissue culture is not hypothetical, and without proper tissue culture procedures, it would be difficult to achieve an efficient and legally binding gene transfer, selection, rejuvenation and testing of putative transformed plants.

2 Meristem Cultures

2.1 What Are Meristem Cultures?

The cultivation of axillary or apical shoot meristems is commonly known as meristem cultures. Meristem cultures involve the growth and development of existing immature or dormant shoot meristems on the explant, and when these shoots are mature or

sufficiently old, the regeneration of adventitious roots from the developed shoots is possible. It usually does not involve the regeneration of new shoot meristems. The objective is to use and maximise existing shoot meristems in explants and culture them eventually to produce many more regenerated plantlets ready for 'weaning' or 'hardening off' in nursery production (Sect. 11).

2.2 Use of Meristem-Cultured Plants

Most cases of meristem cultures are essentially multiple shoot-tip cultures. Nodal explants of various sizes are commonly employed for rapid clonal propagation. In vegetative propagated plants, the size of shoot tips used for culture is not that important. However when the objective is to free the stock from viruses, it is essential that apical meristems should be excised with a minimum of the surrounding tissue to aid virus elimination. The shoot tip may be cut into fine pieces (i.e. fragmented) within limits to obtain more than one plantlet from each tip. Generally, explants taken from actively growing parts of plants at the beginning of a growing season are most suitable to culture.

2.3 Browning of the Medium

In many plants, phenolics may leach into the medium from the cut surfaces of the explants. The phenolics turn dark brown or black and oxidise, and these compounds can be detrimental to the cultures. This problem is very common in woody crop species, particularly when explants are taken from mature tissues. This problem can be overcome in most plants; however in some plants like mango, pistachio and some other woody crop plants, control of phenolics is a major problem since the entire explant can quickly turn black and die. To overcome this excessive phenolic exudate, a number of measures can be taken, such as frequent sub-culturing, a period of culture in liquid media, adding antioxidants like ascorbic acid, cysteine-HCl or citric acid, adding adsorbent materials like activated charcoal or PVP (polyvinylpyrrolidon) and even culturing explant material in the dark where synthesis of phenolics is restricted (Peat and Jones 2012; Tilkat et al. 2012).

2.4 Rooting of Shoots

Explant root induction medium usually contains low salts and reduced sugar content, and in most species, the auxin group of phytohormones (NAA and/or IBA) is required for root initiation. In plants like citrus, a pulse treatment with the auxins

above gives optimum rooting. Shoots are usually rooted in an agar medium, but a recent trend is to root explants directly in vermiculite or potting mix. Rooting in potting media can save costs as rooting and soil transfer stages are combined, and an 'in vitro' rooting stage is eliminated, but it is not always successful. Plantlets with as little as two cm of roots may be transplanted into pots, but more roots are desirable for improved success. Fog or mist is used to keep the humidity high, which is gradually decreased to ambient levels, and simultaneously the light intensity is increased. The plants, after survival under these more controlled conditions, can finally be exposed to full greenhouse environments (Tilkat et al. 2009a).

2.5 Vitrification Process

Some shoots developed in vitro appear brittle, glassy and water soaked, and this process is called 'vitrification'. In many species, vitrification may be present but not visible to the eye, and other symptoms may include the poor development of vascular bundles, little or no wax cuticles, abnormal functioning stomata and reduced uptake of nutrients and sugars. Vitrification is an undesirable consequence of tissue culture and leads to losses of plantlets. Vitrification at times may be overcome by decreasing humidity, increasing agar percentage, cooling cultures, lowering phytohormone levels and use of growth retardants (Al-Taleb et al. 2011; Naik and Chand 2011).

2.6 Somaclonal Variation

Morphological variants may arise at any stage during the multiplication of plant tissue. Such variants occur at a frequency comparable to that in vivo; however the rate of cell division is usually higher in culture, so it may appear as being an increased rate of variation. In commercial ventures using meristem cultures and very rapid multiplication rates, undesirable variant genotypes unfortunately increase in frequency. Visual selection is an important practice to eliminate many of these undesirable variants (Bhatti and Jha 2010). Commercial enterprises and clonal sources of elite material, therefore, prefer to multiply shoots for only a few cycles from any one explant source; after this a fresh batch of cultures is initiated from new field or greenhouse certified plants. When plants are regenerated from callus cultures, new genotypes appear even more frequently. All of these different variant genotypes, no matter how they may have arrived in culture, are grouped as 'somaclonal variation'. Somaclonal variation in plants and the phenotypic/genotypic changes evident can be useful in plant breeding, but may be unstable, and if these are to be used further in breeding programmes, they must be genetically stabilised (Rahman et al. 2012).

2.7 Other Limitations to Meristem Cultures

The production of virus-free material through tissue culture using shoot meristem or apex cultures is an important technology. In the ornamental nursery industry, virus-free material became very popular after its production from fast-growing shoots of many important virus-infected plants (Morel and Martin 1952). Basal media alone can be used to regenerate virus-free crop plants, such as cotton (*Gossypium*) (Gould et al. 1991a), maize (Gould et al. 1991b) and other cereals (Hiei et al. 1997). Tissue culture is considered an essential step for transformation in the majority of protocols used in plants, and consequently higher priority is needed to establish easy, reliable and efficient procedures that can reduce somaclonal variation (Birch 1997). Somatic embryogenesis (Sect. 6) after callus formation can lead to more numerous somaclonal variations or even more permanent genetic mutations, but plants produced through meristem cultures show considerably fewer somaclonal variations or genetic mutations. Meristem (shoot apex) cultures have been reported to diminish the activation of retrotransposon activity in cultured plant cells and tissues, and this potentially avoids creating permanent mutations (Hirochika 1993). Using meristem cultures, the opposite is also possible, and it is easy to induce mutations via conventional means of radiation or mutagenic chemicals. The uses of variation in culture and induced mutations have become popular and have produced new, stable and useful genotypes for many purposes, including crop breeding (Navratilova et al. 2008; Hoang et al. 2009). However the genetic integrity and the morphological stability of any plant culture system must be monitored and tested frequently.

3 Cell Suspension Cultures

3.1 What Are Cell Suspension Cultures?

Leaf tissue and callus are one of the better tissues to isolate single cells suitable for culture. Additionally, leaf tissue contains one of the most homogeneous populations of cells, especially if leaf segments of similar age are chosen. Hence, these types of explants are suitable to act as candidates for raising controlled cell cultures on a large scale. Single cell cultures commonly begin with callus derived from in vitro plantlets, leaves or other actively dividing tissues, and the resulting callus can be successfully used to initiate suspension cultures. Incidentally, often the calli produced in culture can be used to regenerate other organs (e.g. shoot apices and somatic embryos).

3.2 Use of Cell Suspension Cultures

Cell suspension cultures are primarily a tool to study cell growth and to produce secondary metabolites and a method for rapid micropropagation of some economically

valuable and rare plants. By using suspension culture techniques, we can understand cell growth parameters and establish more efficient protocols for plant regeneration. However, in production agriculture and horticulture, the most often use of cell suspension cultures is the production of secondary metabolites in high concentrations as important metabolites for medicine, flavour, essential products and even for use in cosmetics. Cell suspension cultures are also used in the induction of somatic embryos and small shoots for in vitro mutagenesis, selection of mutant lines and especially genetic transformation.

3.3 Cell Suspension Methods

3.3.1 Mechanical Method

Sterilised leaves are macerated (pounded) mechanically. The homogenate is filtered through muslin or large pore nylon nets. Cells are then washed and pelleted by centrifugation at low speed. The cells are collected and debris removed, before being placed in a culture medium (Keng et al. 2010).

3.3.2 Enzymatic Method

Enzymatic digestion can isolate and release maximum amount of cells with minimum damage and injury. This is accomplished by first providing osmotic protection for the cells in a sucrose/mannitol/sorbitol-rich media. Cell wall digestion enzyme mixtures (pectinase/macerozyme) degrade the middle lamella and cell walls of the tissue, and individual cells are set free. Single cell systems can be obtained from callus. Callus growing around an explant is sub-cultured and macerated in the same culture medium to get a mass of friable loose cells. Repeat sub-culturing results in more friability of the callus. Friability is considered a prerequisite for increasing the totipotency of cells in suspension or liquid medium. To obtain a fine free cell isolate, the addition of defined digestion enzyme mixes may help (as in protoplast isolation—Sect. 5). Suspension cultures can be grown as batch or continuous cultures.

3.4 Mass Cultivation of Plant Cells

After selecting cell lines for high yield, efforts are concentrated on mass culture to achieve large-scale production of metabolites. Cell suspension cultures grow to low densities due to availability of low amounts of nutrients. Therefore, different types of fermentors (2 to 20,000 L capacity) have been designed to grow a significantly high density of plant cells (Mulagabul and Tsay 2004).

3.4.1 Airlift Bioreactors

These bioreactors are designed to supply oxygen at the surface of plant cells. Such bioreactors support biomass levels of up to 30 g dry weight cell tissue per litre of medium. The use of bioreactors with modified paddle-type impeller systems can result in cell cultures of up to 75 g dry weight cell tissue per litre of medium, but this appears to be high production (Keng et al. 2010).

3.4.2 Stirred-Tank Bioreactors

Stirred-tank bioreactors with modified impellers (which provide mixing under low shear) have been advocated for large-scale cultivation of fragile plant cell suspension cultures, but may actually not be more efficient than the best airlift type. Oxygen plays a major role in the bioenergetics of plant cells in culture. Most plant cell cultures require O_2, besides CO_2, to improve cell growth and the biosynthesis of metabolites. Hence, these two gases must be provided in an improved controlled manner for their optimal use in the most efficient of bioreactors in both these categories.

3.5 Some Developmental Problems

The mass culturing of plant cells has some biotechnological barriers, such as slow-growth rates and genetic instability of cells as they grow. Other barriers include shear sensitivity, low oxygen transfer, cell aggregation, cell adhesion to the vessel walls and thickened cell walls. Therefore, these limitations and other basic properties of slow cell division and low biomass production must be taken into account when cell cultures are selected for mass cultivation and metabolite production in plants.

4 Callus, Organ and Tissue Cultures

4.1 What Are Callus, Organ and Tissue?

Plants can be regenerated from unorganised callus tissue, derived from different explants by dedifferentiation induced by exogenous growth regulators. Callus is a mass of vacuolated, unorganised cells resulting as a consequence of a ‘typical’ wounding reaction in plants and placed in culture. Callus may be from pollen, anther, bud, fruit, embryo, ovary and leaf or shoot apices. Some of these explants can be termed ‘organ cultures’, where a specific organ is excised and cultured on a suitable growth nutrient medium under aseptic and controlled physical and chemical conditions. The particular organ cultured retains its characteristic structure and

features and continues to grow as usual, unlike true callus cultures where cell organisation of intact tissue is lost. When cultured on a suitable growth medium under controlled conditions, isolated cells usually differentiate and multiply to form callus. This callus can be induced to re-differentiate to give rise to 'embryoids'. Embryoids develop into plantlets and later on into whole viable plants. Plant regeneration from calli, therefore, is possible by either de novo organogenesis or somatic embryogenesis.

4.2 Use of Callus, Tissue and Organ Cultures

Callus cultures facilitate the quick multiplication of limited plant material. In addition, plant regeneration from calli permits the isolation of rare somaclonal variants which result either from existing genetic variability in somatic cells or from the induction of mutations, chromosome aberrations and epigenetic changes by in vitro applied environmental stimuli, mutagenic agents and growth-promoting substances added to the culture (Radfar et al. 2012). Hypocotyl and root explants usually provide excellent material for callus initiation. A method utilising root explants to produce callus cultures has been used successfully to regenerate large quantities of ecotypes and breeding lines of *Arabidopsis* mutant lines for years (Mathur and Koncz 1998).

4.3 Some Developmental Problems

Regeneration and cell division from stem and leaf explant tissue is usually not very successful. Tissue culture protocols, especially those used for callus formation, often fail. The elongated shoots from callus, if produced at all, are usually transferred into root induction media, and root production often fails. However, even in the absence of functional roots, the shoots may flower and seed set on these shoots even in culture. These cultured shoots may have some advantages over normal cultivated shoots in plant breeding applications due to their quick production.

5 Protoplast Culture

5.1 What Are Protoplasts?

Protoplasts are 'naked' plant cells, i.e. cells without a cell wall. There are three ways of isolating protoplasts: mechanical methods, sequential enzymatic (two-step) methods and mixed enzymatic (simultaneous) methods. In the sequential and mixed enzymatic methods, cellulases, hemicellulases and/or pectinases are used to isolate

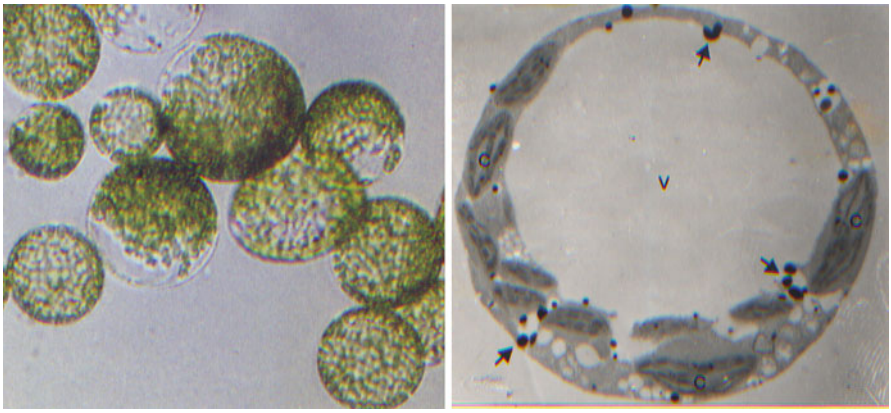

Fig. 12.1 Typical mesophyll protoplasts isolated from *Grevillea* leaves under the light microscope unstained (*left*) and under the electron microscope (*right*) using low magnification. Note the smooth protoplast membrane, osmiophilic deposits (*arrows*), chloroplasts (C) and large vacuole (V). Figures are from the author

the protoplasts. Plant leaves and leaf mesophyll tissue are the most convenient and popular source for plant protoplasts due to cell uniformity and their characteristic visible appearance under the light microscope (Fig. 12.1).

Besides leaves, protoplasts can also be isolated from stems, callus, pollen grains, embryo sacs and roots. Protoplasts are somatic cells that are quite difficult to keep intact and metabolic active, but can be grown into a whole plant. Isolation of protoplasts is quite simple now by enzymatic digestion of the cell walls; however culturing protoplasts into callus, tissue and plantlets is technically demanding, extremely difficult and perhaps near impossible for some species, especially monocotyledon plant species.

5.2 Isolation of Protoplasts

Plant protoplasts are usually obtained following a multistep procedure, which includes:

(a) Surface sterilisation of plant materials, e.g. leaves, roots, stem sections or buds
(b) Removal of the epidermis, or softening the epidermis by physically puncturing it
(c) Pre-enzyme treatment in an osmoticum, carefully adjusted to levels in plant cells
(d) Incubation in cellulose/pectinase digestion enzyme mix preparations in the osmoticum
(e) Isolation/purification of protoplasts by filtration or centrifugation (Davey et al. 2005)

5.3 Factors Affecting Protoplast Culture

There are different factors that affect protoplast culture such as osmotic pressure (adjusted in the growth medium), nutritional requirement (vitamin B_2, carbon and nitrogen sources), growth regulators (as required for callus culture), protoplast density (number of protoplasts per unit volume of medium) and a number of environmental factors (i.e. optimum dim light or darkness, 24–26 °C temperatures and pH range from 5.5 to 5.8) (Compton et al. 2000). Examples of important crop species that have been successful in protoplast isolation and regeneration include many commercial plants important to agricultural and horticultural production (Table 12.1).

5.4 Protoplast Fusion

Hybridisation of protoplasts and even cells is relatively simple by cell fusion methods, which can be affected by either chemicals [e.g. polyethanol glycol (PEG)] (De Filippis et al. 1996; De Filippis et al. 2000) or electric fields (electrofusion) (Hampp et al. 1997; De Filippis et al. 2000). Hybridisation of cells and protoplasts has led to new genotype plants in which desirable traits have been identified and selected. Hybrids and cybrids (where the cytoplasms only are fused) can be produced by fusion methods, and these can be used in many situations, but perhaps the best use of fused cells or protoplasts is to develop and test genetic markers in marker assisted selection (De Filippis 2012).

5.5 Use of Protoplast Cultures

Isolated protoplasts are used for various purposes including biochemical and metabolic studies, fusion of two different somatic cell/protoplast types to get somatic hybrids, fusion of nucleated (containing nucleus) and enucleated (without nucleus) cells to produce cybrids (cytoplasmic hybrid), genetic manipulation and transgenic plants, drug sensitivity, toxicity assays and basic studies in plant physiology and biochemistry (Klima et al. 2009). Isolated protoplasts are also exploited in numerous miscellaneous studies involving membrane function, cell structure, synthesis of pharmaceutical products and toxicology (Sutiojono et al. 2011).

5.6 Problems and Limitations to Protoplasts

A major problem with protoplasts has always been that a number of important crop species still remain recalcitrant to culture. Nevertheless, isolated protoplasts constitute unique plant material for a range of experimental procedures, and recently there has been a marked resurgence of interest in protoplast technology, with a particular

Table 12.1 Crop plant species that have protoplast isolation and regeneration protocols described

Crop plant	Donor tissue and isolation	Culture and regeneration to plant
Monocotyledon		
Hordeum vulgare	Embryogenic, suspension cell	Liquid shake culture, callus to plant
Oryza sativa	Callus, suspension cell	Callus, somatic embryo to plant
Panicum miliaceum	Suspension cell, droplet	Callus, somatic embryo, plant, albino
Pennisetum purpureum	Embryogenic suspension cell	Droplet, solid, somatic embryo, plant
Saccharum officinarum	Suspension cell, droplet cells	Bead, liquid, callus, shoot to plant
Saccharum spontaneum	Suspension cell and callus	Liquid, solid, callus to plant
Triticum aestivum	Embryogenic suspension cell	Liquid, somatic embryo, callus, plant
Zea mays	Embryogenic suspense callus	Drop—nurse, somatic embryo, plant
Dicotyledon		
Beta vulgaris	Embryogenic, callus	Somatic embryo to plant
Brassica campestris	Cotyledon, hypocotyl, liquid	Callus, somatic embryo to plant
Brassica carinata	Cotyledon, hypocotyl, liquid	Callus, somatic embryo to plant
Brassica napus	Leaf, shoot apex, liquid	Callus, somatic embryo to plant
Brassica oleracea	Leaf, shoot apex, liquid	Callus, somatic embryo to plant
Capsicum annum	Leaf, cotyledon, root apex	Liquid, solid, somatic embryo, plant
Citrus sinensis	Callus, semi-solid and liquid	Callus, somatic embryo to plant
Cucumis melo	Leaf, cotyledon	Bead, droplet, somatic embryo, plant
Datura sp.	Leaf, shoot apex	Callus, somatic embryo to plant
Daucus carota	Suspension cell, root, in vitro	Callus, somatic embryo to plant
Fragaria ananassa	Leaf, shoot apex	Callus, somatic embryo to plant
Glycine max	Cotyledon, hypocotyl, liquid	Callus, somatic embryo to plant
Glycine soja	Suspension cell, droplet	Callus, somatic embryo, embryoids
Ipomoea batatas	Leaf, stem internode	Callus, somatic embryo to plant
Lactuca sativa	Stem and internode, liquid	Callus, somatic embryo to plant
Lycopersicum esculentum	Callus, cotyledon, hypocotyl	Callus, somatic embryo to plant
Malus domestica	Callus, suspension, liquid	Callus, somatic embryo, embryoids
Manihot esculenta	Leaf, shoot apex in vitro	Liquid, callus, somatic embryo, plant
Medicago sp.	Leaf, shoot apex, cotyledon	Liquid, nurse, droplet, no plantlet
Morus alba		
Pisum sativum	Leaf, shoot, cotyledon in vitro	Liquid, bead, callus, somatic embryo
Prunus sp.	Leaf, cotyledon, root apex	Callus, somatic embryo, root, plant
Trifolium repens	Leaf, shoot apex in vitro	Callus, somatic embryo to plant
Solanum tuberosum	Leaf, shoot/root apex, in vitro	Callus, somatic embryo to plant
Recalcitrant protoplast	*Avena sativa*	*Dioscorea alata*
Isolation/regeneration	*Lens culinaris*	*Pennisetum glaucum*
	Secale cereale	*Sorghum bicolor*

Different donor tissues and culture methods are indicated, and recalcitrant crop plants are outlined. Data was modified from Roest and Gilissen (1989), Davey et al. (2005) and De Filippis (2012)

focus on the generation of novel somatic hybrid and cybrid plants that cannot be produced through conventional breeding. Somatic hybridisation by protoplast fusion enables nuclear and cytoplasmic genomes to be combined, fully or partially, at an interspecific and intergeneric level to circumvent naturally occurring sexual

incompatibility barriers; however this is still technically difficult. Uptake of isolated DNA into protoplasts provides the basis for transient and stable nuclear and organelle transformation to generate transplastomic plants, but again these technologies are extremely difficult. Difficulties include the recalcitrance of some protoplast systems, with leaf protoplasts of numerous cereals being a very good example of this lack of totipotency (Table 12.1). Protoplasts are the equivalent, in general terms, to cultured animal cells. However, unlike many types of cultured animal or human cells, protoplasts exhibit the unique property of totipotency. It is clear that future research using plant protoplasts will inevitably follow developments in animal cell culture and that closer liaison between animal and plant biologists will increasingly become a feature of current biotechnology. Relevant examples are the exciting developments in the fields of molecular or biopharming in which human therapeutic proteins are synthesised in plants.

6 Somatic Embryogenesis

6.1 Process of Somatic Embryogenesis

Somatic embryogenesis is the process by which embryos form and develop from bipolar structures from somatic cells that parallel the developmental path of normal zygotic embryos. Generally, somatic embryos develop from a single cell. This cell undergoes cell division to form a group of meristematic cells that continue to divide to give rise to developmental stages towards an embryo. Somatic embryos may be primary (regenerate directly from explant/callus) or secondary (regenerate from the tissues of other somatic embryos or a part of regenerated somatic embryo).

