Sustainable Development and Biodiversity 4

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Biotechnology and Biodiversity

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Preface

Biodiversity is a contraction of the term 'biological diversity', and refers to the diversity of 'life'. The purpose of this book is to assess the potential effects of biotechnological approaches particularly genetic modification on biodiversity and the environment. All aspects of biodiversity such as ecological diversity, species diversity and genetic diversity are considered.

With the introduction of genetically modified crops, monoculture of transgenic crops in a large area, and heavy use of chemical fertilizers and pesticides have been shown to have impacts on biodiversity. The introduction of a new trait in a genetically modified plant will depend on many other factors, including whether the introduced gene (transgene) responsible for the trait is turned on (expressed) or off, in the specific cells, and how the transgene expression, and the gene product(s) interact with environmental factors. The main issue in plant biotechnology which concerns us is that genetic manipulation has a direct impact on biodiversity at the genetic level. By these genetic manipulations, novel genes or gene fragments can be introduced into organisms (creating transgenics), or existing genes within an organism can be altered. Transgenics are a major area of concern, which effectively combine genes from different species to effectively create novel organisms. Current rates of disappearance of biological and cultural diversity in the world are unprecedented. Intensive resource exploitation due to social and economic factors has led to the destruction, conversion or degradation of ecosystems. Reversing these trends requires from time to time assessment of loss of biodiversity, and forces us to integrate conservation strategies. Biotechnological tools, particularly micropropagation technique has been helpful in developing protocols for multiplication of economically important plants, as well as endangered and threatened species. Chapters in this volume are written by leading scientists in their fields of specialization that include Impact of transgenic crops on biodiversity, impact of transgenes on non-target species, biotechnological applications to threatened and endangered species, pteridophytes, conifers, non-conifer species of gymnosperms, tree species, genetically modified crops, cryopreservation of diverse species, conservation of forest resources, agricultural biotechnology relevant to health and the environment, and prospects of next generation biotechnology for the production of biopharmaceuticals, biofuels, bioplastics, and biofortification of staple food crops.

We believe that biotechnology can affectively solve the problems related to biodiversity management, protection and conservation. The field in plant biotechnology has been kept wide and general to accommodate a wide ranging topics. This book provides complete, comprehensive and broad subject-based reviews useful for students, teachers, researchers, policy makers, conservationists and NGOs for environmental protection, and others interested in the field of biotechnology and biodiversity.

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Part I
Section A: Genetically Modified Plants and Biodiversity

Chapter 1
Transgenic Crops to Preserve Biodiversity

Chandrakanth Emani

Abstract The rapidly expanding field of commercial transgenic cultivation has its greatest concern related to environmental well being as transgenic crops are seen as a threat to the biodiversity in the agricultural fields. Since transgenic technology is continuing to witness a rapid growth in terms of developing novel varieties, it is imperative to examine whether the developed varieties contribute to preserving biodiversity. Further, it is also necessary to focus future research towards developing transgenic varieties to contribute to preserving and enhancing the biodiversity. The present review aims to present an overview of the current status of transgenic technology in contributing to biodiversity and suggest future research strategies enabling the preservation of biodiversity.

Keywords Crop biodiversity · Transgenic crops · Speciation · Non-target species

1.1 Introduction

Biodiversity as a term is a shortened version of 'biological diversity' where 'diversity' as a concept encompasses the range of variations or differences among entities that have a distinct and independent existence. Thus, biodiversity can refer to the number, variety and variability of living organisms (Groombridge and Jenkins 2002). A broader reference to biodiversity can be as a collective indicator of the numerous forms of life and of ecological habitats on planet earth (Convention of Biological Diversity 2000), where it is a crucial factor that affects both the survival and welfare of our existence as a species. To effectively manage and conduct both qualitative and quantitative research on the various facets of biodiversity, three fundamental hierarchies were delineated as: *genetic diversity* (representing the heritable variation between population of organisms in terms of their DNA base pairs and the genetic code); *species diversity* (representing the 12.5 million global species

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in different taxonomic groups of which 1.8 million species have been described to date) and *ecosystem diversity* (representing the abiotic components determined by soil parent material and climate) (Groombridge and Jenkins 2002).