(a) *Embryogenesis*
Somatic embryos are independent bipolar structures, which are not physically attached to the tissue in culture but are produced from a somatic line of cells. The embryos can grow to complete plantlets. Somatic embryogenesis can even occur spontaneously, and as long as the genetic make-up of these embryos is stable, they can be multiplied rapidly and, therefore, have great potential in tissue culture. However, it is still a method most suitable for breeding and physiological investigations rather than a method suitable for rapid multiplication (Han et al. 2009; Liu et al. 2010).

(b) *Organogenesis*
Organogenesis can be defined as the transformation of a single cell, callus or tissue into an organ-like structure capable of independent growth, but are organ-like and not embryo-like. Plants can eventually be produced from somatic embryos and organs by a sort of independent ‘germination’ in their own defined culture media; otherwise organogenesis is essentially the same as embryogenesis (Murphy 2007; Husaini and Abdin 2008; Basha and Sujatha 2009).

6.2 Important Development Phases in Somatic Embryogenesis

(a) *Development phase*—Somatic embryos develop from a group of meristematic cells undergoing development through globular, heart-shaped, torpedo-shaped and cotyledonary stages.
(b) *Conversion phase*—Immediately after the cotyledonary stage, somatic embryos germinate, becoming the conversion phase. However, most of the time if plantlets are obtained from immediate germination, they are metabolically weak and often do not survive without maturation.
(c) *Maturation phase*—Instead of undergoing germination, somatic embryos undergo biochemical changes and become 'hardened' before germinating into plantlets (Aboshama 2011).

6.3 Factors Affecting Somatic Embryogenesis

Growth hormones, the genotype of explants and age, the form of nitrogen source and other nutrients have an influence on success in embryogenesis. Concentration of other metabolites, like sucrose and maltose, can be important. Generally, embryos are formed most often in herbaceous flowering plants. Totipotent somatic cells in culture must follow the embryogenic pathway detailed above to form somatic embryos or embryoids. The embryos and/or embryoids can regenerate to form complete plants (Onay and Jeffree 2000). Supplementing the embryos with activated charcoal facilitates embryogenesis by perhaps adsorbing unknown inhibitory metabolites.

6.4 Applications of Somatic Embryogenesis

Clonal propagation of genetically uniform plant material, elimination of viruses and the production of metabolites are important uses of somatic embryos. In genetic transformation, somatic embryos provide important source tissue. The synthesis of artificial seeds (Sect. 6.6) and regeneration of whole plants from single cells and/or protoplasts are other important uses. Somatic embryos have been produced in a large number of crop plants of different families for large-scale clonal propagation of elite cultivars and as an alternate approach to conventional micropropagation.

6.5 Classification of Embryos Used

(a) *Zygotic embryos* are directly formed by the zygote.
(b) *Non-zygotic embryos* are formed from other cells other than the zygote.

Non-zygotic embryos are of the following types:

1. *Somatic embryos*. Formed from somatic cells in vitro
2. *Parthenogenetic embryos*. Formed by unfertilised eggs
3. *Androgenic embryos*. Formed by pollen grains

6.6 Advantages of Somatic Embryogenesis

Large-scale production of plants through the multiplication of embryogenic cell lines is the most commercially important application of somatic embryogenesis (Jimenez 2005) with many advantages, including:

(a) The culture of large numbers of somatic embryos, all capable of being regenerated.
(b) During regeneration, root and shoot formation can be simultaneous, thus eliminating the need for an additional and costly root induction phase.
(c) The mode of culture permits easy scale-up and subculture with low labour inputs.
(d) Cultures can be manipulated such that embryo formation and germination can be synchronised, maximising plant output, while minimising labour inputs.
(e) As with zygotic embryos, somatic embryo dormancy can be induced; hence long-term storage becomes possible (Takahata et al. 2004).

Somatic embryogenesis can be directed towards the production of identical plants or to induce variability. Redenbaugh et al. (1991) suggested that it is possible to produce asexual embryos in vitro, and synthetic seed technology is one of the more important applications of somatic embryogenesis. In the synthetic seed system, somatic embryos are encapsulated in a protective alginate matrix that provides mechanical support, and a coated wax film prevents desiccation (Redenbaugh et al. 1986). However, the stage of maturation, viability and vigour of the synthetic or artificial seeds have some limitations (Sect. 10.5), as demonstrated by low germination rates and low seed viability.

6.7 Problems and Limitations to Somatic Embryos

The somatic embryogenic process is initiated with somatic cells, theoretically from any part of the plant; however, in practice substantial differences in totipotency of tissues are found. The cells that are more competent for somatic embryogenesis are generally those coming from young tissues and immature zygotic embryos. Young stems, roots and leaves may be useful as well. Somatic embryos are usually induced by simple manipulation of the in vitro culture conditions. Conditions that have been found to have the most influence on somatic embryogenesis are the culture medium

components and growth regulators (Cerezo et al. 2011). Variations in in vitro response have also been described due to explant source (Sharma and Rajam 1995; Burris et al. 2009) and genotype (Kim et al. 2003; Magyar-Tabori et al. 2010). The effect of light and activated charcoal can be important in plants rich with phenolic compounds, and the effects of other biochemicals including glutamine, proline, tryptophan and polyamines have been studied. In some situations, other limiting factors are that the cells are few in number and even within those totipotent cells are fewer, and as a result the number of explants which respond is very low. Therefore, the development and production of somatic embryos tend to be nonsynchronous (Zucchi et al. 2002). A further limitation is the stability of cell lines. Over a period of time, the proportion of cells that enter or complete embryogenesis decreases, and eventually regeneration may be impossible. This last point could be an advantage as prolonged time in culture can lead to the accumulation of mutations (somaclonal variations—Sect. 2.6) which can cause morphological abnormalities, and hence new cultures must be initiated on a regular basis.

6.8 Adventitious Organogenesis

The successful production of transgenic plants through genetic engineering depends on an efficient regeneration system (Gould and Magallanes-Cedeno 1998; Sharma et al. 2000). Compared to indirect (somatic) organogenesis, direct (adventitious) organogenesis from differentiated tissue generally results in a lower frequency of somaclonal variation and greater genetic and cytological uniformity (Debnath et al. 2006). Adventitious shoot (organ) regeneration can occur via interpetiolar buds, hypocotyls, internodes or leaves as explants (Azad et al. 2005). Among these approaches, young leaves from in vitro shoots can be efficient for regeneration (Azad et al. 2005); however, the overall culture procedure can be complex and slow. In the long run, rapid regeneration of plants directly from totipotent meristematic explants can save time and represents a better strategy to avoid somaclonal variation by decreasing the amount of callus (Khatun et al. 2010; Roy et al. 2011).

6.9 Embryo Rescue

Genotypes of zygotic embryos in tissue culture are useful in plant breeding, when normally these embryos would be aborted or are not viable in the plant itself. This is usually due to poor endosperm development or failure, where the media components in tissue culture replace the nutrients normally present in a productive endosperm. Embryo rescue can increase the chance of incorporating new genes into breeding programmes and the development of elite lines which otherwise would not be possible. This method has already been used successfully in a number of crops, including potato, adding new and valuable genetic material (Malik 2008; Slama Ayed et al. 2010).

7 Anther and Pollen Cultures

7.1 What Are Anther or Pollen Cultures?

Pollen culture (microspore culture) is a technique in which haploid plants are obtained from isolated pollen grains, while anther cultures are obtained from culturing complete anthers. Plants developed by this method contain a haploid set of chromosomes. Anther culture is one of the best breeding methods with numerous advantages, e.g. shortening the breeding cycle by immediate fixation of homozygosity, increased selection efficiency, widening genetic variability through the production of gametoclonal variants and allowing early expression of recessive traits (Basu et al. 2011). The microspore enters androgenesis following two possible pathways (Asakaviciute 2008):

(a) The microspore develops into a haploid callus from which haploid plants can be regenerated.
(b) The microspore directly develops into a haploid embryo, germinating into a haploid plant.

It is therefore important to find genotypes whose anthers form morphogenetically active structures.

7.2 Procedure for In Vitro Anther Culture

7.2.1 Collection of Anthers

Preferably anthers and pollen should be taken from plants grown under controlled and clean greenhouse conditions, or may be taken from plants grown in the field with care and good hygiene.

7.2.2 Transfer of Anthers to the Culture Medium

Flower buds of the appropriate developmental stage are collected and surface sterilised. Anthers from the buds are excised and placed on the surface (or partially embedded) in the culture medium. It is important to note that injury to anthers may induce undesirable excess wound callus formation. To avoid this wound callus step, entire inflorescences may be cultured without pollen isolation. Pollen is isolated from anthers either by squeezing or by float culture:

(a) *Squeezing method*—Anthers (50–60) are placed in a culture medium and squeezed gently with a glass/plastic/Teflon rod to separate pollen from the anther walls. The prepared pollen-rich solution is filtered through a nylon mesh of suitable pore size. The filtrate is centrifuged at low speed; the resulting pollen dense pellet is collected and washed a number of times.

(b) *Float culture*—Anthers are excised and placed in petri dishes containing liquid medium. Anthers float in the liquid medium. The anthers release their pollen grains into the medium in a few days.

The ability of pollen to redirect its developmental pathway from gametogenesis to embryogenesis depended on the stage of maturity of the pollen grains at the time of culture. For rice, the best stage has been described as the uninucleate to early binucleate stages (Jähne and Lörz 1995).

7.3 Difficulties in Pollen and Anther Cultures

The many difficulties of anther culture in rice breeding, for example, have been recorded as genotype dependency, low frequency of callus induction, low frequency of plant regeneration, low ratio of green plants to albino development and low frequency of haploid plants. Several influencing factors have been studied such as the genotype of explants and culture conditions (Hu and Du 2006; Lyra et al. 2011). Determining the development stage of the pollen in anthers is most important in order to optimise the anther culture response of any given genotype (Silva 2010). A number of visual markers based on potassium levels, fluorescence of pollen (Rehman and Yun 2006) and UV fluorescence in a microarray system to assess pollen critical stages have been used as a guide to identify the required stage of pollen development (Tang et al. 2010). Additionally the method of Tang et al. (2010) was able to identify some critical genes important in meiotic events in pollen germination.

7.4 Uses of Pollen and Anther Cultures

Anther culture is an innovative technique that can be utilised for the rapid development of homozygous plants. Haploid cultures, upon chromosome doubling, will yield fully homozygous genotypes. The technique has been used successfully to produce homozygous breeding lines in japonica rice (Gioi and Tuan 2004; Brar and Khush 2007). However, the potential for indica rice anther culture is yet to be fully exploited due to various constraints, which included a recalcitrant genetic background in the indica varieties (Roy and Madal 2005; He et al. 2006). Several other attempts at anther culture of indica rice germplasm have also met with limited success (Silva and Ratanyake 2003; Kumar et al. 2006; Ratheika and Silva 2007; Silva and Achala 2008).

7.5 Double Haploid Cultivars in Crop Breeding

In cereals, albinism is a major obstacle to the production of doubled haploid (DH) lines for breeding purposes. In order to identify quantitative trait loci (QTL) for

green plant production in barley anther culture, a specific population was developed for maximum segregation for the green trait and minimum segregation for the other anther culture variables. A combination of bulked segregate analysis and AFLP methodology was used to identify markers linked to the green plant trait. A linkage map was constructed using AFLPs, together with RAPD, STS and SSR markers in peach (Aftab 2012). For a more thorough discussion and use of molecular markers in QTL breeding, consult the manuscripts of Santos et al. (2007) and De Filippis (2012). Basu et al. (2011) quoted the identification of new QTL for high green plant percentage on chromosome 3H and on chromosome 5H in barley. Up to 65 % of the phenotypic variance for this green trait was explained by the additive effects of these two QTLs. Thirty elite cultivars of barley from different origin, row type, growth habit and end use were selected to validate these green trait QTLs.

7.6 Haploid Plants

Homozygous cultured material is sometimes called 'haploid cultures' because the number of chromosomes in them has been halved; however the number of chromosomes can be doubled later by chemical means. Haploid technology is increasing in importance but has limitations in that the method is difficult and unsuccessful in many plants, where much depends on the ploidy level of plants. For example, tetraploid plants are very difficult to culture as haploid plants for use in a breeding programme (Zhang et al. 2010). The advantages of using homozygous diploid plants after haploid culture are that parents derived in this manner have lower recombination events, and there is a higher percentage of recovery of any viable and fertile plants (Vinterhalter et al. 2008; Islam 2010).

8 Hairy Root Cultures

8.1 Hairy Root Induction

Hairy root is a plant disease caused by the Gram-negative soil bacterium *Agrobacterium rhizogenes*. When the bacterium infects a plant, the T-DNA between the TR and TL regions of the Ri-plasmid in the bacterium is transferred and integrated into the nuclear genome of the host plant. The transformation process produces a valuable morphological by-product, hairy roots, which will form at or near infection sites. The transformed roots are morphologically highly differentiated, stable and can produce extensive secondary metabolites, whereas other plant cell cultures have a strong tendency to be genetically and biochemically unstable and often synthesise low levels of useful secondary metabolites (Merkli et al. 1997; Kittipongpatana et al. 1998; Jain 2001). *A. rhizogenes* can transfer T-DNA from binary vectors, which enables the production of transgenic plants containing foreign

Fig. 12.2 Effects of liquid shake cultures on in vitro shoot proliferation of wasabia (*Wasabia japonica*) after 6 weeks in culture. The effects of different culture media are illustrated, (**a**) 1/4 MS, (**b**) 1/2 MS and (**c**) MS (*top figures*). *Figures below* **A**: MS culture media dilution above in a full liquid media without agarose. **B**: MS culture media dilution above but 8 g/L agarose was included. Figures courtesy of K. Johnson and C.D. Hung (UTS)

genes carried on other plasmids. Improved viral vector systems have increased the efficacy of transient gene expression and have allowed large amounts of immunogenic proteins to be produced in plants (Debnath et al. 2006; Hu and Du 2006). Despite many advantages of these approaches, there are still limitations with ongoing requirements for reinfection and infiltration into plants for long-term establishment, and extensive individual transformation testing is required.

8.2 Establishment of Hairy Root Cultures

Successful establishment of a hairy root culture system for plant species requires several essential conditions. Important conditions include the bacterial strain of *A. rhizogenes* used, an appropriate susceptible explant, an effective antibiotic to eliminate redundant bacteria after co-cultivation and a suitable tissue culture medium and environment for the culture to grow. Optimising the composition of nutrients in the culture media for hairy root growth is critical to gain high production, and it is common for liquid cultures to be superior in growth characteristics and metabolite production than using, for example, a solid or semi-solid culture media (Fig. 12.2).

8.2.1 Reporter Gene

The β-glucuronidase (GUS) gene is usually transferred into hairy roots as a reporter gene and can be easily analysed by histological assays (Jefferson et al. 1987; Christie 1997). So far the GUS system is the most common means of

monitoring most plant transformation systems. Recently, the neomycin phosphotransferase II (NPTII) encoding kanamycin-resistance enzyme has also been successfully used (Han et al. 1993; Qin et al. 1994). In a few reports, both GUS and NPTII have been transferred into transgenic hairy root cultures (Christie 1997; Azlan et al. 2002).

8.2.2 Selection of Hairy Root Lines

Due to the site uncertainty of T-DNA integration into the plant genome, the hairy roots developed often show different accumulation patterns of secondary metabolites. Mano et al. (1989) analysed 45 hairy root clones of *Duboisia leichhardtii* F and found that there were considerable variation in growth rates, alkaloid content and subculture ease amongst the clones. Generally, hairy root cultures are considered to be stable, easy to subculture and relatively trouble-free to maintain.

8.3 Application of Hairy Roots

8.3.1 Functional Analysis of Genes

Debnath et al. (2006) studied transgenic lines of *Lotus japonicus* Regel supertransformed by infection with *A. rhizogenes* containing gene constructs for expression of hairpin RNAs (hpRNAs) with sequence complementary to GUS coding region. The results indicated that GUS activity in hairy root lines was downregulated by more than 60 %. An antisense dihydroflavonol reductase (*DFR*) gene was introduced into hairy roots of *Lotus corniculatus* L and effectively decreased tannin biosynthesis in two of the recipient genotypes (Carron et al. 1994). A review of the metabolites expressed by hairy root cultures has been provided by Hu and Du (2006).

8.3.2 Expression of Foreign Proteins

The production of industrial and therapeutic proteins by plants is an area of intense commercial interest. Three genes from *Ralstonia eutropha*, a type of bacteria necessary for poly 3-hydroxybutyrate (PHB) synthesis, were introduced into hairy roots of sugar beet (Menzel et al. 2003). The 20 transgenic hairy root clones of sugar beet produced up to 55 mg PHB per gram dry weight. The pea lectin gene was introduced into white clover (*Trifolium repens* L.) hairy roots and correctly processed lectin (Diaz et al. 1995). Hairy root plants transformed with virus proteins often show resistance to further virus infection. Torregrosa and Bouquet (1997) succeeded in obtaining grapevine hairy roots with the coat protein of grapevine chrome mosaic nepovirus expressed, but unfortunately, the regeneration of

transgenic grapevines from hairy roots was not achieved. However grafted grapevine plants with transgenic roots could be established in the greenhouse (Sivanesan and Jeong 2009).

8.3.3 Production of Secondary Metabolites

Root cultures normally need exogenous phytohormone and grow very slowly, resulting in low or negligible synthesis of secondary metabolites. The hairy root system is stable and highly productive under hormone-free culture conditions. Metabolic engineering in hairy root cultures offers to improve the production of secondary metabolites by the overexpression of target genes, as demonstrated in red beet (Rudrappa et al. 2005). This approach has been shown to lead to an increase of some (but not all) enzymes involved in the metabolic pathway of targeted compounds. For example, engineering the tropane alkaloid biosynthetic pathway via overexpressing only *pmt* and *h6h* genes in *Atropa belladonna* has recently been demonstrated via hairy root cultures, and the transformed cultures facilitate the accumulation of tropane alkaloids. Further, it was shown that transgenic hairy root cultures of *A. belladonna* could be used in bioreactors to produce pharmaceutical quantities of important tropane alkaloids (Hakkinen et al. 2005; Yang et al. 2011).

8.3.4 Production of Compounds Not Found in Untransformed Roots

Transformation may affect novel and new metabolic pathways and produce new compounds that cannot be produced normally in untransformed roots. For example, the transformed hairy roots of *Scutellaria baicalensis* accumulated glucoside conjugates of flavonoids instead of only glucose conjugates found in normal roots (Nishikawa and Ishimaru 1997). Polyunsaturated fatty acid accumulation and changes in fatty acid patterns were studied in *Echium* sp., and concentrations and ratios of polyunsaturated fatty acids were highly depended on the lines of the transformed cultures and conditions during hairy root culturing (Cequerier-Sanchez et al. 2011).

8.3.5 Changing Composition of Metabolites

Bevage et al. (1997) reported the expression of an *Antirrhinum* dihydroflavonol reductase gene, which resulted in changes in condensed tannin secondary structures, and tannin accumulation in hairy root cultures of *L. corniculatus*. The analysis of selected root culture lines indicated an alteration of monomer tannin levels during growth developed without changes in composition. Similarly, the production and types of pyranocoumarins (Xu et al. 2009), lignans (Chasmi et al. 2011) and silamaryns (Rahnama et al. 2008) were substantially altered by hairy root cultures.

8.3.6 Regeneration of Whole Plants

Regeneration of whole plants from hairy root cultures has been reported in several plant species. The successful regeneration of transgenic plants, however, depended on in vitro culture conditions for each particular plant species. The genotype and juvenility of explants were important for transformed roots to regenerate into somatic embryos following the addition of appropriate phytohormones. Cho and Wildholm (2002) reported that when cultured on a medium with 2,4-dichlorophenoxyacetic acid, the hairy roots of *Astragalus sinicus* L. developed somatic embryos. Shoots were regenerated from hairy roots of *Robinia pseudoacacia* L. following the addition of α-naphthaleneacetic acid and 6-benzyl-aminopurine (Han et al. 1993).

8.4 Potential Problems

8.4.1 Different Regulation of Secondary Metabolism in Related Species

Secondary metabolism in related species may share the same pathway, but their regulation may well differ. For example, tropane alkaloid was increased in hairy roots of some plants of different species and distant genera (Moyano et al. 2003), and the genotypes of recipients affected the expression of transferred genes. The antisense *DFR* gene downregulated tannin biosynthesis in two genotypes (S33 and S50) of the hairy root cultures derived from *L. corniculatus* (Carron et al. 1994), and tannins were lower as expected. However, in the third genotype (S41) of *L. corniculatus*, the transgenic hairy roots actually accumulated higher levels of condensed tannins.

8.4.2 Overexpression of Key Enzymes Does Not Always Improve Secondary Metabolism

Two key enzymes encoding chorismate pyruvate-lyase and HMGR, which were known to be involved in the biosynthesis of shikonin, were transformed into the hairy roots of *Lithospermum erythrorhizon* Sieb (Koehle et al. 2002). However, shikonin accumulation remained unchanged, even with high expression levels of these two enzymes.

8.4.3 Possible Reduction of Chromosome Numbers During Subculture

The primary hairy roots of *Onobrychis viciaefolia* Scop had initially a normal chromosome diploid number ($2n = 28$; Xu and Jia 1996). However, after 4 months of subculture, the percentage of hairy root cells with normal chromosome numbers

were reduced from 85 % to 23 %, and 8 months later, only 4 % of cells had a normal diploid chromosome number.