Human intervention has been responsible for the continual degradation of biodiversity and this led to numerous economic, environmental, and social consequences. The failure to preserve our natural biological resources especially the diverse plant life on which we depend for food, clothing, pharmaceuticals, and more recently energy in the form of biofuels is indicative of a fact that we may be losing potentially beneficial compounds and materials that have not yet been discovered from natural resources (OECD 2005). More recently, research has shown that climate change is having a significant effect on the world agricultural output and thus directly influences world food security (White et al. 2004). For decades, the development of novel crops by both conventional breeding as well as biotechnological crop improvement strategies had a direct influence on world food security (Kropiwnicka 2005). Plant biotechnological strategies that focused on the development of improved high-yielding and disease resistant crop varieties were a result of collaborative efforts between conventional and molecular breeders (Van Buerren et al. 2010). A logical continuation of such research collaborations can now culminate towards focused research studies resulting in preserving and enhancing plant biodiversity.

Plant biotechnological research was witness to many path breaking and application-oriented technologies (Kumar et al. 2009) and it sometimes is a challenge for current researchers to identify contextual research strategies without getting lost in the diverse array of biotechnological strategies that unfold every year. In order to develop novel strategies to preserve plant biodiversity (Pijut et al. 2011), researchers will be best advised to focus on certain crucial aspects of plant molecular biology to better utilize the tools of biotechnology that are available.

1.2 Transgenic Crops

Transgenic crops are a result of the process of *transgenesis* (also referred to as plant genetic engineering) that involves the introduction of a desirable exogenous transgene in crops and ensuring their stable expression in the offspring. The process of transgenesis relies on stable integration, desired level of expression, and predictable inheritance of the introduced transgenes. In the context of introducing novel crop varieties and the strategies that enable introduction and expression of genes of interest across any crop species, transgenic technology is now being hailed as a gene revolution that is similar to the plant breeder-pioneered green revolution of the 1950s (Pingali and Raney 2005; Jain 2010). Green revolution owed its success to identifying strategies that effectively disseminated novel cultivation techniques and distributing the resulting improved germplasm freely to targeted farmer populations thus working towards greater public wellbeing (Jain 2010). This resulted in increased staple crop yields and a simultaneous price decrease that benefitted farmers in Asia, especially for crops such as rice and wheat among farming communities of Asia (Barta 2007). Comparison of the green revolution to the transgenic technology

and christening it as a gene revolution has to overcome the purported empty rhetoric to a concerted identification of strategies similar to the success of green revolution related to the increased yields and the resulting economic benefits to farmers. Further, the major limitation attributed to green revolution in terms of increased monocultures that allegedly led to a decrease in biodiversity may also be a debatable issue. Transgenic research is also seen largely as a private enterprise with varieties available to farmers only on market terms (Pingali and Raney 2005) and this may jeopardize its cause to contribute to increasing biodiversity.

1.2.1 The Current Status of Transgenic Crop Cultivation

Transgenic crops (or the popular term attribute to them being genetically modified crops or GMOs) are increasingly becoming a common feature of cultivated landscapes with the total plantings seeing a significant increase from 3 million hectares in 1996 to 67.5 million hectares in 2003 (James 2003). Transgenic technology has also been showcased as having a positive impact on commercial farming (Carpenter 2010) and as an effective research alternative to meet to the global needs of food security (Schiøler and Pinstrup-Andersen 2009). The pioneering efforts as exemplified by the herbicide-tolerant soybean (Carpenter and Gianessi 1999), insect tolerant Bt-corn (Hilbeck 2001) and the more recent successes of the genetically engineered-vitamin A fortified "golden rice" (Beyer 2010) has led to the approval of a new generation of transgenic crops that produce health benefitting vitamins and vaccines, and economically important enzymes and industrial products (IUCN 2007). The rapidity with which over 16 million farmers of the global agricultural community has taken up the transgenic technology has surpassed all innovative agricultural practices of the past 80 centuries (James 2011; Lawson et al. 2009). Since the first commercial transgenic crops of China of 1992, the countries that rapidly adapted the transgenic crop cultivation were the USA followed by Argentina, Brazil, Canada and India (GM Compass 2009) with the total transgenic crop cultivation area registering an increase from 134 million hectares in 2009 to 160 million hectares by the end of 2011(James 2011). The four decade old global biotechnology industry has the United States leading the world in the rapidity of transgenic technology acceptance with proportions of major transgenic crops as high as 73 % in maize, 87 % in cotton and 91 % for soybean (USDA 2007/2010). In contrast, the opposition to transgenic technology has been so severe in the European Union with only 114, 500 ha that accounts for less than 0.01 % of European agriculture area mostly in Spain cultivates *Bt*-maize (James 2011).

1.2.2 The Advantages and Limitations of Transgenic Technology

The major advantages of transgenic technology as put forth by the proponents of biotechnology center around the careful and planned introduction of insect and herbicide resistance into arable land that would reduce the crop losses due to weeds,

insect pests, bacterial and viral pathogens, thus providing an environmental-friendly agrochemical free atmosphere (Krimsky and Wrubel 1996) that will eventually contribute to a sustainable agricultural environment (Braun and Ammann 2003). This optimistic view is not shared universally across the plant biology scientific community, where certain researchers compare the embrace of transgenic technology without proper methods of risk assessment to the pesticide overuse of the twentieth century agriculture (Krebs et al. 1999; Herren 2003).

Herbicide resistant sugar beet were shown to receive lesser number of herbicide sprays in farm-scale evaluations (Champion et al. 2003), but the same was not observed in case of transgenic oilseed rape and maize. However, research in developing transgenic herbicide varieties has been seen as having a significant potential beneficial effect on environment and biodiversity as the reduction in pesticide use would reduce the pesticide-induced mortality of natural enemies, a key aspect of conservation biological control and integrated pest management (Barbosa 1998; Gurr et al. 2003).Transgenic herbicide resistant crops also aided in creation of precise patterns of weed strips connecting field margins with field interiors, beetle banks, and networks of habitat corridors favoring beneficial arthropods that enabled farmers to develop easier weed management (Garcia and Altieri 2005). Simplification of farming practices has a direct effect on increasing agricultural efficiency in terms of increased yields and profits.

However, the limitations of transgenic plants if looked at through the lens of biodiversity can be summed up as those associated with ecological processes that have an impact on operation and molding of agrosystems (Garcia and Altieri 2005). Some of the major limitations can be viewed as under:

a. The spread or acquisition of transgenes to wild or weedy relatives leading to what sometimes are termed super weeds that may lead to reduction or increase of the fitness of non-target weeds or local varieties and also selection of more herbicide-resistant and noxious weeds.
b. The evolution of resistance of insect pests to transgenic insect toxins and the accumulation of transgenic toxins, which remain active in the agricultural land.
c. The disruption of natural control of insect pests through intertrophic-level effects of the transgenic insecticidal toxins and the unwarranted effects on non-target herbivorous insects.
d. The transgenic vector-mediated horizontal gene transfer and an uncontrollable recombination that will lead to creation of new pathogenic organisms.
e. The escalation of new herbicide use in herbicide-resistant crops that lost their originally introduced resistance crops that may lead to environmental impacts including reduced weed populations and in turn plant diversity that may also result in.
f. The indiscriminate reduction of weed populations due to uncontrolled herbicide sprays in fields planted with herbicide-resistant transgenics leading to declines in bird populations that feed on or shelter in weeds or feed on the arthropods supported by weeds.
g. The reinforcement of genetic homogeneity and promotion of monocultures that would result in crops vulnerable to climate change, pests and disease.

1.3 The Impact of Transgenic Crops on Biodiversity

In the present agricultural scenario, the most direct negative impact on biodiversity is the conversion of natural ecosystems into agricultural land that has a tremendous environmental impact in terms of a significant loss of natural habitats. In overcoming this environmental hazard, transgenic crops have had a significant role to play in the past decade with their potential to increase crop yields, decreasing insecticide use, promoting the use of environmentally friendly herbicides and the facilitation of conserved tillage practices (Carpenter 2010, 2011). The efficient utilization of transgenic crops has a beneficial trade-off in terms of the requirements for lesser land to produce high yielding pest and herbicide-resistant varieties as compared to traditional crop practices that are low yielding extensive agricultural systems requiring more land and pesticide usage. This will also free more land that would otherwise be forcibly be converted to agricultural land, thus minimizing the negative impacts of biodiversity on non-arable lands. Many recent reviews have focused on both well-researched as well as hypothetical scenarios of transgenic crop cultivation related to their impact on biodiversity (Garcia and Altieri 2005; Raven 2010; Carpenter 2011; Jacobsen et al. 2013). The present review attempts to focus on an overview of the most important factors of transgenic crop effect on biodiversity and suggest some application-oriented research strategies.