8.4.4 Co-suppression of Endogenous and Foreign Genes

A higher number of copies of the transformation genes in hairy root cultures do not always result in a greater expression of the target enzymes and a corresponding increase in product. *Catharanthus roseus* hairy roots harbouring hamster HMGR cDNA expressed different alkaloid production patterns (Ayora-Talavera et al. 2002). Transgenic silencing may be a probable explanation, but only in some species. Hairy roots of *Cinchona officinalis* L. transformed with tryptophan decarboxylase (TDC) and strictosidine synthase (STR) produced high amounts of tryptamine and strictosidine at the beginning of transformation (Greerlings et al. 1999). However, 1 year later, they had completely lost their capacity to accumulate alkaloids without observable changes in the cultures.

8.4.5 Morphological Alterations of Regenerated Plants

Plants regenerated from hairy roots often have abnormal leaves and extremely abundant and plagiotropic root systems, reduced apical dominance, reduced internode length and leaf size and an increased ability of leaf explants to differentiate roots in phytohormone-free media (Tepfer 1984; Tayler et al. 1985; Cardarelli et al. 1987; Spano et al. 1988; Hamamoto et al. 1990). In addition, asymmetrical leaflets, variegated leaves and reduced spine length have been observed (Han et al. 1993). These abnormal phenotypes have been explained as possibly originating from genomic disturbances due to either the insertion of foreign DNA or somaclonal variation, rather than from the expression of T-DNA genes in the transformants (Han et al. 1993). An additional disadvantage is that hairy root transgenic plants often show higher mortality than normal plants.

8.5 Production in Hairy Root Culture Systems

Hairy root culture systems are potentially a good approach to the production of secondary metabolites, especially pharmaceuticals, because these cultures have desirable traits, such as rapid growth rates, easy culture and genetic manipulation and most importantly an increased ability to synthesise useful metabolites that cannot be produced by normal plants in high concentrations (De Guzman et al. 2011). Potential problems with the application of the hairy root culture system include a variation in biochemistry among different clones, a possible loss of chromosomes and co-suppression between structural and regulatory genes. Several decades have lapsed since hairy root culture systems were first described; however, the system has

not been utilised globally in industrial production. The present established culture systems are based on flasks or small-scale bioreactors. So far, no successful example has been described of scaled up commercial production. Many attempts have been made to develop economical bioreactors containing airlifting, bubble columns, mist, dual and wave reactors (Du et al. 2003; Lin et al. 2003; Palazon et al. 2003; Souret et al. 2003; Kintzios et al. 2004; Suresh et al. 2004), but few have been commercially adopted. All of the existing hairy root culture systems have met with similar cost-effective problems (i.e. they could not resolve the *Hairy Root Application in Genetic Engineering* contradiction between investment and consumption), which have made hairy root cultures virtually impractical for commercial adoption.

9 Plant Transformation

9.1 Biotechnology and Genetic Manipulated (GM) Crops

Improvement of plant cultivars by conventional breeding involves crossing parents containing part of the desired traits, while selecting progeny of individuals showing better combinations of a superior set of these traits. Additionally, tissue culture techniques have made it possible to upregulate or downregulate genes in breeding crop plants (Özyiğit 2012; Rashid et al. 2012). Some types of gene selection however have limitations, and only some of these can be removed by breeding and tissue culture techniques; therefore in some cases only the use of transgenic plants can provide a path forward for improved plant breeding. De Filippis (2012) has provided a full comparison of differences between conventional breeding, MAS breeding and genetic transformation. Transgenic and GM plants and molecular breeding have opened up a number of important possibilities and improvements in agriculture and horticulture as listed below:

(a) Isolating and amplifying genes and gene families encoding for a particular trait in vitro
(b) Addition of knowledge of genes important in the regulatory signals recognised by plants
(c) Introduction and expression of a modified gene or foreign gene into plant cells and tissues
(d) Regeneration of transgenic plant cell lines into mature fertile plants suitable for breeding

The main advantage of GM technology is that it offers the possibility of adding a new trait or regulation of this trait directly to an existing valuable variety or cultivar. In such GM crops, potential species barriers to foreign gene incorporation and expression do not exist. Applications using this approach have already been well documented in many dicotyledon and fewer monocotyledon plants (e.g. tobacco, potato, tomato, rapeseed, corn, wheat, barley oats, soybean and banana) (Jackson

et al. 2006; Akhond and Machray 2009). Genes for incorporation can be accessed from exotic sources: plants, animals, bacteria, viruses or even humans. Transgenic and GM methods used in plants, however, have some limitations to consider, and there has been limited success mainly because:

(a) Few reliable protocols for introduction and expression of the foreign DNA exist.
(b) The number of documented genes of interest is still limited, although expanding all the time.
(c) Understanding the regulation of gene expression is vital for success, but is limited in plants.
(d) Tissue culture methods for some plant species regeneration are still inadequate.
(e) Long periods of time are required for full technological development and transfer.
(f) Lack of long-term funding for such programmes.
(g) Absence of a co-ordinated approach by the scientific community.
(h) Unfavourable public perception towards transgenic plants.

GM technology was first developed for plants as early as 1983, but the first GM crops reached the marketplace only during the mid-1990. During the next two decades, the development of transgenic technology has proceeded rapidly. The global area planted to biotech crops has increased over the last 20 years at an annual rate of 10–12 %, and the major biotech growing nations are detailed by De Filippis (2012). The biotech crops planted in agriculture are still dependent on four crops (i.e. soybean, corn, cotton and canola) in most countries; however, the number of countries planting biotech crops is expanding all the time. The process of genetic transformation of plants involves several distinct stages, namely, gene insertion, gene integration, gene expression and stable inheritance of the new DNA (genes), and details of the steps involved are adequately covered in a number of books and reviews (Smith et al. 2001; Herman 2009; Neuman and Kumar 2009).

9.2 Advantages of Genetic Manipulated (GM) Crops

Potential advantages of GM technology include increased yield and food security, and less application of pesticides and practices to reduce soil erosion, which ultimately would help small farmers in saving costs and to improve their quality of life. The technology is easy to disseminate, but developmental costs are high. Golden rice producers have recently been granted patent licences by Monsanto without any cost to them; hence they can now grow freely this transgenic rice to help meet the requirement of vitamin A deficiency in humans. More crops such as wheat, rice and sugarcane, having improved characteristics, are now in different phases of regulatory testing as engineered crops and are on the pathway to commercialisation. A large area of farmland presently inappropriate and unavailable for crop

production (e.g. salt-affected soils) will hopefully become useful for cultivation with suitable GM crops. Fruit crops at this stage do not appear to have as much significance and are not as developed in GM technology as cereal or staple food crops such as wheat, corn, rice and soybean in most countries.

9.3 Requirements for Genetic Transformation

Tissue culture is used in all present practical transformation procedures. It is not just a hypothetical or wish requirement, but without proper tissue culture procedure, it is difficult to achieve an efficient gene transfer, selection and growth of selected transformant lines. Therefore, improvement in tissue culture systems is essential to develop more efficient transformation, which at present is very genotype dependent. For example, potato and apple are two model horticultural crops in this postgenomic era (Shulaev et al. 2008). Tissue culture has been extensively used for raising multiple clones (micropropagation) of some apple rootstocks and some potato cultivars. Besides this, tissue culture has also played a central role in virus-free planting material, cryopreservation of genetic resources, development of synthetic seeds and improvement and availability of elite lines for breeding programmes through the use of transgenics (Dobranszki and Teixeira da Silva 2010; Zhai et al. 2010). A number of commercially important plant species are routinely transformed by different biotechnological methods. Methods available for plant transformation are arranged in three main groups: use of biological vectors (virus- or bacteria-mediated transformation), direct DNA transfer techniques (chemical-, electrical- or laser-induced permeability of protoplasts or cells) and use of non-biological vector systems (microprojectiles, microinjection or liposome fusion). Today, a number of transgenic food crops such as soybean, maize, canola, sugar beet, sugarcane and alfalfa are available, and the most preferred method is still *Agrobacterium*-mediated transformation. Many important plant species (legumes, cereals and woody plants) and varieties still remain recalcitrant to the *Agrobacterium* method and unresponsive to plant growth regulators (Choffe et al. 2000; Wang and To 2004). In some crops, regeneration is found to be restricted to only a few cultivars, and this can be limiting in application of such expensive and complex methods (Firoozabady and DeBoer 1993; Zhang and Blumwald 2001; Kumar et al. 2006).

9.4 Agrobacterium *System*

The soil bacterium *Agrobacterium tumefaciens* can be considered a natural gene transfer vector. It is still one of the most efficient methods employing the natural occurring ‘crown gall’ disease process of DNA transfer. *Agrobacterium* transformation results from the stimulation of cell division by gene products encoded by a

segment of the plasmid DNA (T-DNA), which is transferred from the bacterium to the plant. The T-DNA and *vir* regions required for this transfer are located on the tumour-inducing plasmid (Ti). The *vir* system will process and transfer any DNA between the two flanking regions on the T-DNA. The strain of *Agrobacterium* used as a carrier for DNA into plants has been modified (disarmed) to remove gall-inducing (tumour-producing) genes and engineered to carry the gene or genes of interest, together with a selectable marker. Inside the plant, the T-complex is imported into the nucleus of the host plant, the T-strand becomes stably inherited into the plant chromosomes, and the genes (including the foreign genes) are expressed (Broothaerts et al. 2005). The *Agrobacterium* system has the ability to integrate into a variety of plants; however monocotyledons have shown some resistance to transformation, but now this has become a routine method even in selected monocotyledon crops (Smith and Hood 1995). More problematic is that the site of integration of T-DNA is random, and the integrated DNA can be rearranged or truncated, but the biggest problem of all is making sure that the carrier bacterium itself does not persist internally in the transgenic plants causing 'crown gall' disease (Basha and Sujatha 2009).

9.5 Biolistic Method

A number of reports have been published for plant transformation of shoot cultures and shoot apices using a 'gene gun' (Genga et al. 1991; Finer et al. 1999). Particle bombardment of suspension cultures can produce high-copy-number transformants, but can be an expensive method (Finer and McMullen 1990). The gene gun method was developed in the mid-1980s and has been widely used as a more direct gene transfer system in a number of plants, including previously hard to transform monocotyledons (Southgate et al. 1995). The technique is based on the rapid acceleration of a metal microprojectile to deliver nucleic acids, including specific genes (or DNA sequences) into an intact nucleus, or even just into plant cells and tissues. Biological material and compounds apart from DNA could be delivered by this method, and a commercial particle gun delivery system for gene transfer is commercially available (Biolistic PDS-1000 gene gun). Due to the genotype-independent physical nature of this DNA delivery method, the protocols employed are simple, efficient and similar regardless of the nature of the foreign DNA or the target cells. It is a highly valuable and adaptable method potentially applicable over many different types of cells and tissues.

During the last two decades, it appears that microprojectile bombardment has become more routine and reliable; so much so it is replacing the *Agrobacterium* method quickly (Hansen and Wright 1999). However, there are still problems to be resolved, particularly more direct and efficient targeting of the nucleus, tissue culture subsequent problems related to totipotency and low regeneration of plants in crops like soybean. The results of the biolistic method can be a complex pattern of transgene integration that can be undesirable, but can be mitigated by using 'agrolistic' approaches. Agrolistic transformation allows integration of the gene of

interest without undesired plasmid virulence vector gene sequences of *Agrobacterium*. In other words, deliver by biolistic means *Agrobacterium* containing the foreign DNA and only some virulence genes (e.g. *vir* D1 and *vir* D2), but not *vir* E2. It has been demonstrated that only these two *vir* D genes are required for in planta stable transformation once inside cells, tissues and organs (Hess and Dressler 1989; Beranova et al. 2008). Many laboratories still prefer the *Agrobacterium*-mediated method of genetic transformation, induction of somatic embryos and organogenesis (Firoozabady et al. 1987; Lyon et al. 1993; Thomas et al. 1995; Jin et al. 2005).

9.6 Polyethanol Glycol and Their Direct Gene Transfer (DGT) Methods

(a) *Protoplast transformation*—protoplasts can be transformed either by the *Agrobacterium* system, by biolistic methods or by direct DNA uptake using electrofusion or PEG treatment and subsequent fusion and endocytosis. These transformation methods have been used routinely to study transient gene expression of the transgenes. Transgenic plants have been obtained only in a few cases, and plant regeneration has been demonstrated only where suitable protocols were already available for culturing of protoplasts to complete plants (Shillito 1999; Murphy 2007).

(b) *Chloroplast transformation*—chloroplast transformation has generated much interest and sometimes has been called an 'environmentally friendly' approach to plant genetic engineering. Chloroplasts provide a system where it is possible to avoid outcrossing of transgenes to weeds or other nontarget plants, thus reducing the potential transfer, toxicity, sterility and other undesirable effects of transgenic pollen to nontargeted species. Biolistic gene delivery is probably the most reliable and reproducible method to use; however, the method has only been described in a limited number of crop species (Daniell and Dhingra 2002; Jain and Saxena 2009).

(c) *Floral dip method*—a technique that could circumvent the time-consuming steps in many of the previous methods and is a simple method to master. There are a number of such methods, and they are all placed into the general category of 'in plant transformation'. One such method has been well developed in *Arabidopsis* and has achieved relatively high rates (0.5–3 %) of transformation, and there are reports of success in a number of other crop plants (e.g. *Brassica campestris*, *Medicago truncatula* and radish) (Liu et al. 1998; Trieu et al. 2000; Curtis 2005).

(d) *Pollen-mediated transformation*—transformation of pollen has attracted significant interest, and there have been a number of reports claiming success (Harwood et al. 1996, Heberle-Bors et al. 1996) by incubating pollen in DNA solutions and electroporation (Hess and Dressler 1989), direct particle bombardment (van der Leede-Plegt et al. 1995) or co-cultivation with *Agrobacterium*

(Langridge et al. 1992). Many of the methods were later proven to be unreliable, with only the report of Smith et al. (1994) having described transgenic plant production and could be considered reliable. A recent report by Beranova et al. (2008) has described an enhanced *Agrobacterium*-mediated transformation system using flax pollen.

Tissue culture techniques play a primary role in all the transgenic methodologies that have made it possible to transfer important and foreign genes from one plant to another. In general, these new gene technological advancements have placed plant biotechnology into a new and exciting category with future possibilities that can hardly be overestimated (Twyman et al. 2002, 2003).

9.7 GM Crops in Agriculture

Genetic research has been initiated and described for genes responsible for tolerance to salinity, drought, temperature, insects, pesticides and even improvement in transformation efficiency in crop plants. The complex physiology of stress tolerance, genetic architecture and variation between or within species makes research difficult, and it has been slow to achieve the desired amount of stress tolerance, primarily due to the poor knowledge of these mechanisms operating at cellular and whole plant level (Ashraf 2004; Munis et al. 2010; Ni et al. 2010). For example, Horsch et al. (1986) developed the first transgenic tobacco plant expressing foreign phytohormone biosynthetic genes in tissue culture, but since then only about 100 transgenic plant species and cultivars have been described which show enhanced resistance to a number of biotic and abiotic stresses. In addition, these transformed plants overexpress various other agronomic traits, such as growth, leaf and seed size, yield, number of tillers and floral organs which could be used in significantly improving crop breeding achievements outlined in Table 12.2 (Ashraf 2010; Bhatnagar-Mathur et al. 2010; Cerdeira and Duke 2010; Wang et al. 2010; Wojas et al. 2010; Ashraf et al. 2012).

9.7.1 Transgenic Plant Production

The production and planting of genetically modified plants is increasing daily. In 1996, only 1.7 Mha of land was under transgenic crops, but in 2000 the area had increased to 44.2 Mha, and in 2008 it had increased further to 125 Mha (James 2007, 2008). The world area of transgenic crops has grown rapidly, and development of transgenic biotechnology has promoted the commercialisation of genetically modified crops in a number of countries (Aftab 2010). Around 25 countries are contributing to the production of biotech crops, but the major portion of GM crops are still produced in the USA, where 62.5 Mha is under transgenic plants (James 2010). The major biotech crops cultivated are tomato, wheat, alfalfa, rice, soybean, maize, canola, squash, tobacco, cotton, sugar beet, petunia, sweet pepper,

potato, squash and carnation (Ashraf and Akram 2009; James 2010). Other transgenic crops are on the approval pathway and will hit the market soon. According to Panos Media Release (1999), there are, or will soon be, three generations of GE crops. The first generation of crops are those that have shown resistance to environmental stresses such as herbicide resistance, insect resistance and drought tolerance. The second generation of crops may provide nutrient-rich seed or leafy vegetables for food, whereas the third generation of crops are those which generate biofuels, pharmaceuticals and essential metabolites (Aziz et al. 2002, 2005). The GE crops that have been widely adopted and planted until now mainly belong to the first generation of crops (Table 12.2). GE crops in the second- and third-generation phases are still yet to come in a substantial way.

9.7.2 Yield and Production

Increased yield is a major aim in agriculture, and improvement in yield and stability of crops is a major area of research by improving tolerance to biotic and abiotic environmental cues. In this context, substantial progress has been made in enhancing crop yield worldwide using advanced molecular biology tools (Ashraf and Akram 2009; Chen et al. 2010; Oliver et al. 2010; Ashraf et al. 2012). Table 12.2 outlines some of the genetic improvement traits applicable to crops, and examples showing the success in improving yields are provided.

9.7.3 Insect Damage

The *Bacillus thuringiensis* (Bt) toxin was the first protein described as non-toxic to humans and animals, but toxic to insects (BANR 2000). Subsequently, genetically engineered corn, cotton and tomato have been approved for planting under field conditions, and the area under Bt crops has increased steadily since 1996 (James 1999). Other insecticidal proteins worthy of testing have been discovered, including other toxins, lectins, protease inhibitors, antibodies, wasp and spider toxins, microbial insecticides and insect peptides (Kozlov et al. 2010; Kim et al. 2009; Joshi et al. 2011).

9.7.4 Herbicides

Many herbicides can create undesirable environmental impacts. Chemicals such as glyphosate have been widely recommended for use, because soil microorganisms are able to degrade glyphosate. By introducing glyphosate tolerance genes into crops, herbicides can now be applied over the top of crops during the growing season to increase production, and this has resulted in decreased herbicide applications up to 25 %. Plants expressing transformed herbicide tolerance genes accounted for 71 % of all transgenic crops planted, such as soybean, corn, cotton and canola; the four are the major transgenic crops planted (Liu et al. 1999; James 2008, 2010) (Table 12.2).

Table 12.2 Genes described for possible use in transgenic crop plants that have important properties of promoting plant growth and development, phenotype changes and resistance to insects and diseases

Plant group	Gene, enzyme and protein	Phenotype and expression
Transcription	ARGOS	Enlarged organs
	AINTEGUMENTA	Increase size of floral organs
	MEGAINTEGUMENTA	Increase size and higher weight
	Growth regulating factor 1,3,5	Increase leaves and cotyledons
	ANGUSTIFOLIA	Increase leaf size
	NAC1	More roots, larger leaves, thicker stems
	ATAF2	Increase biomass, larger leaves
	PEAPOD	Larger leaves and cotyledons
	DREB1A	Tolerate drought, salt and cold
	OsNAC10/SMCP1	Osmoticum, better salt/drought tolerance
	A1fin1, Tsi1	Osmoticum, better drought/salt tolerance
Cell cycle	CyclinD2	Increase rate of leaf initiation
	CyclinD3	Leaves with more cells but smaller
	ABAP1	Larger leaves with more cells
	CDC27a	Increase growth and organ size
Hormone metabolism	AtGA20-oxidase	Promotes growth/biomass, xylem length
	HOG1	Increase leaf size and seed yield
	IPT	Increases leaf biomass
	DASS	Increases plant fresh weight
	ARF2	Longer inflorescence stems, larger leaves
	AVP1	Increase number/size of leaves and roots
	SPDS	Resist salinity, high spermidine synthesis
	IMT1	Resist drought, salinity, higher myoinositol
	TSRF1/F2	Tolerate salt, drought, ethylene antagonist
	W6, LeERF2	Ethylene antagonist, resist drought, freezing
	AtOAT	Ornithine amino transfer, higher ornithine
Photosynthesis	PEP carboxylase	Higher photosynthesis, stomata conductance
	Pyruvate orthophosphate kinase	Increase photosynthesis, tiller number
	Cytochrome C6	Higher photosynthate, increase leaves/roots
	Rubisco activase	More vegetative growth and number of seed
	Glycolate dehydrogenase (glycolytic cycle enzymes)	Improved carboxylation/oxygenase ratio, greater biomass
Sulphur metabolism	APS1, APS2, ATP sulphurylase	Increased uptake and utilisation of sulphur
	SMT, cysteine methyl transferase	Elevated levels of organic sulphur, growth
	CyS, crystathione synthase	Increased sulphur and tolerance to sulphide
	SATm, serine acetyltransferase	Mitochondrial amino acid metabolism
Gene silencing (micro RNAs)	miR396	Larger leaves and generally more leaves
	miR319	Larger leaves but crinkled edge leaves
	miR156	Increase total leaves and side shoots
	miR159, miR393	Fungi and bacteria resistance, better growth
	miR160,miR164,miR166,miR177	Resistance to viruses and increased growth
	miR395, miR399	Increase sulphur metabolism and growth

(continued)

Table 12.2 (continued)

Plant group	Gene, enzyme and protein	Phenotype and expression
Metabolites	bet Ac, choline dehydrogenase	Higher osmolite against salinity and cold
	COD1, COX (choline oxidase)	Increase osmolite for cold, light stress
	BADH1 betaine	Increase osmolite due to salinity
	TPS/TPP trialose	Resist drought, salinity, temperature
	HAL1, HAL3 (binding peptides)	Osmotic tolerance to salinity
	PPO polyphenol oxidase	Water stress and drought tolerance
	SAMDC polyamine synthase	Tolerate drought, salinity, fungal pathogens
	PSCS carboxylate synthase	Resist cold, drought and salt
	ProDH proline	Increases osmolite due to salinity
	ME-leaN4/LEA 3	Protective proteins for salt and drought
	SOD (Fe/Mn) superoxide	Antioxidation for salt, drought protection
	GSH glutathione	Antioxidant to protect against cold and salt
	AtNDPK2/AtNHX1 kinases	Vacuole antiporter for salt, cold resistance
	Ascorbate peroxidase	Antioxidant to protect against drought, salt
	DnaK (heat shock proteins)	Protective proteins against salt and drought
	AtHsp17.6A(heat shock protein)	High protection against water stress
	ZPT2-3 (zinc finger proteins)	Protection against water stress and salt
	Cysteine protease (SPCP2)	Protects against salt and drought
	WCOR15	Protection against extreme cold
	KatE (catalase)	Salt and oxidative stress response
	DHAR1 (dehydroascorbate)	Antioxidation products, stress response
	MsALR (aldolase)	Drought and heavy metal resistance
	Gly1, Gly2 (glyoxylate)	Resistance to salinity and temperature
	Cnb1, OsCDPK (calcineurin)	Membrane damage and salinity
	AtNHX1 (Na antiporter)	High resistance to salt stress
	SOS1 (Na antiporter)	High resistance to salt stress
	SPCP2 (cysteine protease)	Protects against salt and drought
Diseases	Cry1Ac, Cry2A, Cry9A	Yellow stem borer, Asiatic rice borer
	Shti, GNA	Yellow stem borer, Asiatic rice borer
	Protease inhibitor Pin2	Yellow stem borer
	Hrf2 hairpin protein	Resistance to Sclerotinia
	Pi-d2	Tolerance to rice neck/leaf blast
	P35	Resistance to tobacco mosaic virus
	GbTLP1	Resistance to Verticillium
	St PUB17 (UND/ARM)	Tolerance to Phytophtora
	RB resistant gene	Resistance to potato late blight
	Ta-Tip (traumatin like)	Powdery mildew and Fusarium blight
	Chitanase + glucosinase	Resistance to Rhizoctonia and 'damping-off'
Insects	Cry1Ab, Cryp1Ac	Tolerant to potato tuber moth
	Cry1Ab, Cryp2A	Resistant to rice lepidopters
	Magic 6 peptide	*Spodaptera frugiperda*
	Cry1Ac, Cry1Ac gna	Lepidoptera insects

Data was modified from De Filippis (2010), Roy et al. (2011) and Ahmad et al. (2012)

9.7.5 Abiotic Stress

Abiotic stresses such as salt, drought, flood, extreme temperatures and oxidative stress often diminish plant growth and final yield. Agricultural productivity could be increased dramatically if crops were 'redesigned' to better cope with such stresses (Table 12.2). Transgenic plants that regulate cytoplasmic solutes such as mannitol, betaine and proline have been used to alleviate stress in plants (Hasegawa et al. 2002). Plants with elevated amounts of glutathione and oxidative metabolites (e.g. ascorbic acid) can also be used to reduce abiotic stress (Xiang et al. 2001; Yadav et al. 2005).