1.3.1 Impact on Speciation and Impact on Traditional Crop Cultivation

One of the biggest concerns expressed against transgenic crops is their potential to reduce species abundance or the levels of genetic diversity within cultivated varieties that include traditional land races as the focus will be on a small number of high value cultivars (Ammann 2005). Initial studies conducted on transgenic cotton related to field genetic uniformity (Bowman et al. 2003) showed a significant reduction in uniformity as compared to a study conducted on conventionally bred glyphosate tolerant cotton (Sneller 2003) that showed no little impact on diversity. However, the scope of examining the consequences of transgenic varieties on diversity needs to be expanded to consider the impacts at three levels, namely, the crop, farm and landscape levels (Carpenter 2011) to accommodate all the levels of agricultural biodiversity from the gens to the ecosystems. When examined under this umbrella, it is seen that transgenic crops increase the crop diversity by enhancing underutilized alternative crops and making them widely domesticated (Gressel 2008) as seen in orphan crops such as sweet potato (Bhattacharjee 2009). In case of impacting farm-scale diversity, transgenic crops had no significant effect on non-target soil organisms and weed communities (Carpenter 2011). At the landscape level, introduction of transgenic corn, soybean and canola resulted in reduction in encroaching into non-arable lands, and also helped in environmental friendly expansions of arable lands (Bindraban et al. 2009; Brookes et al. 2010; Trigo and Cap 2006).

Further, an area-wide suppression of target pests by Bt corn and cotton led to pest management benefits in other cultivars such as soybean and vegetables (Carpenter 2011). Research over the past decade has shown that the commercial cultivation of transgenic crops had a positive impact on biodiversity through increased yields that resulted in an alleviation of pressure to encroach on non-arable land, increased use of environmentally friendly herbicides, reduction of insecticide use adoption of conserved tillage and a general increase in agricultural sustainability (Carpenter 2011). An enhanced knowledge on the part of farmers who are now better educated in agricultural practices also takes care of the fact that the purity of traditional varieties will be maintained as most of the farming community grow both traditional and improved types in the same field (Bellon and Berthaud 2004). In fact, the situation faced due to transgenic crop commercialization is no different from the times when agriculture was exposed to conventionally bred commercial crops (Ellstrand 2001). There is a mechanism in place to study the impact of transgenes in the form of an environmental impact statement requests by the USDA (Aphis-USDA 2007) that precedes the release of a new transgenic plant.

1.3.2 Impact on Natural Environment and Populations

Mathematical modeling predictions showcased an undue ability for herbicide tolerant transgenics in affecting the diversity and numbers of natural organisms that play an important role in controlling pests and diseases (Watkinson et al. 2000). The persistence of a transgenic plant released into the natural environment and any unforeseen competition with native species is dependent on the introduced transgene's invasiveness (Conner et al. 2003; Hancock and Hokanson 2001). A proper framework in assessing the stable and predictable transgene expression that enables a transgenic crop to grow better in an environment would focus on its ability to outcompete a wild species in terms of crucial factors such as reproductive success as it would be a measure of a decrease in biodiversity (Hancock 2003). Studies till date that monitored engineered traits such as insect resistance (Romeis et al. 2008) and stress tolerance (Nickson 2008) have not shown any evidence in terms of the transgenic crops invading unmanaged habitats or outcompeting wild species. Monitoring agencies mandate a strict contextual environmental risk assessment of every transgenic crop (Hancock 2003) and tiered tests are in place to assess the potential environmental risks related to fitness changes in hybrids between transgenic crops and wild relatives (Raybould and Cooper 2005). In UK, a well researched study to quantify the effects of herbicide tolerant crops such as sugar beet, maize and oilseed rape on bird and animal populations was carried out, where the impact of transgenic and conventional herbicide tolerant crop was compared. A 5 year study of 266 field trials showed a non-uniform pattern (DEFRA 2007) where in case of sugar beets and oil seed rape, the conventional varieties harbored more insects due to presence of weeds. In case of maize, there were more weeds and the late herbicide application resulted in more butterflies and bees. The differential environmental effects

were attributed to new weed control strategies practiced by farmers as opposed to the mere presence of the transgenic crops (Lemaux 2009)

1.3.3 The Myth of GM-Induced Superweeds

The potential scenario of a transgenic herbicide tolerant trait introgressing into a native wild relative gave rise to the concept of a hypothetical superweed that can take over entire ecosystems and be completely resistant to existing herbicides (Chapman and Burke 2006). This challenge, though not based on fact, was not limited to transgenic crops but was first observed as a gene flow from conventional domesticated herbicide tolerant crops to its wild relatives (Ellstrand et al. 1999; Itoh 2000). This never led to the exaggerated scenarios of environmental disasters, but only limited the effectiveness of existing weed control strategies and hampered weed management options. Conventional breeders did manage the situation efficiently with strategies such as revolving dose sprays to effectively delay the evolution of both quantitative and major monogene resistance traits acquired within field populations (Gressel et al. 1996; Gardner et al. 1998). Evidence for herbicide tolerance trait tolerance from transgenic crops resulting in resistant weeds was seen in well documented studies (Watrud et al. 2004; Nandula et al. 2005; Warwick et al. 2008; Lemaux 2009). As in conventional breeding, this could be still be attributed to a single herbicide overuse (Lemaux 2009), and this was effectively overcome with developing transgenic crops that have herbicide tolerance with alternate mode of action that can be used in crop rotations to slow the resistance in weeds (Behrens et al. 2007).

1.3.4 Impact on Non-target Species

Transgenic crops could potentially impact unintended target species such as beneficial pollinators, soil organisms and endangered species and such indirect effects were seen as threats mainly from the Bt crops (Marvier et al. 2007; Duan et al. 2008; Wolfenbarger et al. 2008). However, this concern could be equally applicable to both transgenic crop fields as well as fields sprayed with conventional pesticides (Whitehouse et al. 2005; Cattaneo et al. 2006). As with most challenges in a transgenic field, the potential for the negative impact on non-target species is assessed closely monitored by regulatory agencies before commercial approval as can be illustrated with Bt cotton trials in India (Bambawale et al. 2004) and China (Pray et al. 2002; Huang et al. 2002, 2005) where a tiered approach focused on a comprehensive risk assessment related to the introduced transgenes and direct exposure to their expressed products and the indirect exposure through feeding patterns and accumulation of expressed gene products in the release environment. No significant differences were observed between conventional and transgenic fields and the studies indicated that Bt cotton cultivation had an overall beneficial effect on biodiversity as compared to regular applications of insecticides (Pray et al. 2002; Gepts 2004).

1.4 Harnessing Transgenic Technology to Promote Biodiversity

Transgenic technology has been successful in pyramiding beneficial genes into single crops (Zhao et al. 2003), introducing single genes to combat multiple stresses (Kasuga et al. 1999) and the ability to develop environmentally friendly disease resistance (Lorito et al. 1998; Emani et al. 2003). As an effective tool to increase biodiversity, transgenic technology has to now focus on identifying effective strategies both in identifying novel genes, model crop systems and effectively exploit the expanding applications of bioinformatics and systems biology.

1.4.1 Identifying Effective Experimental Model Crop Plant Systems

The identification of model plant species to study the genetic, biochemical and molecular basis of plant biodiversity is still not fully realized. Plant molecular biology has christened *Arabidopsis thaliana* as a model system due to its complete genomic sequence in the public domain, easy transformation protocols, short generation times, availability of expressed sequence tags (EST), microarray and proteomics data, and a large set of well-characterized mutants as exemplified by the *Arabidopsis* information resource (TAIR) database (http://www.arabidopsis.org). Efforts geared towards identifying model plant systems by utilizing the molecular and genetic data available on TAIR through bioinformatic analyses can be a good starting point for researchers. The successful completion of complete genomes of rice (Yu et al. 2002; Goff et al. 2002) and sorghum (Paterson et al. 2009) and more recently banana (D'Hont et al. 2012) opens the doors for analyses of specific genes as illustrated by studies made in *Arabidopsis* (Denby and Gehring 2005). A comprehensive database for model experimental plants among edible crops, forest species, pharmaceutically important plants and biofuel crops aimed at data related to transformation protocols, ESTs, microarray, experimental mutants, transcriptome and proteome data in line with the TAIR database will be an effective resource for breeders aiming to develop novel plants to preserve biodiversity.