9.7.6 Enhanced Nutrition

Vitamin A deficiency can adversely affect the eyes in humans and cause increased childhood mortality. Globally, 21 % of children have been reported to suffer from vitamin A deficiency (Sommer 2001). The expression of vitamin A genes in rice (already developed) may be a viable alternative to help eradicate vitamin A deficiency. No other agricultural technology can report to be capable of overcoming such deficiencies in humans. Increased levels of lysine and threonine in cereals, higher methionine levels in leguminous plants and an increase in vitamins A and E in crucifers and rice are now economically possible by the use of transgenic technology and tissue culture (Sun 1999; Ye et al. 2000; Potrykus 2001; Zimmermann and Hurrell 2002; Anand 2010).

9.8 Molecular Pharming

The future of biotechnology and tissue culture could be even more promising. The agricultural biotechnology revolution depends on successful and continued research and development, as well as a favourable regulatory public and private sector (Altman 1999; Huang and Wang 2002; Icoz and Stotzky 2008; Jain and Brar 2010). Initially, agriculture was targeted to improve the production of plant-derived foods in terms of better quantity and quality with current technologies, but other biotechnology areas are now receiving considerable attention (Huang and Wang 2002; Yamaguchi and Blumwald 2005; Carpenter 2012). In recent years, through plant genetic engineering, it has become possible to use biotech plants for the production of therapeutic recombinant proteins, the most important of which are useful in plant-based vaccines (Ma et al. 2003). Table 12.3 lists a number of such advances and products in research and developmental stages and the possible plants and metabolites used in investigations. Interest in producing such proteins in plants comes in part from the problems associated with existing animal/microbial bioreactor systems with limited production and the relative ease of higher vaccine production in plants without storage, purification and lower costs.

Table 12.3 Transgenic crop plant species that contain expressed recombinant medicinal and health important proteins and polypeptides

Crop/plant	Recombinant protein/polypeptide	Biopharmaceutical purpose and use
Alfalfa	Recombinant α-amylase enzyme	Starch hydrolysis in high starch alfalfa
	Mannheimia haemolytic GS60 antigen	Bronchopneumonia in cattle and sheep
Apple	Cyncitial virus (CSV) E-protein	Respiratory virus infection in infants
Banana	Hepatitis-B surface antigen	Human transmitted infection (dormant)
Cherry	Hepatitis-B surface antigen	Human transmitted infection (dormant)
Canola	Hirudin peptide, *Hirudo medicinalis*	Leech salivary glands, anticoagulant
Carrot	Urease (ureB) protein	Oral vaccine, pandemic, chronic gastridis
	HIV-1 subtype Cp24 protein	First drug protein to reduce risk in HIV
Corn	LT-B, heat labile toxin	Enterotoxigenic *E. coli* strains, toxins
	Gastroenteritis virus	Vaccine for numerous viruses combined
	Trypsin	Better digestion by pancreas
	Avidin	Egg protein binds to biotin (essential)
	CT-B toxin	Cholera heat labile toxin
Lettuce	Hepatitis-B surface antigen (HBsAg)	Human transmitted infection (dormant)
Papaya	SPvac test vaccine	Novel synthetic vaccine for testing
Pea	Hemorrhagic disease virus CTBVP60	Vaccine against hemorrhagic disease
Potato	Human lactoferin	Human milk, binds to iron in intestine
	Norwalk virus	Vaccine against gastroenteritis, children
	Hepatitis-B surface antigen	Human transmitted infection (dormant)
	Cholera Cytox A and Cytox B subunit	Stomach outer membrane vaccine
	HIV-1 surface coat protein	Possible entry point to a vaccine
	HRV-VP7 (human retrovirus)	Severe diarrhoea in infants and children
	Newcastle disease virus (NDV)	Contageous fatal disease of poultry
	Salmo solar (sasol protein)	Skin protein colour product enhancement
	Human papilloma virus (HPV)	Protection against cervical cancers
Rice	Salmo solar (salmon) (sasol protein)	Skin protein colour product enhancement
	Japanese encephalitis virus	Mosquito born, transmitted to humans
	LT-B, heat labile toxin	Enterotoxigenic *E. coli* strains, toxins
	Diabetes miletus	Cronic condition, lack of insulin, pancreas
	Human α-antitrypsin	Inhibitor serine proteases, helps digestion
	Japanese cedar pollen peptide	Severe allergy, spring hay fever in humans
Soybean	LT-B, heat labile toxin	Enterotoxigenic *E. coli* strains, toxins

(continued)

Table 12.3 (continued)

Crop/plant	Recombinant protein/polypeptide	Biopharmaceutical purpose and use
Tobacco	Streptococcus mutans surface protein	Mouth microbe, tooth decay, prevent caries
	Human serum albumin	Most abundant liver product in clinical trial
	Human protein C	Vitamin K—glycoprotein in blood clotting
	Human lactoferin	Human milk, binds to iron in intestine
	Hepatitis-B surface antigen (rHBsAg)	Human transmitted infection (dormant)
	Measles virus vaccine	Eliminate live virus/preservatives, concern
	Ig G (Hepatis b virus)	Main immunoglobin, plasma, immunise
	Tet C (tetanus vaccine antigen)	Fragment of tetanus toxin, subunit vaccine
	Bacillus anthracis (PAprotein antigen)	Anthrax, protective antigen to toxicity
	MV-H (measles virus agglutinin)	Measles therapy, prevent cell attachment
	HIV-1 subtype G (GAG protein)	Transduction domain, subtype G virus
	HIV-1 Tat protein	Transduction domain, antigen, adenovirus
	Tricosanthin a (Tricosantes kirowii)	Plant with anti HIV properties in tubers
	Foot and mouth virus, epitope VP1	Prevent cell attachment, vaccinate cattle
	Plasmodium yoelli (prot 4/5) parasite	Surface proteins, protect by vaccination
	Recombinant Norwalk virus	Vaccine against gastroenteritis, children
	Japanese cedar pollen peptide	Severe allergy, spring hay fever in humans
	DTP subunit vaccine against toxins	Diptheria and tetanus recombinant vaccine
	Rabies virus, glycoprotein N293	Possible immunisation capsid protein target
	Human papilloma virus (HPV)	Protection against cervical cancers
	LT-B and ETEC, heat labile toxin	Enterotoxigenic *E. coli* strains, toxins
	Cholera Cytox B subunit	Human transmitted infection (dormant)
Tomato	Rabies virus, monoclonal antibodies	Immunisation against T cell response
	Rabies RSV fusion protein	Capsid glycoprotein, immunisation
	Cholera Cytox A and Cytox B subunit	Stomach outer membrane vaccine
	Hepatitis-B surface antigen	Human transmitted infection (dormant)
	NVCB (Norwalk virus, casid protein)	Vaccine against gastroenteritis, children
	Pneumonic plague	Bacteria Yersima, primary spread to lungs
	Bubonic plague	Bacteria Yersima, secondary lymph spread
Faba bean	Human cytomegalous virus (HCMV)	Salivary glands, danger in organ transplant
Peperomia	LT-B, heat labile toxin	Enterotoxigenic *E. coli* strains, toxins
Green alga	*Staphylococcus aureus* (golden staph)	Infection skin/nose, serious public health

The possible use of these products as biopharmaceuticals and vaccines is outlined. Data was modified from Anand (2010), Rojas et al. (2010) and Ahmad et al. (2012)

Mammalian cell production systems are expensive and cannot be easily scaled up; in contrast, bacterial-based systems can be scaled up. However, often the recombinant proteins for vaccines, important to higher animals and humans, are not properly processed by bacteria, which can lead to intracellular precipitation and non-functional proteins (Streatfield and Howard 2003). Plant-based systems can be scaled up easily allowing large amounts of proteins to be purified at an industrial level. In some cases, it may even be possible to omit purification altogether, as plant material containing recombinant enzymes can be added directly to animal feed or industrial processes with safety. These plant-based systems can benefit both livestock and humans (Pascual 2007). The recombinant proteins in plants which can serve as a cost-effective production system and some of the advances required are listed in Table 12.3. Besides, some plant tissues are the best sites for long-term storage of vaccine antigens without processing or purification. Edible plant tissues can be suitable for oral administration, thus minimising the costs and labour incurred in the delivery of injectable vaccines (Streatfield 2006).

The economics of protein production in plants is complicated. The actual cost will depend on numerous factors; amongst these are the cost of growing the plant, transport costs, processing, extraction and protein purification. The cost of proteins produced in plants may significantly reduce the costs of vaccine production if standard extraction and purification methods can be used in plants. Two major strategies have been adopted for the production of various proteins in plants: firstly, the stable integration approaches where plant viruses are used as transient vectors and, secondly, the stable transgene expression approaches, in which the transgene is regulated by a strong constitutive promoter (such as 35S promoter). This latter approach is perhaps the most suitable for the bulk production of soluble proteins in leaves, although yields can be low (Streatfield 2007). A more sophisticated approach has been to target gene expression and protein production to specific plant cells, tissues and edible organs leading to higher yields. Plant virus capsids have also been used as carriers of recombinant proteins, particularly in vaccines (Sainsbury et al. 2010). In one approach, the coding sequences for epitopes or proteins that have been introduced into the coat protein of a plant virus genome show considerable promise. Another approach used is to construct plant viral vectors to produce recombinant proteins that are targeted to endoplasmic reticulum for processing. Plant viruses are allowed to replicate in the host plant, and through serial passage it has been demonstrated that enough protein can be produced to be an effective treatment method (Table 12.3).

9.9 Limitations and Concerns

Limitations involved with *Agrobacterium* transformation are that it is laborious and time intensive, which may take as long as 8–12 months to obtain embryogenic calli from transformed plant cells. It can lead to the development of only a few embryos, there are problems in embryo germination and growth, and the method can produce low percentage and abnormal plantlets and ultimately result in low transformation efficiency and plantlet regeneration. Because of these limitations and problems, it

can take over 1 year to regenerate plantlets from even the most responsive genotypes, and improved tissue culture protocols are required to address this. There is evidence that the *Agrobacterium* strain, co-cultivation duration, temperature, bacterium density, addition of acetosyringone and other metabolites during co-cultivation and embryogenic calli production can affect transformation (Firoozabady et al. 1987; Jin et al. 2005). The presence of antibiotics/herbicides resistance marker genes and foreign DNA in transgenic plants adds to the concern regarding general acceptability of GM technology, and transgenic plants as a whole are perceived as posing a threat to consumer's health and safety to the environment. The *nptII* gene encoding neomycin phosphotransferase II and phosphinothricin acetyltransferase encoding bar genes are extensively used as selectable markers in plant transgenic research (Degenhardt and Szankowski 2006). These marker genes may potentially improve resistance against aminoglycoside antibiotics (such as genticin, kanamycin and neomycin) and resistance to herbicides (L-phosphinothricin, glyphosate and bialaphos), respectively, and alternatives to these antibiotic and herbicide resistance marker genes are urgently required. Research is proceeding into the use of marker-free plants for future transgenic research and has gained popularity in the hope of better acceptability among consumers.

10 Tissue Culture Preservation

10.1 Conservation of Tissue Culture Material

Conservation of tissue culture material is often required to protect valuable plants and germplasm, and this is most important for vegetatively propagated plants, i.e. plants not normally propagated from seeds. Tissue culture material is easily available in small tubes, is free of pathogens and can be multiplied quickly. Tissue culture conservation has proceeded in two ways (De Klerk 2002):

(a) *Cryopreservation*—cells and tissue are in a frozen state where the methodologies developed for some plant material have been difficult, and water content of the tissue can be a problem; the complete resolution of this tissue vitrification process can be time consuming and costly.
(b) *Lower temperature*—cells and tissue are kept at temperatures above freezing, but at a temperature where growth is considerably reduced; however this method can also be costly on material and space for the new culturing conditions required (Akdemir et al. 2012).

10.2 Encapsulation

As the diversity of wild plant types and cultivars is constantly reduced by anthropogenic pressures, such as land clearance, charcoal burning and overgrazing (Barghchi and Alderson 1989; Padulosi and Hadj-Hassan 2001), both wild and cultivated plant

germplasm are constantly under threat of genetic erosion and extinction, yet comprehensive gene banks for the majority of plants have not been established. Therefore, it is important to develop alternate conservation strategies for the medium- and long-term preservation of plant germplasm in which slow-growth storage or ultra-low-temperature strategies can be used. However, only a few reports have been described concerning success in crop plant conservation with either slow-growth storage or cryopreservation of plant cells, tissues and organs (Ozden Tokatli et al. 2008, 2010; Akdemir et al. 2012).

The concept of encapsulation was first applied to somatic embryos (Murashige 1977) and extended to other vegetative propagated plants (Bupat et al. 1987) by using cultured buds and nodal segments as encapsulated explant material. Several methods of encapsulation have been tested for production of synthetic seeds (*synseeds*), including gelation and interfacial polymerisation (wax coat) to avoid excess dehydration (Redenbaugh et al. 1986, 1987). The gelation technique has proven to be the most effective because it can form sufficiently hard capsules, which guarantees better somatic embryo viability (Redenbaugh et al. 1991). Na-alginate is by far the most commonly used compound, due to its ability to form hydrogels in the presence of divalent cations; it is non-toxic and low cost. These features have led to encapsulation technology being applied to a wider range of explants of agricultural importance (Piccioni and Standardi 1995; Lambardi et al. 1997).

10.3 Slow-Growth Storage

Slow-growing plant material can increase the intervals or time between subcultures and therefore can be used as a method for germplasm conservation and storage. In slow-growing species near-standard tissue culture conditions can also be used for medium-term conservation; however, in most cases modified environmental conditions and/or reduced strength culture media are necessary, with decreased light intensity or even no light for extreme reduction in growth (Engelmann 2004). The potential of slow-growth storage of important agricultural crops from microshoots, for example, has yet to be investigated. Apart from the use of microshoots for the conservation of germplasm, another use is to produce synthetic seeds (*synseeds*), which can resemble the germination of natural seeds, a tissue culture approach which has diminished labour costs in commercial micropropagation. The ‘synseeds’ can be used for long-term preservation of germplasm with the most recent developments of the ‘encapsulation–dehydration’ and the ‘encapsulation–vitrification’ techniques being promising (Panis and Lambardi 2006).

10.4 Cryopreservation

Cryopreservation refers to the storage of biological specimens at ultra-low temperatures (196 °C) in liquid nitrogen (LN) in a cryogenic medium (Withers and

Engelmann 1997). At this ultra-low temperature, all cellular divisions and metabolic processes are virtually stopped, and it is the only sound technique that allows the conservation of specimens for theoretically unlimited time (Engelmann 2004). For crop plants, cryopreservation protocols were initially developed for seeds by using dehydration and one-step freezing techniques (Ozden Tokatli et al. 2007). Using this technique, a maximum of 90 % germination after cryopreservation was obtained following simple drying in silica gel and direct immersion in LN; however it showed mixed results with tissue culture material. Recently a different cryogenic approach has been described for tissue culture, vitrification and freeze protection followed by one-step freezing, in which excised buds from in vitro grown shoots were pretreated with glycerol, DMSO or sucrose to avoid freeze damage followed by LN freezing. This method shows promise with higher survival rates and has been successfully applied for the cryopreservation of axillary buds (Ozden Tokatli et al. 2008).

10.5 Artificial Seeds

The concept came into practical use in the 1970s, and the term 'artificial seed' was coined by Murashige, but also known by alternate names like *manufactured seeds* and *synthetic seeds*. Initially Murashige proposed encapsulation of somatic embryos to produce synthetic seeds, but the concept was further advanced by Redenbaugh (Plant Genetics Inc., California) and Kitoo and Janick (Purdue University). Redenbaugh et al. (1991) then patented the artificial seed technology.

10.5.1 What Is an Artificial Seed?

Artificial or synthetic or manufactured seeds are artificially encapsulated plant propagule (somatic embryos, embryoids and sometimes shoot buds) in a suitable matrix, containing substances like nutrients, growth regulators, herbicides, insecticides, fungicides and mycorrhizae fungi which will allow and aid the seed to germinate and grow into a complete plant under in vitro or ex vitro conditions. Synthetic seeds retain growth potential after storage, and in simple words synthetic seeds usually contain an embryo produced by somatic embryogenesis enclosed within an artificial medium that supplies essential nutrients and encased in an artificial seed covering. Therefore a typical synthetic seed has the following component parts (Anand and Bansal 2002):

(a) Plant propagule (somatic embryo or shoot bud)
(b) Matrix (synthetic gametophyte or nutrient medium)

10.5.2 Artificial Seed Production

In some agricultural and horticultural crops, seed propagation is not successful due to:

(a) Heterozygosity of seeds particularly in cross-pollinated crops of variable viability.
(b) Minute or microscopic seed size which will lose viability quickly, e.g. orchids.
(c) Presence of reduced or incomplete endosperm that may prevent germination.
(d) Some seeds require mycorrhizal fungi association for germination, e.g. orchids.
(e) No seeds are formed at all.

Therefore crop species having these undesirable seed problems must often be propagated by vegetative means like micropropagation or embryogenesis (if possible). Based on tissue culture technology established so far, two types of synthetic seeds may be produced:

(a) *Desiccated*: Synthetic seeds produced from somatic embryos either naked or encapsulated in hydrogels, alginates or polyoxyethylene glycol (Polyox) followed by their desiccation. Desiccation can be achieved slowly over a period of 1 or 2 weeks sequentially using chambers of decreasing relative humidity. Alternatively desiccation can be rapid and cheap by unsealing the containers and leaving them opened on a bench overnight. These types of synseeds are produced only in plant species whose somatic embryos are considered desiccation tolerant.
(b) *Hydrated*: Synthetic seeds produced in plant species where the somatic embryos are recalcitrant and sensitive to desiccation. Hydrated synthetic seeds are commonly produced by encapsulating the somatic embryos in hydrogel and alginate capsules. Gel agents like agar, alginate, polyco, carboxy methyl cellulose, guar gum and sodium pectate can also be used to save costs. Among these, alginate encapsulation has been found to be more suitable and practicable (i.e. cost-effective). Therefore alginate hydrogel is frequently preferred as a matrix.

The concept of artificial seed technology has been applied commercially and successfully to crops like *Azadirachta indica*, some orchids (*Dendrobium*, *Spathoglottis* sp., *Cymbidium*, *Phalaenopsis*), alfalfa, cotton (*Gossypium hirsute*), *Santalum album*, cacti (*Echinocereus* sp.) and lettuce.

10.5.3 Advantages of Synthetic Seed

Synthetic seed technology is designed to combine the advantages of clonal propagation with those of seed propagation for new plant breeding lines produced through biotechnology. The characteristics of synthetic seeds that are agriculturally important are:

(a) High volume and a large-scale propagation method
(b) Maintains genetic uniformity of seeds and plants
(c) Direct delivery of propagules to the greenhouse or field, thus eliminating transplants

(d) Lower cost per plantlet germinated
(e) Rapid multiplication of plants in the field
(f) Ease of handling and high viability while in storage
(g) Serves as a channel for new plant lines produced through biotechnology
(h) Allows economical mass propagation of elite plant varieties

10.5.4 Limitations

Limited production of viable micropropagules that are useful in synthetic seed production can be experienced. Asynchronous development of somatic embryos and improper maturation of somatic embryos can make the method inefficient for germination and conversion (i.e. growth) into normal plants. At times, the lack of dormancy and stress tolerance in somatic embryos limits the storage of synthetic seeds, and somaclonal variation which may alter the genetic constituent of the embryos has been a problem.

11 Plantlet and Nursery Production

11.1 Forcing Plants in Tissue Culture

In tissue culture of woody plants, forcing epicormic or latent buds in a wide range of adult plant species of both temperate and tropical plants may be an advantage for procurement of clean, juvenile explant material. Forcing may be accomplished under different environmental conditions such as fog, mist or greenhouse, and the media used may include sand, sawdust, perlite, vermiculite or any combinations of these. The explants derived from such forced shoots have shown better potential for either rooting directly under greenhouse conditions or further manipulation under in vitro tissue culture conditions for axillary bud activation, shoot apex proliferation, multiple shoot formation and somatic embryogenesis. Over the past few decades, plant tissue culture studies have made considerable progress in these areas (Aftab et al. 2008a, b); however the focus has been on herbaceous plants. It is now possible to maintain totipotency in woody plant material in vitro, and this has paved the way for several advancements in tissue culture and genetic engineering of previously recalcitrant woody crop plants (Aftab and Iqbal 1999; Aftab 2012).

11.2 Facilitation of Rooting

The in vitro initiation of roots for regeneration of plantlets has been found to be especially dependent upon the auxin type and the concentration used in the multiple shoot media (Onay 2000; Ozden Tokatli et al. 2005; Tilkat et al. 2009a). Reports on the

tissue culture of some woody mature plants via somatic embryogenesis are few, but low incidence of root development has been reported frequently. Poor plantlet reproducibility during the rooting stage and the poor establishment of in vitro propagated plants after acclimatisation may result in secondary fungal infections being present (Onay 2000; Tilkat et al. 2009b). Abnormal in vitro morphologies related to callus production of cultured plant tissues are also a hindrance to commercial rates of root induction of in vitro plants, and losses of up to half the stock plants can be present (Tilkat et al. 2009a). Although a high rate of rooting (up to a reported 90 %) can be obtained on a medium supplemented with auxins (Tilkat et al. 2008), the plantlets at times can be prone to severe callusing and hyperhydricity due to the auxins.

A new and simplified root induction protocol was described by Tilkat and Onay (2009), by washing the cut ends of the microshoots before rooting. There was evidence of a significant difference in the frequencies of rooted shoots between the results obtained from shoots excised from the washed, compared to those from the unwashed microshoots, which appeared novel. This method may provide a cleaner environment for microcutting production by solving related problems like release of phenolics and other metabolites, which can inhibit rhizogenesis. Improvements obtained in rooting percentages ranged from 68 % to 88 % by this method (Tilkat and Onay 2009). Improved root induction had been reported for seedling material of pistachio (Onay et al. 1996) and clearly indicated that not only growth substances but also hygiene exudates and other factors may be limiting root initiation; and this direction in research merits more investigations. The rooting responses in plants are significantly influenced by the application of auxins and time, where over 90 % root production from shoots can be obtained with auxin dips of as little as 5 min (Tilkat and Onay 2009). However it is fair to point out that most dipping methods are not suitable for routine use as roots developed from callus are often delicate and vulnerable to pathogen attack once plantlets have been transferred to the soil. A quicker dip approach of less than a minute for the rooting of pistachio under in vitro conditions has been reported to reduce infection (Aftab 2012). Once healthy and strongly rooted plantlets are obtained, further acclimatisation of woody and nonwoody plants under greenhouse protection and fine mist (i.e. near 100 % humidity) can be one of the less hazardous procedures in plantlet ‘hardening’ (Onay et al. 2007; Tilkat et al. 2009b).

11.3 Addition of Metabolites

Antioxidants such as citric acid or reduced glutathione when applied to the root initiation medium and even to the shoot elongation medium can enhance rooting percentage. On the other hand, antioxidants such as diethyldithiocarbamic acid, reduced glutathione, polyvinylpyrrolidone (PVP) or 2-mercaptoethanol can also have inhibitory effects on rooting when added to the medium. The root-inducing property of *Agrobacterium rhizogenes* (Sect. 8) has been used to induce rooting in difficult to root woody shoot cultures of Golden Delicious apple (Patena et al. 1988),

and although this method can produce 'weak' roots, it is being explored in other genotypes of apple for better and stronger root development (Radchuk and Korkhovoy 2005; Zhu et al. 2005).

12 Conclusions and Future Perspective

Tissue culture protocols continue to have drawbacks, which include the lack of suitable and totipotent explant sources, such as present in mature apical shoot tips in many important woody crop plants. The elimination of brown phenolic exudates, the forcing of mature lignified stem sections and lignotubers, initiation from axenic leaves and petioles and the initiation of embryogenic cultures need to be further investigated. Virus-free germplasm, the maintenance and protection via encapsulation of somatic embryos, cryopreservation of axillary buds and plantlets for storage and the facilitation of rooting are other developmental areas requiring more research and improvement. None of these techniques are generally in widespread use in commercial micropropagation and nursery production, as commercially they appear not to be relevant; however all of these areas appear to have great potential in developing a better knowledge and understanding of plant tissue culture, and with this additional knowledge and improved technology, they may become important methods for adoption in crop plant improvement and breeding programmes.

Future developments in plant biotechnology depend on further progress surrounding these tissue culture limitations. Culture contamination, hyperhydricity of cultures, somaclonal variations, presence of chimeric tissues, silencing of transgenes and reduced vigour are some of the more important problems unresolved during tissue culture-mediated biotechnology intervention. A wider selection of antibiotics and aseptic compounds effective in inhibiting growth of bacterial and fungal contaminants is urgently required that have little effect on the viability of explants. Various strategies need to be explored to combat the problem of excessive phenolic exudation in the culture medium. Frequent sub-culturing of explants on fresh medium, use of small-sized explants, culturing on liquid medium for a short time and the use of antioxidants such as ascorbic acid or citric acid or absorbents are strategies that need investigation. Interestingly, some somaclonal variants of plants have been exploited to develop useful traits such as fire blight resistance in apple cultivars and enhanced resistance to *Phytophthora cactorum* in rootstocks (Rosati et al. 1990; Kim et al. 2008), but more research is still required. The associated high cost of tissue culture and maintenance of stocks is a major limitation in large-scale, commercial exploitation, and related to cost–benefit analysis, better plant bioreactors are urgently required for some types of plant cultures. Automation of micropropagation protocols using robotics and bioreactors can be useful in this regard. Although huge problems of costs, technology and logistics are associated with automation, at least the use of immersion culture systems has been shown to be effective in shoot multiplication in a semi-automatic plant tissue culture system (Dobranszki and Teixeira da Silva 2010).

With the increase in crude oil prices, climate change concerns and limited reserves of fossil fuel, attention has been diverted to crops for alternate renewable energy sources for biofuel and biomass. Among the potential biofuel crops are corn, oil crop plants and *Jatropha curcas* L, a non-domesticated shrub, gaining importance. However, only one of these (*Jatropha*) does not compete with edible food supplies. Economic and agronomic selection of plants better suited to biodiesel production needs to be promoted, and a worldwide search for such crops is needed that do not interfere with food security (Qaim 2011), and the germplasm of these plants selected is suitable for crop improvement and breeding (Sitther et al. 2012). However, the lack of adequate knowledge, genetic variation and non-availability of improved varieties in some cases may well limit the prospects of some of these plants being used as successful energy crops. For sustainable biodiesel production, there is an urgent need to identify plant species capable of growing in intensive plantation systems on marginal and unproductive land with a minimum of cost inputs.

Genetic diversity based on morphological traits such as oil content, seed weight, growth and other agronomic traits has been well studied. Most studies indicate the existence of considerable genetic diversity amongst plant accessions and cultivars. Genetic diversity can be assessed simply and economically using molecular markers such as RAPD or ISSR (Hoang et al. 2009; Hung et al. 2011). Further studies are required in crop plants to demonstrate whether molecular and gene diversity correlates with morphological and physiological diversity and if cultivars produced by interspecific breeding have retained enough genetic diversity. A suitable method to quickly determine this is to use tissue culture material, not only to confirm the hybridity in interspecific crosses and diversity for future breeding stocks but also to determine if additional genetic inputs are required from wild sources of plants in achieving breeding objectives (Dhillon et al. 2009).

A number of bottlenecks are also present in the application of ‘hairy root’ culture systems, and future research should focus on the establishment of effective and economical scaled up culture systems that can reduce the consumption of nutrient material and costs and increase growth during the culturing process. The main aims in ‘hairy root’ cultures should be to obtain the biggest benefits and yields of desired secondary metabolites. If such a breakthrough can be achieved, the application of ‘hairy root’ culture systems will be of great benefit not only to tissue culture and plant biotechnology but also to the pharmaceutical, agriculture and essential products industries.

GM crops have introduced farmers to improved agricultural products and yields while reducing the use of pesticides, and transgenic crops hold potential for pharmaceuticals and biomedical requirements (Cho et al. 2006). Agrobacterium-mediated and other direct DNA transfer methods are at present being refined for wider application, such as plant-based vaccines for cholera (Sharma et al. 2008) and rabies (Roy et al. 2010), and this direction in biopharming research is important to promote. Public issues and health concerns on plants, animal and human life and well-being due to GM foods can be unpredictable. However, plant genetic engineering can be considered either a progress or a threat to agriculture and mankind depending on the way it is used, and safety issues are properly addressed.

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Chapter 13
Agricultural Pollution: An Emerging Issue

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1 Introduction

Agriculture is a source of economic development and livelihood on one hand, but pollution due to it can lead to a number of environmental and health hazards. The nature of pollutants and the way they behave in environment are of high importance. Agricultural pollution is defined as the phenomena of damage, contamination and degradation of environment and ecosystem, and health hazards due to the by-products of farming practices. The relationship of agriculture with the biotic and biotic factors of environment forms a loop known as PSR loop:

- Pressure (P): stress on environment from agricultural activities causes changes in the state or condition of environment.
- State (S): condition of the present environment and its resources.
- Response (R): as shown by the society to the stresses on the changing environmental conditions.

There is a need for reliable information about our environment, composition and properties of variety of agricultural pollutants, and their mode of action to understand pollution hazards that resulted due to agriculture. The following chapter focuses on the causes of agricultural pollution, its effects on environment and farm workers, and what actions should be taken in response to it.

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2 Air Pollution and Agriculture

Air pollution is the term used to describe the contamination due to some unwanted materials: solid, liquid, or gaseous substances present in the environment. Agricultural field is related to air pollution in two ways:

- Nonagricultural resources give rise to air pollutants that can affect agricultural crops directly.
- Agricultural activities give rise to pollutants affecting air, environment, and other areas.

It has an adverse impact on crop's production, quality, and yield. Crops can be badly affected, but the severity depends upon the amount of pollutants and certain other conditions that are unfavorable for growth of the crops (Agrawal 2005). These pollutants can be toxic chemicals, greenhouse gases, and other harmful airborne particles. Some of these pollutants are described below:

2.1 Ozone

Ozone is considered to be an important pollutant, and its harsh effects on the growth of crops were firstly observed in 1944 (Roy et al. 2009). It is formed by the complex photochemical reactions occurring in the troposphere involving nitrogen oxides, carbon monoxide, and volatile substances. By burning fossil fuels and through gasoline engines, these substances are produced which contribute to the ozone formation (Guderian 1985). It can cause damage to many plant species such as cucumber, grape, tomato, onion, potato, radish, and tobacco crop (Griffiths 2003). It enters through stomata, which are small openings in the leaves, as cuticle is impermeable to it (Del Valle-Tascon and Carrasco-Rodrigue 2004). Its symptoms usually appear on the upper part of the leaves causing chlorosis (yellowing of leaves), fleck formation (>1 mm diameter), and foliar injury in the crops leading to decrease in the crop production. In a study, it was observed that dicot plants (cotton, peanuts) are more affected by ozone concentration than monocots (corn and wheat) (Heagle 1989). It carries out its action by inhibiting photosynthesis, increasing the senescence process and causing other cellular and metabolic damage (Miller 1987; Runeckles and Chevone 1991). Within the chloroplast, it stops the activity of ribulose bisphosphate carboxylase (Rubisco) resulting in the decrease in the fixation of carbon and thus less production. The fluidity, permeability, and ATPase function of the infected membrane are also lost (Heath and Taylor 1997).

2.2 Sulfur Dioxide

It is a primary pollutant emitted in the air directly and is a mixture of sulfur and oxygen compounds. This gas is mainly produced by combustion of fossil fuels, coal, oils, and other industrial heating processes (Emberson 2003). Soybean is the

most affected crop due to sulfur dioxide pollution. Because of its good solubility and hydration property, it is easily taken up by the stomata of plants, and this can lead to two forms of injury, milder or acute form and severe or chronic injury. In case of acute injury, necrotic lesions are seen on both sides of the leaf along the veins and margins occurring due to the uptake of high sulfur dioxide concentrations in a shorter time period. While in chronic injury there is an exposure to the sulfur dioxide concentrations for a longer period, which leads to chlorosis (Mudd 1975). Crop plants such as alfalfa, barley, radish, spinach, and tobacco are sensitive to this gas (Cohen et al. 1981). It is also the main reason of acidic rain that damages the root and shoot system of plant species and drains out many important minerals and nutrients from the soil and the crops (Tabatabai and Olson 1985). Oxygen and sulfur dioxide gases react to produce sulfur trioxide, which further reacts with water vapors present in the air to form sulfuric acid or acid rain. Sulfuric acid and sulfurous acid both can cause indirect damage to trees and plants (Matsubara et al. 2009).

2.3 Fluorides

Fluoride is considered to be the most important pollutant after ozone and SO_3 (Telesiński et al. 2011). Fluoride is present in the form of hydrogen fluoride releasing from heating rocks, clays, kilns, and from factories producing fertilizers such as aluminum and phosphate fertilizers (Khan 2012). It can cause many changes in the physiology of the plants. Persistent exposure of fluorine-contaminated water badly affects the yield. Its high level prevents the germination of seeds, decreases the photosynthesis process, changes the structure and membrane permeability, and may also induce other alterations in physiological and biochemical structure of crops (Gautam et al. 2010). Its low levels can also lead to metabolic disturbances. It causes yellowing of leaf margins in both monocots and dicots (Gautam and Bhardwaj 2010). It inhibits the enzyme's functions such as glucose-6-phosphate dehydrogenase, malate dehydrogenase, peroxides, and ATPase, an important enzyme of chloroplast (Treshow and Anderson 1991). Fluorine gas causes damage to peach, grape, plum, sweet corn, and barley (Griffiths 2003).

2.4 Greenhouse Gases

These gases absorb infrared radiations of sunlight which are reflected back into the atmosphere and in this way maintain the Earth's temperature. This process is known as greenhouse effect, but due to the imbalance between the sources and sinks of these gases, their concentration in the atmosphere is increasing day by day which is a potential threat to our Earth's population, and now they are becoming the major contributor of changes in the atmosphere and climate (Preston and Leng 1989). These gases not only affect agriculture but also contribute to the production of these gases. It is an important fact that 20 % of these gases are produced through

agriculture pollution. These gases mainly include carbon dioxide (CO_2), nitrous oxide, and methane, usually produced from wetlands. Agricultural sources resulting in the production of these gases are described below along with the description of greenhouse gases.

2.4.1 Carbon Dioxide

Carbon dioxide is beneficial for the photosynthesis and respiration of crop plants and their growth as well. This is called carbon fertilization, but its elevated concentrations are harmful for the growth cycle, physiological structure, and chemistry of plants. This gas is responsible for 60–70 % of greenhouse effect (Hatano and Lipiec 2004). It is mainly produced by industries, burning of fossil fuels, and by the manure. Addition of organic substances into the soil surface enhances nutrient level in it. Its decomposition results in CO_2 production. It enters through stomata of the plants and can cause necrotic lesions on the leaves of tomato and cucumber (Griffiths 2003). The elevation of carbon dioxide causes increase in the concentration of nonstructural carbohydrates inside the leaves, which can be a reason of reduced nitrogen amount in plant tissues (Ainsworth and Long 2005). The ability of stomata to conduct gases also decreases. In grain crops, not only the quality is badly affected but protein and mineral quantity is also decreased by a certain percentage (Ainsworth and McGrath 2010). Farmers are trying to grow more crops, and for this purpose they use fertilizers to enhance the yield of crops. As a result more carbon dioxide is released by plants during respiration, which ultimately has some drastic effects on the climate.

2.4.2 Nitrogen Oxide

It comprises of nitrogen dioxide (NO_2) and nitric oxide (NO), which play vital role in causing pollution and nitrous oxide (N_2O). These oxides are mainly produced by the reaction between oxygen and nitrogen present in the atmosphere occurring at high temperature (Emberson 2003). Nitrous oxide is produced by the microorganisms present in the soil if large amount of nitrogen is present and is not used by the plants (Doll and Baranski 2011). Nitrous oxide is also released by cultivating nitrogen-fixing crops and by the use of manure in the cropland (Aneja et al. 2009). These oxides are also contributed by fuel combustion in vehicles and by using synthetic fertilizers, which induce conversion of nitrogen into nitrous oxide species with the help of microorganisms present in the soil (Sanders 2012). It may be a causative agent of acidic rain which infects growing crops like *Nicotiana tabacum* (tobacco), *Daucus carota* (carrot), and *Pisum sativum* (peas). On the upper surface of the leaf, it forms gray- and green-colored patches resulting in lesions. Within greenhouses, propane burner is used for provision of carbon dioxide, which results in the release of these oxides ultimately affecting the pepper plant (Law and Mansfiel 1982). Plants require nitrogen from these oxides for protein production. It is suggested that for effective growth of crops, its concentration should be low to avoid

negative impact on plant development. On radish and alfalfa, nitrogen dioxide causes mild injury symptoms, whereas disease symptoms are not visible in case of corn. Sulfur dioxide and ozone close stomata and stop the process of photosynthesis, while nitrogen dioxide slows down food-making process.

2.5 Soil Erosion

Agricultural soils are used for growing crops to meet the increasing demand of food. However its quality is affected due to its inappropriate use and this is called as land degradation caused by increased cultivation, industrialization, deforestation, and inappropriate use of fertilizers. It affects the farming of crops and biodiversity as well. Excessive grazing, desertification, nutrients removal, increased salinization, and soil erosion either by water or wind can lead to degradation of soil (Maqsood et al. 2013). It is the removal of upper layer of the soil nutrients and minerals, which makes the soil fertile for crop's better growth. Erosion by wind and water may lead to soil erosion. Wind carries out small particles with it, which degrades the soil. Wind erosion destroys the seeds that are sowed underground for crop yield. It changes the structure of the soil, decreases the productivity of crops, and reduces their quality.

3 Impact of Agriculture on Air Quality

This part focuses upon the impact of agricultural technology on air pollution. Different processes are carried out in this field, which badly affect the environment.

3.1 Agriculture Burning

It is the process of burning waste material coming from agricultural practices and is carried out for clearance of land, shrubs, pests, and production of better quality crops by getting nutrients from the land. The by-products of this process include certain chemical substances, smoke, and particulate matter, which pollute the air and are harmful for health. This also releases carbon, carbon dioxide, carbon monoxide, and sulfur dioxide, which not only affect atmosphere but also the crops (Jenkins et al. 1996). These contaminants result from a combustion process carried out at low temperature (Werther et al. 2000). Residual waste of rice and wheat usually contributes to the production of many gases (Venkataraman et al. 2006). Agricultural burning is usually performed for the management of crop's wastes, but it causes pollution. There should be some guidelines for farmers to be followed, while performing such activities.

3.2 Use of Fertilizers

Fertilizers are added to the soil to increase fertility and nutrient quantity of the soil for better crop production. These can be chemical or mineral fertilizers, and nitrogen, phosphorous, and potassium are present as primary nutrients in these fertilizers. They have a very important role in the production of corn. If increased quantity of chemical fertilizers is applied to plants, it affects the air and releases nitrogen oxides such as NO, NO_2, and N_2O causing air pollution (Savci 2012). The use of fertilizers has been decreased in the developed nations of the world because of their impact on the environment, but is still used in excessive quantity in underdeveloped countries. Fertilizers result in the emission of 1.2 % of greenhouse gases into the environment (Kongshaug 1998). Ammonium fertilizers result in the emission of ammonia gas. Ammonia is converted to nitric acid through oxidation process resulting in the acidic rain, which then affects the crops. During nitrification and denitrification of soil, nitrous oxide is produced. Nitric acid is also responsible for the emission of nitrous oxide, which has already been discussed above.

3.3 Rice Field as a Source of Methane Gas

The fields in which rice is grown are flooded with water (paddy fields), which are an important source of methane gas production (Zhuang et al. 2009). These fields provide favorable conditions to the methanogenic bacteria like humidity, organic substances, and environment limited in oxygen supply. When organic matter is decomposed, carbon dioxide, hydrogen gas, and acetate are produced. The methanogenic bacteria carry out the conversion of these substances into methane gas, which ultimately pollutes the air (Sandin 2005).

3.4 Particulate Matter

It is the mixture of sulfate, organic and elemental carbon, solid compounds, dust, nitrate, smoke, and small droplets of liquid (Jacob and Winner 2009). Their diameter ranges from >2.5 μm to <10 μm. It can also be resulted from wind erosion, tillage process performed to prepare land for agricultural purposes, by burning of crops, and can be formed during the reactions of sulfur and nitrogen oxides. They badly affect the vegetation by interfering with the pesticides. Besides this, alkaline dust may increase the alkalinity of the cultivating land, inhibiting the crop growth and death of leaf tissue (Lemke et al. 2004).

4 Water Pollution by Agriculture

According to the recent reports of Environmental Protection Agency (EPA), agriculture is the sole reason for the disturbance of rivers and streams, more accurately the third largest source of pond, lake, and reservoir pollution. In accordance with the data published by National Summary of Assessed Waters Report in 2010, approximately 53 % of global rivers and streams have been declared unfit for the designed use (Rabotyagov et al. 2012).

It would have been easier to evaluate the impact of agriculture on aquatic system if the constituent activities of agriculture had regular and quantitative impressions. This could be a helping hand in deciding the designs for motivated systems that would in turn enhance the agricultural practices and for curtailing the environmental consequences. However, it is not true in this case. The relationship of both aquatic and agriculture systems is quite complicated, and the mesh that they create has multidimensional aspects. The most important interaction in this relationship is between catchment area and the receiving water. The whole Earth surface, which is usually agrarian or agricultural, constitutes catchment basin for the natural water communities. Any activity going on in catchment area would affect the natural waters. For the sake of understanding, this relationship can be compared to the relationship between home and the waste container. All the "doings" going on in home would be depicted in contents of the waste container. The Royal Commission of Environmental Pollution (RCEP) published the 7th report called "Agriculture and Pollution" in 1979. The report discussed the impacts of various contributors used in agricultural practices such as fertilizers and pesticides; however, at present "pollution" has taken wider perspective because of the increased understanding of the functioning of complex system (Moss 2008).

To evaluate ground zero effects of agriculture on receiving water, immaculate landscape is selected with no previous agriculture settlement. Such immaculate area is usually chosen so that natural flora that flourishes is grown according to the particular environmental conditions of that area. This would in turn help in natural selection of plant varieties, and this landscape would now be able to withstand the harsh conditions for the production of plant species in that specific area (Moss 2008). Some elements like phosphorous, nitrogen, and a few other minerals are known as scarce elements, which have been largely depleted from landscapes due to excessive deforestation and extensive agriculture (Likens et al. 1977). The undisturbed natural landscapes are more capable of retaining soil particles and minerals. Moreover, naturally selected species have efficient root system and microorganisms capable of retaining soil particles and minerals by forming protective soil crusts. Obviously, such landscapes will be affected by natural disasters like volcano, hurricanes, and tree falls, but such natural events are inevitable. Moreover, the aftereffects like exposure of soil and its erosion can be faded by successful succession of species that eventually restabilizes the soil surface (Moss 2008). Carbon in particular is abundant on Earth, and its provision by natural terrestrial vegetation to the aquatic system often balances the conservation of mineral nutrients. The headwater

streams are replenished by the leaf fall sources and wood debris for their primal functioning. Leaf debris composed of rich cellulose, lignin, and tannins are the primary energy sources of streams shaded by forest covering (Moss 2008).

4.1 Agriculture as a Destroyer

Before giving insight into the water pollution caused by agriculture, let us first get an overview of other biological and physiological disturbances caused by agriculture on this planet. Agriculture disturbs natural soil and nutrient conservation mechanism. It displaces the sources of wood debris, terminates the predators like wolves and bears to protect the domestic stock, and may completely alter the complex biological and physiological flood system in order to promote irrigation and drainage system. Moreover, it may cause huge alteration in prey-predator relationship by favoring the production of specific fish species due to nutrient enrichment and hence resulting into aberrant web chains (Vanni et al. 2005). It also introduces unknown species likes biocides, which cannot survive longer due to underdevelopment of defensive mechanisms. Such species have spent less evolutionary time and hence cannot be kept in natural aquatic habitats such as maintaining ponds (Williams et al. 2004), wet meadows, and fens (Wheeler 1980). In short, agriculture has no positive effect on ecological functioning and biodiversity of aquatic habitats. Landscapes selected and used for agriculture pose a serious threat to water biodiversity (Moss 2008). Yet it is an inevitable fact that agriculture is a basic necessity for human beings, and to feed huge human population, agriculture can never be ignored. It is estimated that in next 50 years further 10^9 hectares of natural landscapes will be used for agriculture, which will increase eutrophication by two- to three-folds (Tilman 2001) to approximate the conversion of 5×10^9 ha land from natural ecosystem (Millennial Ecosystem Assessment Board 2005). This includes one-third of all landscapes impossible to harvest or at least very difficult to cultivate, for example, deserts, tundra, and taiga. Here it is a high time to redesign the agricultural systems and to promote the flow of natural resources from natural ecosystems as much as possible. Certain activities can be promoted like atmospheric control, hazard regulation, water cleansing and its storage, provision of bug control facilities, promoting natural grazing, and lumber production (Moss 2008). It is certainly a policy challenge to keep an accord with environmental legislation as well as keeping productive and intensive agriculture (Sutton et al. 2011). Therefore, eco-efficiency has evolved to a rampant topic in both environmental and agriculture disciplines nowadays (Picazo-Tadeo et al. 2011).

4.2 Agriculture and Aquatic Ecosystems

Before discussing the comprehensive impacts of agriculture and its regulation, let us first define agriculture and its impacts. Agriculture in context means modifying

natural landscapes for the production of supplies that can either be used as food (both animal and human) or for other purposes like market or means of livelihood. Other uses of agriculture include forestation, crop production, and production of organic material for food and fuel for domestic animal. In fact agriculture can be carried out in varying intensities, and that is the reason for the pollution of freshwater and marine water ecosystems by agricultural alteration of natural terrestrial ecosystem. It affects the water chemistry and overall water quality with excessive enrichment of food chain (Moss 1996; Pretty et al. 2003; Moss 2004), biocide percolation (Corsolini et al. 2002; Van den Brink et al. 2002; Cold and Forbes 2004), erosion of soil with bulks of suspended loads (Brodie and Mitchell 2005), and modification of water cycles (altered evaporation as well as transpiration rate and hence overflow and variation of river flowing patterns with water loses) (Williams and Aladin 1991). Some other deleterious effects include extinction of exotic species used (usually fish and crustacean) and substantial alteration of habitat due to several river engineering practices like embankment, channelization, and channel modification.

There have been growing evidences that agricultural intrusion into natural systems may cause an increase in disease vectors like naucorid insects belonging to arthropod family Naucoridae, which transmit *Mycobacterium ulcerans* causing Buruli ulcer. Such bugs are found mostly in Australia and tropical Africa (Merritt et al. 2005). Increased incidences of malaria have been noted, strongly linked with ever increasing irrigated agriculture (Kebede et al. 2005). So in a nutshell, pollution is not merely the inclusion of some harmful things into environment that kill organisms, but it is defined as the cluster of man-made interfering activities that damage the delicate ties of natural ecosystem (Moss 2008).

4.3 Types of Impacts by Agriculture on Water Systems

The assessment of various impacts of agriculture on water systems is not easy because the relationship between agriculture and its impact on water bodies is quite complicated as described earlier. Generally various agricultural activities like application of chemical fertilizers, livestock and poultry breeding, aquaculture, and rural population are responsible for increased chemical oxygen demand (COD), ammonia–N, nitrogen, and phosphorus levels which are released into the receiving water systems (Wu et al. 2013).

4.3.1 Impacts on Surface Water Quality

Agriculture raises various problems like waterlogging, erosion, salinization, and desertification. In addition to this, it may also bring up the issues of water quality degradation by agrochemicals, salts, and toxic leachates. In recent years, the salinization of water bodies is a more widespread phenomenon and possibly has greater concern than that of soil salinization. In the past few years, some trace elements like

selenium, molybdenum, and arsenic have gathered attention, and their presence in agricultural drainage causes pollution that poses a threat for the progression of few irrigation plans.

4.3.2 Impact on Enrichment of Water

Excessive application of nitrogen- and phosphorus-based fertilizers on agricultural lands of Europe as well as other developed countries has led to enrichment of nitrogen and phosphorous in surface water, groundwater, and soil (Volk et al. 2009). This process of leakage of these nutrients into water bodies ultimately causes eutrophication, and according to one report, about 55 % of excessively enriched surface water in Europe is caused by agriculture mostly by the loss of these nutrients from soil into surface water (Vörösmarty et al. 2010; Sutton et al. 2011). Similarly, such type of pollution contributed by agriculture is recognized as major factor for the poor water quality in the USA (Sharpley et al. 2008). A study was conducted in Shanghai, China, to analyze the concentration of nitrogen and phosphorous in groundwater and surface water. These results showed that this nonpoint source-polluted groundwater and surface water was unfit for drinking purposes. The concentration of nitrogen in surface water was 6.3 mg per liter, while in groundwater, 16.85 mg of nitrogen per liter was calculated. Further studies confirmed that pollution of groundwater is caused by nitrogen-based fertilizer used in peach orchard (Shen et al. 2012).

Farmers apply these nitrogen- and phosphorus-based fertilizers to increase the production or output of the crops. These two nutrients are required for the growth of the plant; however, plants use only the required amounts of these nutrients, and excess nutrients are usually associated with leaching and excessive runoff transfer from land to water bodies (Tunney et al. 2010). The damage done by such nutrient losses to the water bodies would be very much site specific and would depend on various factors like unstable interaction of water systems, type of soil, chemistry of atmosphere, and fertilizer practices on farm level (Doody et al. 2012). Pig farming has emerged rapidly in the province of Hungyen, Vietnam, few years back. With this widespread trend of pig farming, agricultural efficiency flourished in Vietnam economically and was officially encouraged by the Vietnamese government. Four types of such pig farming systems run in the country, i.e., (1) VAC system, which is formed by combining fruit cultivation and fish farming with pig farming; (2) AC system, which is a joint system of fish and pig farming; (3) VC system, a blend of pig farming and fruit cultivation; and (4) C system, only comprising of pig farming (Ho et al. 2013).

However recently it was indicated that such farming systems became a potential source of pollution of surface and groundwaters in the Red River Delta and its vicinities. The excreta of pig are a supplier of sufficient amounts of nitrogen, phosphate, and potash (0.5 %, 0.3 %, and 0.4 %, respectively). The surface water is dangerously polluted following the contravention of recommended water quality in each of the farming system. The parameters like DO (dissolved oxygen), BOD (biological oxygen demand), COD (chemical oxygen demand), and ammonium and phosphate concentrations exceeded the set values by Vietnamese water standards.

Similarly exceeding level of ammonium that breached the standards set by national technical regulation of Vietnam contaminates groundwater. Overall the water pollution was highest in C and VC systems followed by relatively low pollution in VAC and AC systems (Ho et al. 2013).

4.3.3 Impacts on Public Health

Agriculture has major impact on water quality; thus, the polluted water causes various waterborne diseases. According to the World Health Organization (WHO), about four million children die each year due to diarrhea, a waterborne disease. The bacteria-causing disease called coliform is excreted by human which gets mixed in drinking water through poor water management. Surface runoff and nonpoint source pollution may consequently contribute towards high levels of pathogens in surface as well as groundwater bodies (Ongley 2005). According to another WHO report, nitrogen level in groundwater has drastically increased due to excessive farming. Reiff in 1987 made some important discussions about agricultural impact on water quality. He described that due to reservoir construction for irrigation and hydroelectric power production purposes, there is strong evidence of increase in malaria (particularly in Latin America) and schistosomiasis infecting two hundred million individuals in 70 tropics and subtropics. Farmers and children that bathe in infected water are at more risk. Contamination by other nonpoint source pollution may also pose serious health threats.

Microbial contamination of food crops results from either using polluted water for irrigation or its direct contact with the food. Diseases that are commonly associated include typhoid, cholera, ascariasis (caused by *Ascaris lumbricoides*), amoebiasis (amoeba, *Entamoeba histolytica*), giardiasis (protozoan, *Giardia lamblia*), and enteroinvasive *Escherichia coli*. Mostly these diseases are caused by consuming ground crops such as cabbage, carrots, or strawberries. Other complications include hormonal disturbances in human, animals, and fish. Due to immense importance of endocrine and its secretions in early days of development, toxicological effects of polluted water badly affect reproductive system.

4.4 Agriculture and the Aral Sea Disaster

The world's biggest example of rich land and water system destroyed by excessive agricultural practices followed by poor management is Aral Sea and its drainage basin. Although water quality in that area had many other impacts, but agriculture still remains the major contributor. Aral Sea drainage basin surrounds many countries including Southern Russia, Tajikistan, Uzbekistan, and partly Turkmenistan, Kirghizstan, Iran, Afghanistan, and Kazakhstan. According to 1987 census, population recorded was 23.5 million, and in 1990, 34 million is recorded. Total area of the basin is l.8 × 106 km^2, while the irrigated area is 65.6 % (1985) (Ongley 2005).

5 Climate Change and Agriculture

Climate change is referred to as changes and variations that occur in climate and persist for a longer period of time ranging from a few years to many decades. The reasons for this change in climate can be many; it can be due to natural processes occurring in the earth's atmosphere or anthropogenic changes (effect of humans on environment). Agriculture has obtained a central role while studying the potential effects of climatic change (Decker et al. 1986).

The consequences of agriculture on climate and the outcome of climate change on agriculture are interlinked to each other. The most important link between these two is "the greenhouse gases." As much as GHG affect the agriculture and crop production, studies on agriculture's contributions to trace gas emissions have increased in the past few decades (Adams et al. 1990). Agriculture practices such as use of nitrogen fertilizers and synthetic pesticides emit approximately one-quarter of global anthropogenic greenhouse gases (Scialabba and Lindenlauf 2010). A greenhouse gas is the one that absorbs and ejects radiations within a thermal infrared range resulting in the greenhouse effect. Water vapor, methane from wetlands, carbon dioxide from burning fossil fuels, and ozone and nitrous oxide from microbe's activity in the soil are included in these gases. These gases occur naturally in the atmosphere in low quantity, but their concentration is increased manifold due to human impacts such as industrial and agricultural activities. Agriculture discharges a variety of GHG such as CO_2, CH_4, and N_2O (Cole et al. 1995; Paustian et al. 2004) into its surrounding environment. These trace gases result in environmental changes such as rise in temperature, changes in precipitation, and extreme weather conditions. Increased GHG level will cause Earth's average temperature to raise approximately 0.3° centigrade every decade (Houghton et al. 1990).

In the twentieth century, global warming is mainly because of the anthropogenic increase in GHG (Crowley 2000). A high concentration of greenhouse gases produces radiative forcing which tends to warm the surface of the earth (Houghton et al. 2001). The increased concentration of greenhouse gases has led to increased warming of the earth due to positive radiative forces. Increased emission of carbon dioxide is attributed to the expansion in land used for agriculture besides fossil fuels and burning of green plants and forests. Expansion of agriculture has resulted in soil degradation, decrease in soil organic carbon and nitrogen, and increase emission of atmospheric carbon dioxide, nitric oxide, and methane either by converting natural systems into agricultural system (deforestation, biomass) or by soil management practices (use of fertilizers) (Tavi and Lal 2013). High concentration of carbon dioxide and methane has the most significant contribution to the warming. Carbon dioxide release is mostly because of microbial decomposition or burning of soil organic matter (SOM) and plant litter (Janzen 2004; Smith 2004). Methane emission, due to enteric fermentation, is one of the most significant sources of GHG emissions from agriculture. It mostly accounts for 4–5 % of the world anthropogenic gas emissions (Scialabba and Lindenlauf 2010). Methane (per mole) contributes to an estimated 3.7 times to global warming of carbon dioxide (CO_2) (Lashof

and Ahuja 1990). Use of nitrogen fertilizers in rice crops is the major contributor of methane in atmosphere. It has been studied that CH_4 emission from fertilized rice crops is 3 to 5 times more as compared to unfertilized crop (Cicerone and Shetter 1981). Though use of the nitrogen fertilizer helps in increased crop production, but these benefits cost us significant environmental loss such as increased atmospheric N_2O and other reactive nitrogen gases in atmosphere (Ussiri and Lal 2013). Increased N_2O emission is due to the use of different fertilizers and sodium-, nitrogen-, and potassium-containing pesticides. Different microbes or bacteria transform nitrogen in soil, manure, and fertilizer to generate nitrous oxide. Apart from these biological emissions, agricultural practices such as land clearing, cultivation, and irrigation cause a vast change in the global and local land cover characteristics. Expansion of agricultural land into natural ecosystems has an important impact on climate. Challenges due to climate change arising from increased farming practices can be fought by agricultural communities, thus leading to reduced GHG emissions by simple strategies such as farming with perennials, livestock production in climate-friendly manner, increasing soil carbon level, and providing protection to the natural habitats (Scherr and Sthapit 2010). Although many programs have been formed to mitigate the emission of GHG, increased food demands have kept the release of these gases at higher rate. The application of different practices including reduced tillage systems, management of crop residues, nutrient and pest management and their control, agroforestry, use of biochar as soil amendment, and other agricultural technologies are some recommended managing practices which will help in reducing the impact of agriculture on climate and environment (Tavi and Lal 2013).

6 Soil Pollution

Soil pollution is the presence of toxic compounds and materials, xenobiotic chemicals, minerals or other salts, radioactive substances, or agents that are responsible for causing different diseases in the soil. These pollutants have negative effect on plants, humans, and atmosphere. The most common soil contaminants can be categorized into four types (Alloway 1990):

(a) Agricultural pollutants
(b) Industrial pollutants
(c) Municipal pollutants
(d) Nuclear pollutants

Soil can be polluted by a large number of pollutants, besides waste disposal on land; the pollutants can be agricultural (pesticides) or industrial (different kinds of hazardous chemicals) that can cause land pollution (Aelion 2002). In this chapter, we will only discuss those pollutants that originate from agricultural practices. The pollution of agricultural areas in different countries is due to the overuse of fertilizers, pesticides, herbicides, insecticides, etc. A huge quantity of chemicals is applied to soils, which results in the increase level of heavy metals such as

cadmium, arsenic, and lead (Atafar et al. 2010). The use and variety of pesticides have increased drastically around the globe with increased consumption of food, relative to increased crop production. This large utilization of pesticides has resulted in their misuse, thus posing serious environmental pollution and health risks. Pesticides can be any substance or combination of different substances that are intended for prevention, destruction, or repelling any pest (EPA 2009). In order to assure increased productivity to meet the required need of food in population, the use of pesticides is very necessary.

However, their use in excess or abuse results in serious complications (Pimentel 2009). Pesticides and its by-products generated after their degradation can escape into the environment, soil, or rivers, ultimately leading to the accumulation of toxic substances. Pesticides such as DDT (dichlorodiphenyltrichloroethane), chlorinated hydrocarbons, dieldrin, and organophosphates are absorbed by the soil particles where they accumulate and cause contamination of root crops grown in soil. Consequently, the use of such contaminated crops causes the pesticide particle present in crop to get an access to human biological systems causing different diseases. Organochlorine pesticides (OCPs) are subtypes of persistent organic pollutants (POPs), which are more bioaccumulative and highly toxic. The presence of OCPs in different soils including cultivated and vegetable fields is detected even after the ban on their use in 1983 (Wang et al. 2008).

Pesticides decrease the fertility of soil by contaminating them with different heavy metals. Cadmium-, mercury-, and lead-containing pesticides were prohibited in 2002. An estimated total input of 5,000 and 1,200 tons of Cu and Zn, respectively, were applied in agrochemical form to agricultural area in China (Luo et al. 2009). Contamination of different agricultural soils and crops with heavy metal is a serious complication in agricultural industry. Heavy metals such as cadmium (Cd), zinc (Zn), lead (Pb), and copper (Cu) are very common in upper layers of soil especially in rice fields. Heavy metal accumulation in fields and vegetation results in hazardous effects on human health when consumed in food chain. Copper is widely used in animal feeds, and there is an increased threat of pollution in the soil by the addition of such manure in crops (Xiong et al. 2010). Fertilizers containing high level of sodium and potassium decrease the soil pH, destroy the soil structure, and reduce the efficiency of field crops (Savci 2012). Phosphate fertilizers are an important cause of cadmium metal accumulation as compared to other fertilizers. The sources of heavy metal, apart from fertilizers, are other agrochemicals such as pesticides, livestock manure, and use of polluted water for irrigation (Longhua et al. 2009).

7 Genetic Engineering Leading to Gene Flow and Plant Contamination

Genetically modified crops or GM crops are generated when their DNA is modified by inserting desired genes for favorable characters with the help of genetic engineering techniques. These techniques are extremely precise and specific, unlikely mutagenesis in which the plant is subjected to radiations or chemicals to create

mutations in DNA. Genetically modified crops lead to improved shelf life and nutrition, herbicide and stress resistance, and increase in productivity. But GM crops remain a controversy. Advocacy is from both sides, and both the groups, in favor or opposed, have their own reasons. The possible commercial and industrial scale cultivation of GM crops in Europe presents enormous risks and challenges for ecology (Gray 2004). The recombinant biotechnology and products formed from this technique have brought serious and hazardous problems of biosafety (Liu and Zhu 2001). The use of GM crops has raised the concern on their safety and the potential effects on health and agriculture (Brookes and Barfoot 2010; Zdjelar et al. 2011). There is an increased threat of potential allergenicity of food products having foreign genes (Mishra et al. 2010).

The risk of genetically modified crops is the absence of barriers to the spread of transgenes or gene flow through sexual reproduction. This can be due to spread of these transgenic genes to the weedy species by processes such as hybridization (Kaiser 2010). Genetic engineering may lead to an increase in the possibility of escape of transgene (Bergelson et al. 1998). Gene flow is an important pathway for the transgene to escape from biotech crops to their wild relatives. Gene outbreak from crops to similar wild-type species can be pollen or hybridization (Ellstrand and Elam 1993). These transgenes able to break out in the environment can cause ecological risks. These foreign genes, resistant to biotic and abiotic stresses, can lead to unpredictable environmental issues. Crops such as rice, soybean, and millets have their wild-type species and weedy relatives present in the agricultural ecosystem. The release of the alien gene variety into environment will result in crossing over with wild-type species (Bao-Rang et al. 2001).

Crops are present along with their weedy biotypes, besides their wild relative species, especially in those plant and crop species that share the similar genomes and ploidy level with their wild types, simultaneously in the same field. Examples of such types may include rice (*O. sativa*) whose weedy type is *O. spontanea* or soybean (*Glycine max*) whose wild type is *G. gracilis.* Because of their close proximity, gene flow among crops and their weedy and wild relative species is common and frequent. Sexual reproduction and vegetative propagation are the means by which the transgenes are going to persist and scatter to the wild and weedy population where they are going to express themselves in a natural way in the wild populations. The effect of this gene flow is different depending on the traits and characters encoded by the transgenes; if they encode for character such as high protein content, vitamins, and good grain and seed quality, the ecological risk will be less. In contrast, if it encodes for resistance to biotic and abiotic stresses and herbicide resistance, the ecological problem will be drastic (Bao-Rang et al. 2001). Transgene breakout to wild species population through outcrossing or hybridization causes contamination of the natural populations and can even lead to the extinction of endangered species of the wild types in agriculture ecosystems (Kiang et al. 1979; Ellstrand and Elam 1993).

Dispersal of transgene conferring characters that enhance survival and reproduction to wild or weedy populations such as dispersal of transgenic herbicide-resistant gene can act as a serious threat in supervising and overcoming weeds and nonessential plants (Snow et al. 1999; Hall et al. 2000). Seeds of traditional corn, canola,

and soybean varieties are contaminated with low amount of sequences of DNA that are derived from transgenic varieties (Mellon and Rissler 2004). Seed contamination has great consequences for two important reasons:

- Seeds are reproductive and transfer genes to future generations. Transgenic sequences entering the seeds and contaminating them will reside in them overtime in those plants where they are not expected and are problematic to control.
- Seeds form the foundation of our food systems and food chains. They constitute the base on which we are able to make crops better, or they can be the source where we will go back in case crop fails. However, if these seeds are contaminated inconsiderably with genetically engineered sequences, then there will be a huge problem in understanding their chemistry, and their manipulation will be difficult.

Seed contamination gives rise to food safety concerns in the future, and contamination of traditional plant varieties makes their trade or commercial importance negligible for consumer who wants pure and natural types.

8 Health and Agriculture

Since the late 1900s many changes have been brought in the agricultural sector for improving health and safety conditions of farmers and other people working in the agricultural sector. These changes include improvement in technology being used, awareness of health hazards among individuals, and personal protection. Food Quality Protection Act (1996) and Worker Protection Standard (1992) are the examples of the regulatory approaches taken in order to prevent occupational and environmental health hazards that can be caused due to agricultural practices. The current conditions show that there is still a need for research and awareness of agricultural health and safety. Steps need to be taken to educate farmers and other individuals related to agricultural industry.

8.1 A Dangerous Occupation

Farming has been declared a highly precarious industry according to a study done by the Bureau of Labor Statistics. Agricultural workers face injuries, illnesses, and fatalities due to physical exertion, contact with animals, use of machinery, and a high rate of exposure to toxic materials (US Department of Labor, Bureau of Labor Statistics 2007).

People who work in farms have an average age of 54.3 years. The reasons are many including:

1. *Aging*—as a farmer ages, he becomes more susceptible to adverse effects, and because he has been facing toxic chemicals for years (occupational exposure), he might contract respiratory and musculoskeletal diseases.
2. *No background knowledge*—the farm workers that are hired take jobs in agricultural sector as an entry-level job. They lack background knowledge of how operations are carried out in agricultural farms.
3. *Language barrier*—if a language barrier exists, it may hinder information exchange on essential matters like agricultural safety and work practices.
4. *Ethnic variation*—dissimilarities in ethnicity may also lead to potential health hazards in certain races due to genetic reasons.
5. *Hiring of farm workers by labor contractors*—technically farm workers should be employed by farm owners not farm labor contractors. Hiring done by contractors who are oblivious of conditions and risks present in farm raises new health and safety concerns.

8.2 Physical Diseases and Illnesses

Due to the arrival of technical revolution in the agricultural sector, the living and working conditions improved drastically, but it also brought forward a number of inhalative hazards. These hazards include biological, chemical, and physical hazards. Biological hazards include tuberculosis, tularemia, and Q fever. A number of allergenic dusts originating from vegetable crops have also been observed. Pesticides and fertilizers form a part of chemical hazards, whereas physical hazards are present in areas where agricultural machines are repaired. Some examples include asbestosis and silicosis. A number of unspecific dusts also cause physical hazards.

8.3 Dusts

8.3.1 Inorganic Dusts

There are many methods used in agricultural operations that increase exposure to inorganic dusts. They include field activities like tractor tilling, haying, plowing, and harvesting. Inorganic dust mainly constitutes of silicates either crystalline (quartz) or noncrystalline (diatomite) originating from diatomaceous earth. Silica can also be present in aerosolized during rice stubble burning. Out of the total exposure to dusts, 15–43 % of exposure is of inorganic dusts. Silica causes a restrictive lung disease called pneumoconiosis. Abnormal radiographic findings are associated to people that have pneumoconiosis. Inorganic dusts play a minor role, are relatively non-toxic, and thus cause a few respiratory illnesses as compared to organic dusts. It has also been observed that respirable quartz dust from agricultural origin is less pathogenic than that from other industrial origin.

8.3.2 Organic Dusts

Most studied agricultural respiratory diseases are those caused due to exposures to organic dusts coming from confined animal feeding operations (CAFOs) and grain processing. Swine confinement facilities have been associated with mucous membrane inflammation syndrome, sinusitis, bronchitis and non-immunogenic bronchospasm. Organic dusts cause ODTS (organic dust toxicity syndrome), asthma, asthma-like syndrome, farmer's lung [farmer's hypersensitivity pneumonitis (FHP)], and chronic bronchitis. They also impose biological hazards due to the presence of proteins that can be proinflammatory and allergenic. Toxic exposures both additive and synergistic can damage respiratory health (Von Essen and Donham 1999).

8.3.3 Grain Dust

It consists of animal dander, vegetable product, insect fragments, rodent and bird feces, microorganisms, pollens, endotoxins, and pesticides. Forty percent of respirable organic dusts range from <0.01 to 100 μm in size. Respirable dusts can be defined as the dust particles that have a diameter of 4.0 μm or less. They have the propensity to penetrate into the gas exchange unit in humans that is the terminal bronchioles and alveoli. Grain dusts are produced due to processes like harvesting, processing, production, transfer, storage, and grain cleaning. According to OSHA (Occupational Safety and Health Administration), there is 10 mg/m^3 (upper limit) permissible exposure levels (PELs) for grain dusts.

8.4 Allergens

Grain and animal confinement contain a huge lot of possible allergens like animal dander, grain dusts (wheat sorghum and soy), fungal molds, insect fragments, pollens, and bacteria. Exposure to allergens in enclosed areas leads to problems in upper respiratory tract to asthma and bronchial hyperreactivity (more common in swine confinement), wheezing, and non-IgE-induced asthma-like syndrome. Some of the many examples of allergens found in agricultural settings are food proteins in animal feeds, type 1 allergens in pig saliva, and urine and epithelium and storage mites in *Acarus siro*, Lepidoglyphus destructor, and *Glycyphagus domesticus*.

8.5 Endotoxins and Inflammations

Heat-stable lipopolysaccharides in G-negative bacteria cell walls have been associated to flu-like symptoms in farm workers. Both animal and human activities

produce endotoxins. Conditions in CAFO (concentrated animal feeding confinement) specifically swine and poultry, grain elevators, livestock farming, potato processing, cotton industry, and animal feed industry promote endotoxin production. The gram-negative bacteria that produce endotoxin in agricultural areas are *Bacillus, Pseudomonas*, *Corynebacterium, Vibrio, Pasteurella,* and *Enterobacter*.

Endotoxins can cause organic dust toxic syndrome (ODTS). Among the people who work in swine confinement barns and other agricultural settings, ODTS is very common. It is an acute, noninfectious febrile flu-like illness that may seem similar to hypersensitivity pneumonitis. It is characterized by dry cough, shortness of breath, chest tightness, fatigue, myalgia, chills, and fever. Inhalation of endotoxins in the swine confinement barns is the most common cause. Lung inflammation and systemic inflammatory reactions are observed which are self limiting and die off in a matter of days. People suffering from ODTS and exposed to endotoxins for an extended period of time are at a high risk of chronic bronchitis and other respiratory diseases. Fungal and bacterial spores can synergistically cause evolution of ODTS. Inflammatory pulmonary reactions may also result. The permissible exposure levels are not known for endotoxins, but Donham recommends 1,000 ng/m^3 to the upper limit value of endotoxins. This value has been specifically mentioned for chronic exposure in swine confinements.

8.6 Microorganisms

Farming, animal production settings, and husbandry are good reservoirs of infectious agents and are responsible of zoonosis. Swine influenza is mainly spread through hog confinements. Psittacosis (an infection by Chlamydia psittaci) is caused in poultry workers specifically those who work with turkeys and ducks. Zoo workers and veterinarians can also develop psittacosis. Sheep, cattle, and goats infected with rickettsia (*Coxiella burnetii)* spread Q fever that may result in atypical pneumonia. Community outbreaks over half a mile are possible via aerosolized bacteria like *C. burnetii* (a storage infectious organism). *Mycobacterium bovis*, the bacteria responsible for tuberculosis, if present in farm animals can be transferred to farm workers. Aerosolization of rodent saliva, urine, droppings, and contamination by Hantavirus can cause Hantavirus pulmonary syndrome. The responsible agent of this disease is Sin Nombre virus (*Bunyaviridae* family). The vectors for this virus could be rodents found in confinement settings.

8.7 Toxic Gases

Nitrogen oxides cause silo filler's disease. Major source of this gas is silage fermentation and heavily fertilized soil. Once lethal levels are reached, nitrogen oxides may stay for weeks later. Low-level exposure causes pulmonary decompensation

and chronic pulmonary diseases due to fibrotic scarring. Acute high-level exposure may result in acute hemorrhagic pulmonary edema and death. Increased NO_2 levels in air have shown to exacerbate allergic responses once the allergens are inhaled (Gennaro et al. 2013). Swine waste produces gases like hydrogen disulfide, ammonia, carbon dioxide, and methane. Hydrogen sulfide at low levels acts as a respiratory irritant and as an asphyxiate at high levels. It is very easily recognized due to its rotten egg-like smell. Its PEL value is 10 ppm; concentration above 150 ppm can lead to olfactory paralysis, and concentration of 500 ppm causes death. It has been reported that about 1,000 ppm concentration of hydrogen sulfide has been achieved when manure pits are emptied. Ammonia is produced mainly in animal and poultry confinement areas. It is an irritant of mucous membrane and respiratory tract. Being exposed to ammonia gas can lead to upper respiratory tract irritation, chronic obstructive pulmonary condition, sinusitis, and mucous membrane inflammation syndrome. With time, tolerance may develop. Prolonged exposure below the PEL of 25 ppm can cause adverse effects on human health.

Carbon dioxide and methane are asphyxiates and do not cause adverse effects generally. Coming mainly from animal respiration, carbon dioxide is an indicator of ventilation. Its acceptable concentrations are below 5,000 ppm. Methane at levels less than 5 % can be potentially hazardous. Carbon monoxide is produced via kerosene heaters or gasoline-powered machines like washers. In the presence of poor ventilation, it takes no more than 3–5 min for carbon monoxide to reach toxic levels. Its toxicity can cause coma, chronic neurologic difficulties, respiratory arrest, and death. Lagoons and manure pits are rich in these toxic gases. A person entering an area having high levels of the abovementioned gases may lose consciousness within seconds and collapse. Those who survive develop pneumonia caused by polymicrobials called drug lung.

8.8 Crop Protection Chemicals

Pesticides are more recognized for affecting the human nervous system. They also affect lungs. According to a research in the USA and Sweden, it was shown that work-related asthma is a major issue in agricultural workers, and its causes include not only plant and animal allergens but also commercial pesticides (Paul et al. 2013). Paraquat (herbicide) induces free radical production that leads to fibrosis. Roundup (glyphosate) if intoxicated causes chemical pneumonitis. Bronchoconstriction is the result of exposure to organophosphates and carbamates, which is temporary. Very few pesticides have been reported to cause pulmonary diseases. Acute exposure to pesticides can cause hypersensitivity reactions, whereas chronic exposure may lead to serious health problems like onset of cancer or neurological issues (Amoguis et al. 2010). According to a study done on Ethiopian farmers, the most common symptoms observed due to pesticide toxicity were 58.8 % headache, 38.2 % vomiting and salivation, 36.5 % nausea, and 12.5 % sneezing (Karunamoorthi et al. 2012).

A number of pesticides function as endocrine disruptors (ED). ED by bluffing as endocrine hormones in human body interferes with the natural physiological functions and disrupts them. Some of the examples are brominated diphenyl esters, polychlorinated biphenyls, and phthalate esters (Ozen et al. 2011). Atrazine, an herbicide, is harmful for the reproductive development of humans. It also has a major role in causing cancers like adrenocortical carcinoma, ovarian cancer, and placental cancer by increasing aromatase expression (Jessica et al. 2012). A systemic insecticide Phorate (PHR) causes problems in nerve impulse transmission by inhibiting cholinesterase enzyme (Timoroglu et al. 2012).

8.9 Farmer's Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis (allergic alveolitis) is an alveolar inflammation in the lungs due to hypersensitivity reactions to inhaled antigens (mostly organic dusts). It shows a number of clinical expositions. It is very common among farmers. It fits in none of the four types of allergies, i.e., it gives an immunologic and cell-mediated response: antigen–antibody complex formation after an extended exposure which is a type III reaction and at times formation of granulomas which is a type IV reaction. Farmer's lung or farmer's hypersensitivity pneumonitis is caused mainly due to the inhalation of biological dusts and agricultural products like mold spores, straw, feed, and hay dusts. Specific antigens that cause Farmer's lung are spores of *Micropolyspora faeni, Saccharopolyspora rectivirgula, Aspergillus* species, and *Thermophilic actinomycetes*. FHP shows ODTS-like symptoms. Long-term exposure to allergens of FHP can cause fibrotic changes in lungs and induce emphysema. In acute phase, alveolar infiltrates and desaturation of oxygen can be radiographically demonstrated. Antibodies against molds and thermophilic bacteria called precipitins are also detected. It is more prevalent in cold, moist climates. FHP is more prevalent in nonsmokers, whereas smokers have a more chronic form of FHP.

8.10 Agriculture and Cancer

Cancer incidence is low in agricultural workers overall, but some studies suggest that certain types of cancers prevail in farmers more commonly. These cancers include leukemia (cancer of white blood cells), multiple myeloma (cancer of plasma cells in bone marrow), non-Hodgkin's lymphomas (NHL), and skin, prostate, brain, lip, and stomach cancer. Agricultural workers are exposed to a number of pesticides and other chemicals that increase chances of cancer in them. In most of the countries, a family shares the agricultural operations, which not only puts the agricultural worker but also the rest of the family members on risk to have cancer. People living near farms are indirectly exposed to these harmful chemicals. These chemicals can also become a part of dust particles and contaminate air.

8.11 Prevention

Health, farm labor, and agricultural productivity depend on each other mutually. Just like farm labor and health have a significant impact on agricultural productivity, the agricultural systems, machinery, and settings can affect farmer's labor and health. Damage to farmer's health would impair him to experiment and innovate, so prevention is necessary. Following steps can be taken to decrease health issues faced by the farmers:

- Respiratory masks should be used to prevent diseases like asthma and FHP.
- Face shields and helmet containing powered air purifying respirators should be used for sensitized individuals.
- Frequent use of chemical cartridges.
- Having occupational stressors, e.g. when working hours are long or economic conditions are worse; farmers don't bother using personal protective equipment or take other preventive measures.
- Dust production can be prevented via using mist, oil, and wet methods to clean surfaces.
- Fats should be used in feeds.
- Proper ventilation is important.
- Manure should be stored outdoors in leak-proof container.
- Use of disinfectants.
- Use of urea preservative for hay and straw can decrease mold and bacterial growth.
- Moisture should be added before hay chopping.
- Well-trained health-care providers should be appointed.
- Monitoring of dust, endotoxins, and gases should be done using accurate and cheap devices.

9 Biodiversity and Agriculture

There exists a continuous conflict between these two streams that is agriculture and biodiversity since ancient days, but this conflict rose at its peak in the late twentieth century. Both the conservationists and agriculturists are running against the tide. One deems agriculture to be the source of victuals and survival for others, and the others deem it the mass destruction of wildlife and thus disturbing the natural balance of the ecosystem. In this section, we will make an attempt to analyze both the perspectives in the light of facts and figures. We will also make an attempt to resolve this conflict between the two in an appropriate manner. So to begin with let's address the claims side by side. If we peep into the history, we find that in the olden times agriculture was not as much extensive as it is today. It was relatively simple, labor demanding, and was not mechanized. However, by the end of the twentieth century, there was revolution in the field of agriculture known as agricultural revolution

especially in the developed world. The reason is that it provided a lot of space for the use of high-tech farming based on industry. The industrialized farming posed an enormous threat to biodiversity in a number of ways. In this discussion, we will be looking at the great and intensive harms to biodiversity by this contemporary industrialized, high-tech farming. This is one of the aspects which in developing countries is entirely overlooked. No one bothers what are our losses and how to cut them down. What are the challenges and how to counter them? Apparently prodigious agriculture is only good for humanity with no harm especially for the developing countries in terms of food security, economic growth, and improved quality of life especially in those people who practice farming routinely, but it is important to explore the underlying threats to this increased industrialized farming and agriculture. The primary forests have decreased to 20 % and natural grasslands and savannah by 40 % due to the deterioration brought about as an aftermath of agricultural intensification and other activities of man. On the other hand, cultivated lands have aggrandized to 390 % and pastures to 490 %. According to the estimates, almost 39 % of the earth has been cultivated and transformed to an agricultural land (Goldewijk 2013).

9.1 Contemporary Agriculture-Intimidating Biodiversity

Actually the ways and means of agriculture that are in practice today and in recent past few decades are highly industrialized and commercialized from every aspect, and they are distressing the natural balance of the ecosystem in a variety of ways, for instance, clearing up of vast lands, alongside hedgerows, copses, or wildlife corridor, and displacing time-honored assortment of highly productive but consistent and genetically identical seeds. Shortly, the desire for getting vast leveled land for cultivating the crops of our own desire, for our own benefits, and self-serving turns the native green of that locale eliminated even more. The natural green fragments of earth containing in them a myriad of wildlife—rich in biodiversity—are prone to extermination. The contemporary man is unfamiliar that his unforeseen jettisons for biodiversity will in long run cause him to pay for it. As we all know that if we are going to harm the natural balance of ecosystems existing on earth, in turn ecosystem will not provide its services in a way that it was providing us since our earliest days. Furthermore, we are making use of soaring amounts of pesticides, insecticides, and chemical fertilizers which is another important factor in causing setback to the already afflicted biodiversity. In this case those lands are massively afflicted where the ultimate goal of the growers is to increase its agricultural yield.

In this era, healthy ecosystem and biodiversity proliferation are crucial for prolonged endurable agriculture. It appears as if both agriculture and prolific biodiversity are the two paradoxes of this modern era. Primarily, the agricultural products and livestock upon which the agriculture of our epoch—the so-called novel agriculture—depends are vital to planet Earth's genetic resources. In addition to this, agriculture is not just leveling of vast areas of land, but it demands some extra things as

well. These additional demands of agriculture are termed as environmental services. Environmental services include supply of water, the revolving of nutrients in the soil, pollination and biological ways of controlling pests, and sequestration of carbon. These demands of agriculture are being put into practice by both green resources such as plants, trees and forests, and also by animals including microorganisms like bacteria for nitrogen fixation, smaller insects and birds for pollination, and higher animals that serve the purpose.

On a serious note, if the animals and plants are exterminated by one way or the other, the environmental services that they probably can provide will no longer be in our hands because of their ultimate extinction from our planet Earth. The entire pattern of life will get disturbed at all levels if we will continue to play with our ecosystem insanely. We need to manage this appropriately.

9.2 Agriculture and Biodiversity: The Greatest Paradoxes

Firstly it is of no doubt that agricultural intensification and expansion has contributed a lot in tempering the wind of poverty to shorn lamb, but it occurred at the cost of compromising biodiversity and environment (Tilman 1999). Natural ecosystems suffered an immense damage or deterioration, and the services provided by these ecosystems have drastically reduced to nil as if they were nonentity (MEA 2005). Biodiversity losses are the direct consequences of this agricultural revolution, and there is a variety of species of all kinds that are at the verge of extinction due to these massive agricultural practices that have happened to drastically affect all the scenario of the last century and the upcoming century. According to the data received from IUCN, agriculture is the main cause of bringing all kinds of species either at the verge of extinction or extinction in the world. This is what has had happened in the past and now what would happen in the future. By 2050, it is estimated that world food demand is going to be twofold more (Tilman et al. 2002). Further estimations reveal that land for agricultural use would also be increased (say by 25 %) in developing parts of the world to accomplish the demand of food (Balmford et al. 2005). This is also apparent that in the equatorial regions of the world, biodiversity would suffer an immense loss (Scharlemann et al. 2005). Furthermore, the requirement of raw material for bioenergy is also increasing with increasing industrialization (Field et al. 2008). Crops like sugarcane and oil palm form an unfavorable habitat to a variety of species that contribute to biodiversity (Petit and Petit 2003; Aratrakorn et al. 2006).

9.3 Repercussions of Industrial Agriculture on Biodiversity

The information obtained on the basis of facts and figures gathered by making a study on the relationship between industrialized agriculture and biodiversity has

immensely ameliorated the statistical figures obtained from current findings and data recordings along with those provided by the Consultative Group on International Agricultural Research and the United Nations (UN) Food and Agriculture Organization (FAO). Statistical studies reveal the fact that agriculture and livestock rearing covers 28 % of the total surface area of the Earth. It is a harsh reality as well that 41 % of the agriculture being practiced is by making an extensive use of heavy machinery and fertilizers that are potently chemicals. It is noteworthy that since the 1700s, 50 % of the temperate, tropical, and dry subtropical forests of the world are now transformed to agricultural lands. And 25 % of the transformation has occurred in the previous half of the century.

The land that has been servicing mankind for growing staple food crops decisive for food security remained reasonably consistent, i.e., approximately 1.3 billion hectares for the last 25 years. The good thing is food production has attained sustainability. This is because of the intense research in the field of agriculture for the production of high-yielding crops for developing part of the world. This procedure of growing high-yielding plants is popular as green revolution. Another ballpark figure suggests that another three billion hectares of land that is at present not under cultivation, that is not agrarian, will be transformed to agricultural land in near future. Another thought-provoking event that is going to happen to this apparently all-good process is an inadvertent effect on biodiversity. But such trends and agricultural practices must not be allowed to proceed unmodified and unaltered because such changes on massive level are posing perniciousness to biodiversity.

9.4 Intensive Use of Chemicals: Bullying Biodiversity

The intensive farming practices have curtailed landscapes in the entire Europe. This has marked with severe loss of biodiversity. Recent studies have revealed that intensive farming practices have adversely resulted in loss of plants, beetles, and birds particularly in the Western Europe. Drastic effects have been observed in the Western Europe. The intricate landscape, which has been maintaining the biodiversity for decades, has been oversimplified by this intensive action of farming the vast area of land. The cause of these effects is aluminum (Al), which is one of the key components of all these massive intensification practices.

The effect of Al on plants, ground beetles, and breeding birds in eight major European countries in the cereal fields has been studied. These countries include Estonia, Germany, France, Poland, Spain, Ireland, the Netherlands, and Sweden. As this has occurred because of Al, let's have a look for the sources of Al – the main element of modern intensive farming. Al has been accumulated in farmed zones from fertilizers, pesticides, tillage operations, and other mechanical methods that have been employed for weed control. Depending on the amount of Al that has been employed on each specified zone of land as per requirement and need, they are classified as low, medium, and high levels in each region.

Furthermore, the area under cultivation is also categorized into three main tiers. These tiers are field, farm, and regional. Here in particular analysis, Al was found to affect drastically the population of plants and birds, but one amazing thing observed was that it does not affect the population of ground beetles. This analysis reveals that the effects of Al on different species are like chalk and cheese. Furthermore, the mobility of species also shapes the diversity of landscape. The three major effects of Al on the diversity of landscape are perceived as follows:

- Entire variety of plants gets affected if the concentration of Al is towered up in the cultivated area. Plants are not mobile, so they are more affected by this intense accumulation of Al.
- Birds in the farms and regional zones are notably affected by the intense accumulation of Al; on the other hand, with less intense accumulation of Al, proliferation was observed in the population of birds. If agricultural practices are carried out at low pace with much lesser intensity as our farmers usually do, then natural habitats of many species will be less affected though yield must be comparatively low, but still the biodiversity of birds will be maintained to a greater extent.
- The abundance of ground beetle occurring in fields perhaps supported by field margins and nearby selectively natural regions would provide habitats for the production and survival of beetles in the cultivated areas (Flohre et al. 2011).

The above-mentioned points clearly state how intensive agricultural practices affect biodiversity of different categories of cultivated areas. Here, we considered the consequences of Al on the three major tiers that are very important to be considered individually as well as collectively. We also need to consider the effects of some more elements and ingredients that are associated with contemporary agricultural practices.

9.5 Biased Distribution of Water Between Farmed Lands and Nature

Agriculture largely banks upon irrigation which is another deleterious factor as per biodiversity is concerned. The extensive use of water for the productivity of grains is also the gist of green revolution. In reality we all know that today, most of the water drawn from surface as well as grounds is being used to serve the purpose of agriculture rather than serving the industry and household. However, by 2025, it is thought that the agricultural consumption of water will decrease worldwide, but it had been predicted by some scientists that it is not going to happen for the developing parts of the world. They will entail even 50 % more water in these coming years.

Waterlogging and salinity in many parts of the world are the two undesired and redundant effects of agriculture. Agricultural use of water—irrigation—has also resulted in running down of water in various natural reserves of water. Over 50 % of world's wetlands have been wiped off altogether because of the agricultural use of

water during the last century. This threat of transforming wetlands to dry lands has continued since then, and 3,500 of water bodies are still prone to risk globally. Among these, 25 % are living water bodies.

In the rivers located in the tropical regions of different countries like Asia and Africa, there is a wide range of living species including plants and animals that have managed to adjust themselves to the frequent overflowing and submerging conditions. At present, due to the construction of dams that are being mostly erected to supply water to the agricultural lands, the vast diversity of living entities are at the verge of extinction. The construction of such dams perturbs the normal flooding processes and endangers the biodiversity in these rivers. There is a discord between the two—upon the consumption of water. There is a dire need of water for human use particularly as per agriculture is concerned and for sustaining biodiversity. The Pangani River of northern Tanzania very well elucidates this point. This river flows via most biologically diverse regions of the world. These regions include the prolific fauna and flora of the eastern arc mountain and a large number of shoreline where the water from the river runs into the seawater.

The River of Pangani and its surrounding region supply water to fulfill its requirement as well as enough water buttressing biodiversity. However, this balance has been disturbed by the continuous withdrawal of water for irrigation purposes. This can be compensated with the more efficient use of water for agricultural practices. Reviewing the Pangani River example, approximately 85 % of water gets squandered on its way to the users because of various reasons such as pipe leaks and other orifices in the supply line. This situation can be ameliorated by the application of efficient water use systems as that have been practiced in Middle East where there are water availability constraints and shared water systems are very rampant (Committee on Sustainable Water Supplies in the Middle East 1999). However, present-day situation is very alarming, and solutions based on modern technology are not possible alone to solve the dilemmas occurring in agricultural practices that have impact on world's biodiversity.

9.6 Agriculture and Biodiversity: On Common Lines

This means that contemporary agricultural practices have to change to become more amiable towards biodiversity. One suggested line of action that is deemed somewhat practical is that it is for the agriculturists, farmers, and cultivators to leave some portions of land adjoining cultivated and farmed land uncultivated so that strips associated with natural habitats are maintained. To preserve biodiversity, this will serve as a region for the growth of different plant species. Another advantage of this would be that these species are going to control the erosion of soil and support pollinators and other insects. Another line of action is to use native species for cultivation. There are certain species that are leaning towards extermination by contemporary agricultural practices. For instance in West Africa, researchers are trying to persuade farmers to grow specie of the tree found in the wild commonly known as bush

mango. Researchers are assisting them to make this really practical. One thing noticeable for this specie is that the duration between planting and fruiting is just 4 years in comparison with the normal specie that takes almost 12 months. This makes this species a really appealing nominee to be made a component of the current farming practices.

Government may play an essential role in this regard as it can educate farmer to make use of things that are environmentally favorable for their land (this has been at present practiced in European Union countries). This will in turn lead to the production of somewhat lesser in quantity but sustainable yield, as mentioned earlier favoring the creation of wildlife corridors. Financial support should be provided by the government and other stakeholders for appreciating minimum use of chemical fertilizers, pesticides, and insecticides, and other chemicals should be as lessened as possible, promote hedgerow and other plants that are local to that particular area. This in turn is going to attract species of that particular locality. But obviously it is an unanswered question of how the developing countries of the world should give incentives to their farmers as they are short of funds and other resources. However, an attempt should be made in order to improve the situation. Everyone should make an individual effort to work this out and improve our surroundings.

9.7 Amelioration of Agricultural Praxis-Minimal Chemicals

Biodiversity has been threatened by agricultural praxis making use of chemicals for decades. This situation can be handled by making minifying use of chemicals. We rely on extensive use of chemicals for our good agricultural produce. One of the interesting facts and figures estimated by FAO has revealed that presently the food production has drastically doubled. The reason for this doubling is the massive use of chemical fertilizers in the world (Tilman 1999). Annually seven times rise in use of nitrogen fertilizers has been observed. The preeminent use of fertilizers has threatened the biodiversity. The massive uses of chemicals of which most of them are highly toxic are used to encounter pests in the cultivated area. It has been observed in some cases that the use of chemicals has significantly increased the yield in the fields. The use of pesticides has resulted to cause an unforeseen dilemma to the world. The haphazard, random, unselective, and unsystematic use of chemicals has led to various issues such as crop plants get resistant to pests, and they also develop the potential of resurgence, which could be very damaging.

Some of the crops get worn out due to this reason. So the crop losses due to increased pest's resistance have risen throughout the world with the growing use of pesticides (Oerke 1994; Lewis 1997). Both national and international policies are to be denunciated that they are responsible for the aggressive utilization of chemical for short-term gains. For instance, international trade organizations often support use of pesticides at subsidized rates. Nontoxic chemicals and pesticides do exist which if and when used are considered to be the safe alternatives as a substitute to these injurious and fatal chemicals and pesticides. One of these safe alternatives

includes biopesticides, which have gained support by most biologists of the world. The main components of biopesticides are bacteria, viruses, plant extracts, etc. But their use has not been in practice due to some regulatory constraints. Some international organizations only support the use of broad-spectrum chemicals.

It is a known fact that most of the developing countries of the world import these chemicals and pesticides from the developed countries of the world. This also puts a great stress on country's economy. These chemicals are used on export crops; however, their use does not improve local food security conditions. Moreover, it is found that wherever in the world these chemicals are used, they tend to move out of the farms via seepage and trickle down into adjoining areas and thus contaminate them as well. We have to search for some other ways that are environment friendly and are healthy agricultural practices. Biological control is also one of the safe alternatives in this regard. In East Africa, the destruction of nearly 80 % of maize and other crops like sorghum by stem borers is regulated by growing some particular type of grasses in the vicinity of crops. The moths that are being attracted by the chemicals secreted by the grasses are thus distracted from the maize. A unique kind of grass has been found to have a self-defense mechanism against invading pests as it secretes an adhesive and gummy material that captures the approaching insect (Khan and Mengech 2001).

Another alternative is the use of GMO crops, but its use is highly contentious and a debatable issue. It is the most hot topic encountered by the biologists, especially agriculture biotechnologists all around the world. Such crops are designed to be herbicide resistant, and moreover, they are even designed to produce their own pesticides. GMOs are also considered ominous to biodiversity by conservationists, as it can manipulate the natural genetic makeup of the plants and can lead to serious problems. Moreover, it can cause a great setback to the world's biodiversity. The development of contemporary and well-adapted agricultural techniques is the need of an hour. This includes the development of new crop varieties, various inputs, and techniques. These will serve as good sources of production but minimize the effects of farming practices on the atmosphere. For instance, the fixation of nitrogen by nitrogen-fixing bacteria and amalgamated farming techniques is found to nurture soil flora and fauna and also make sure that the nutrients remain there on the farm without trickling them elsewhere and thus avoiding the pollution of the waterways.

9.8 National Seed Policies Require Amendment

Reformation, amendment, or amelioration in national seed policies by different countries of the world on its own part is imperative. The development of biodiversity-friendly agricultural practices is the need of an hour. The diversity in different food crops as well as animals has been curtailed due to massive industrial scale agricultural and meat-producing activities. According to the recent estimates by FAO, nearly 5,000 varieties of farm animals have exterminated at present all through the world. And 30 % of those that persisted are at the verge of

extinction. There are countless grounds on which these extinctions are based upon; for instance, sometimes there are commercially only few varieties in demand. Obviously, this results in decreased interest of farmer to conserve the varieties that are not in use at that time.

In the previous times, when small-scale agricultural practices were common, seed conservation was done as olden man was well aware of the significance of different varieties of crops and vegetables. But at present neither the farmers, cultivators, growers nor the holders of agribusiness and government take the responsibility of conserving the natural ecosystem. Today in the modern world, the countries where small-scale agrarian industries are more prevalent are even intimidated by this upcoming issue. Countless perils are there in government seed policies, for instance, how the different seed dealers like farmers, cultivator, grower, or agriculturists go for the selection of seeds; how they maintain, preserve, and shelve the seeds; how they are going to market or trade a seed; or probably how they are going to opt for some seed exchange programs. In the past, it was comparatively easier for the farmer to exchange seed with each other, and thus conservation of biodiversity was achieved. But current rules and regulations enforced by world trade organization (WTO) strictly put a ban on such practices, for instance, patented seed harvested by one farmer cannot be passed on to other freely. This regulation, however, caused a great setback to the customary seed conservation method that was being in practice for years. These policies require to be ameliorated, and if amelioration is not possible, they are to be repealed and new biodiversity-friendly policies should be made.

9.9 Agricultural Policies Influencing Biodiversity in Europe

In the wake of World War II, the European Union Common Agriculture Policy (CAP) in 1962, at present, is considered as a great setback to biodiversity by conservationists. This policy promoted plenteous and profuse use of agriculture in order to combat upcoming shortage of food. In consequence of World War II, issues regarding food security were roaring in the air, so this policy devised intensive agriculture practices using modern techniques to prevent their nations from hunger, but in doing so they did not pay heed to one of the underlying damage that was prodigious damage to biodiversity and ecosystems serving us from millions of years.

However, in the mid-1980s, there has been an increasing demand for amelioration of CAP policy and encouraging environment-friendly policies and trends, which are expected to continue even more. To enhance and implement biodiversity-friendly approaches, incentives were given to the farmers in European countries (Donald et al. 2002). Agri-environmental schemes have been launched to pragmatically ensure biodiversity-friendly attitude and thus conserving our natural ecosystems, which are imperative for our survival and existence. Although the agri-environmental schemes in the UK are effective as per biodiversity is concerned

(Carey et al. 2005; Evans 1997; Hanley et al. 1999; Hodge and McNally 1998; Wakeham-Dawson and Aebischer 1998), however, they are practically implemented only exceptionally limited and constrained manner.

The conservation of agricultural as well as wild biodiversity is essential for normal and healthy activities of human beings but also for the sustenance of the natural ecosystems. A plethora of details are there in the world scientific literature, and also in our discussion as well that agriculture, agricultural pollution, and biodiversity are totally in contrast with each other and their coexistence seems to be impossible and an unattainable objective yet, it is apparent that by collective efforts of agriculturists, farmers, law makers, and conservationists, both can be brought up on common lines. However, still if no heed is being paid on this issue and negligence is being continued as such by all the concerned parties, then certainly biodiversity is jeopardized (Walcott 2004).

10 Agricultural Sustainability

According to Malthusian view, the rate of increasing number of people is more than the earth's space. And this would lead towards environmental destruction and issues of food security. The current population of planet Earth is approximately six to seven times more than the population number in the times when Malthus had given his prediction about the principles of population growth. So, the agricultural sustainability is the need of the hour to ensure solution of problems like food security and ecological degradation, because these issues are directly linked to the agricultural sustainability. The threats to agricultural sustainability can also arise due to the perception of only getting immediate profit and production motives. For agricultural sustainability, the plans should be designed to get production benefits in the long run. For instance, there should be precautionary measurements for conservation of soil fertility and soil structure in the land used for cultivation. We can include cultivation of such pulses and *Sesbania rostrata* in the crop rotation sequence, which can fix soil nitrogen for the replenishment of nitrogen source in the soil. The agricultural sustainability can only be achieved, if we give priority to the long-term benefits of ecological stability and conservation of biodiversity over short-term benefits of yield gains and commercial goals. In short, the agricultural sustainability means the productivity in continuity without any fatigue, and for this the agricultural practices should be eco-friendly (Kesavan and Swaminathan 2008). The practices that are required for agricultural sustainability should have three characteristics: no harmful effects on ecosystem, easily available for farmers, and increase in food production (Pretty 2007). The herbicides with novel characteristics should be produced to overcome the resistance of weeds to already available herbicides (Duke 2012). Today food security is a major problem. To solve this problem, sustainable food production and organic farming can be proved very helpful (Paoletti et al. 2010).

10.1 Agricultural Waste Management

The agricultural waste management is a complex of many things like the waste treatment, the patterns used for agriculture, and the rural progress. In addition to farmers, a number of other parties have also an important role to play in agricultural waste management. A variety of nonnatural wastes are produced by agricultural industry which includes agrochemicals like pesticides, fertilizers, silage films, horticultural films, unused medicines, syringes, needles, and machinery wastes. These nonnatural agricultural wastes are associated with the environmental and human health hazards. So, these wastes should be treated and recycled to control the agricultural pollution. Another way is the consumption of reusable containers. A comparative study on the strategies of waste management has been done between European region and five other countries (Sakai et al. 2011). According to a research performed in Indonesia, Cassava waste pulp can be used as a good superabsorbent. Cassava is an important crop grown in Indonesia. It is modified by copolymerization to be used as a superabsorbent (Mas'ud et al. 2013).

10.2 Pest Management

Pesticides are applied to control the pests like weeds, insects, and diseases because these pests are the cause of reduction in agricultural yield. On the other hand, the use of these pesticides is a source of agricultural pollution. So, it is essential to design, establish, and implement such technologies, which have application of precision agriculture to pest management. The feasible use of pesticides can be helpful in preventing human diseases and ecosystem destruction (Rossi et al. 2012). An experiment has been performed in which oils are extracted from leaves of two plants (*Haplopappus foliosus* and *Bahia ambrosioides*) and then tested for their insecticidal activity against housefly (Urzúa et al. 2010). Another study has been done which describes the role of DNA-tagged nanoparticles of gold in controlling pests (Chakravarthy et al. 2012).

10.3 Soil and Water Quality

The properties of soil and water are interlinked with each other in a way that if one thing is in poor condition, then the other must be affected. Healthy soil keeps the water clean, and similarly clean water keeps the soil in a healthy condition. The soil quality is defined as the amount of soil fit for sustainable agricultural production. The water quality is assessed by the quantity of hazardous chemicals and sediments present in the water. Again the soil and water quality can be maintained by the eradication of excess pesticide and fertilizer use. The industrial effluents also have to be

properly disposed and not poured in the water used for agriculture purpose. According to an estimate by the Agricultural Department in the USA, the contamination by pesticides has the potential of affecting 54 million people who use this water for drinking purposes.

10.4 Eco-agriculture

The eco-agriculture is the combination of agricultural sustainability and biodiversity conservation. Investments need to be done to produce the methods and technologies that can enhance output and decrease their costs, while conserving the biodiversity. It is also essential to provide opportunities to the farmers for increasing eco-agriculture proportionally. Otherwise economic and social problems faced by farmers will affect the biodiversity conservation. There should be a sufficient number of community-level organizations for the eco-agriculture. The strategies should be made to acquire adequate benefits from eco-agriculture. Another important aspect is to produce and share knowledge about eco-agriculture. The priorities have to be set for establishing an understanding of interactions between conservation and production areas. For an eco-agriculture system to be most successful, fully documented information is needed. The linking of biodiversity conservation with sustainable agriculture is very important to bring the eco-agriculture at a global level. Unfortunately, the current knowledge is not sufficient for the purpose noted above. It is required to consider and coordinate the research and policy communities for the development of such strategies which can overcome the challenges of the twenty-first century (Scherr and McNeely 2008).

10.5 Manure Recycling

Farm animal feces and urine together are called as excreta, which can be used to produce manure. The benefits like supply of nutrients to crops, refining soil structure, and moisture-absorbing ability of the soil can be obtained by using manure. If the excreta are collected in semiliquid form, then it is called as slurry. Slurry is also used like manure to increase the soil fertility. Using nitrogen present in excreta can do the nitrogen cycling in the environment. Based on the variation of species, the nitrogen content of manure and slurry can also differ. This is due to different strategies of excreta production and storage in different species of animals. When animals are grazing, about half of the excreta from cattle are dropped on the grass. The remaining half is collected in the houses. Due to storage of manure and slurry, nitrogen losses can occur because nitrogen may be released from slurry to the air in the form of ammonia gas. Moreover, if the slurry is mixed to avoid the hard covering formation or to reduce the smell problems, aeration is done. These all can cause losses of nitrogen. The availability of nitrogen in excreta

returned to land does not remain hundred percent till the season of crop cultivation since nitrogen can be lost from the soil by evaporating as ammonia in the air or by combining with the organic matter of soil. Due to droppings of excreta produced during grazing in the winter season, the much of nitrogen is washed into the soil and remains available to the crops for a long period of time. The best response of crop to the nitrogen content is attained with application in the spring season. But nitrogen in the organic form may be unavailable to plants even in spring season. Some amount of this nitrogen can benefit in the coming years. It is evident that in some conditions diluted slurry applied to grass may be more resistant to the nitrogen losses than concentrated slurry. In autumn and early winter, the loss of nitrogen most probably occurs by dissolving in the rainwater (Gostick 1982). In China, a study was done on beneficial effects of leaf dew as a natural fertilizer. It gives many important nutrients like nitrogen and phosphorus to the plants and helps in improving their growth (Xu et al. 2013).

10.6 Compost Application in a Cropping System

The agricultural pollution can be prevented by using compost in the cropping system. The compost is produced from manure and other agricultural by-products. It has many advantages like reduction of soil erosion, refinement of soil texture, and decreased use of fertilizers. Its most important functions are to provide nutrient source and to suppress plant diseases. Compost prevents soil erosion as it provides a structure to the soil to which it is added. It has beneficial microorganisms that actually work in suppressing the plant diseases. In different places, different volumes of compost are used to attain the same desired results.

11 Conclusions and Future Perspective

Three "P"s of agricultural Policy, agricultural Production, and agricultural Pollution are correlated with each other. It is considered that agriculture is affected by the environmental pollution, but there is always the other side of the story too. Two aspects of agriculture have been discussed in detail. One is the pollution caused by the agriculture and the other is the impact of pollution upon agriculture. It has been seen that there is a complex relationship between the two and the resulting consequences indicate that it is difficult to handle such complications. There is no doubt that agriculture sector plays an important role in the economy and food industry of a country. Many kinds of staple crops, grains, and fruits are being produced from this sector, which are making major share in the export industry. But with the passage of time, this sector is becoming troublesome for the surrounding environment. Agricultural pollution not only affects air, water, and soil, but problems related to health and biodiversity have also been observed through the use of fertilizer,

pesticides, organic matter, and greenhouse gas emissions. There will be an alarming situation when agricultural pollution will minimize the agricultural yield itself.

There is an increasing public concern regarding agricultural pollution and its impact on the environment. There is a need to maximize the agricultural production to overcome the increasing demand of food. Nowadays, farmers are using new techniques to increase the crop productivity and quality, but despite all this, this industry is not following rules and regulations that have been implemented in other industries. So there should be a primary focus to strengthen the regulatory programs to prevent the agricultural pollution and its drastic effects on the environment. Proper policies should be made on local to global level to minimize its effects on our surroundings and to improve yield, quality, the agricultural practices, and the well-being of humans and biodiversity.

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