1.4.2 Plant Databases for Targeted Gene Discovery

Existing databases such as TAIR (http://www.arabidopsis.org), Gene Ontology (http://www.geneontology.org), Plant GO slim (http://www.geneontology.org/GO.slims.shtml) and the more recent TRY (http://www.try-db.org) with over 3 million trait records for 69,000 plant species with the integrated whole genome profiling information can be utilized towards concerted efforts aimed at identifying a vast number of target genes related to preserving plant biodiversity. The identified target

genes with crucial molecular functions related to preserving and enhancing plant biodiversity can then be evaluated for their biotechnological potential by genetically engineering them into popular cultivars and forest species.

1.4.3 The World of Small RNAs

A more recent development in the form of the discovery of microRNAs involved in an array of molecular processes in popular crop plants such as rice (Li et al. 2010) and sorghum (Zhang et al. 2011) is opening an entirely new and effective field of plant molecular research (Nelson et al. 2003). Techniques are now available in designing and silencing miRNAs for various traits across plant species (Ossowski et al. 2008; Warthmann et al. 2008; Schwab et al. 2006) and a dedicated web site makes the technology readily accessible (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). Researchers can effectively use the technology to design artificial 21-mer microRNAs (amiRNAs) that can be genetically engineered and function to specifically silence single or multiple genes of interest across plant species according to the previously determined parameters of target gene selection.

1.5 Conclusion

Charles Darwin in his monumental work *The Origin of Species*, focused on biodiversity to unravel the biological mechanics behind the rich variety of life forms on our planet (Darwin 1859). Darwin attributed the evolution of diverse species on earth to the ability of plants and animals adapted to their environment to breed and pass on their characteristics to their offspring. In the revolutionary conclusion to his classic theory, Darwin reflected on the crucial principle of the importance of relationships between species and contemplated an "entangled bank" where various life forms live in unison. This unraveled the fact that no species including our own *Homo sapiens* can exist in isolation from other living things. Every species on earth is dependent on natural processes for its own survival and in doing so contributes to the natural balance of the environment that translates into the very survival of our planet. We as human beings can thus be the agents of change to the preservation as well as degradation of the rich biodiversity on our planet. With the ever growing field of plant biotechnology that has a diverse array of technological applications to choose from, a planned contextual strategy to best utilize the available state of art techniques will richly benefit future researchers and their studies. The planned research strategies will help fulfill our existence as a human race in being the very agents of change that are responsibly preserving and enhancing the rich plant biodiversity to benefit planet earth, while systematically exploiting its resources to better our lives.

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Chapter 2
Genetically Modified Crops in Africa

Georgina D. Arthur and Kwasi S. Yobo

Abstract Hunger and malnutrition are flammable pertinent issues that hinder progress of a nation and become an increasing risk. Biotechnology and food security have very good relationship both in the present and the future, concurrently embracing technology that offer new opportunities with increase crop and animal production. Additionally, they offer capacity building, collaboration, research and ensure sustenance. There is the need to engage and address exploration of new techniques and encourage various scientific and community debates with the support of respective governments. The way forward is to review biotechnology tools including biosafety processes, policies and proper implementation to sustain biodiversity.

Keywords Biotechnology · Environmental safety · Africa · Food security · Biodiversity · Genetically modified organisms · Biosafety

2.1 Introduction

West Africa, East Africa Central Africa and Southern Africa form the Sub Saharan region of the African continent. The majority of Agricultural practices in this region are characteristic of typical developing region where agriculture is still an economic backbone. The agriculture economy employs about 60 % of the workforce and contributes an average of 30 % of gross domestic product (USAID 2003). Agricultural growth rates for SSA declined in the 2000s and food insecurity is still a concern, as the prevalence of malnourishment has only dropped from 34–30 %

Dedicated to all who are pursuing to sustain biodiversity

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in two decades. Agricultural development in sub-Saharan Africa (SSA) also faces the daunting challenge of climate change and increasing climate variability in most vulnerable areas (Thornton et al. 2011). The productivity of crops grown for human consumption in SSA is very much at risk due to the incidence of pests, especially weeds, pathogens and animal pests (Oerke 2006).

Staple crops cultivated by means of organic methods such as the cassava, maize, sorghum, millet, cowpea, groundnuts amongst others are at risk due to crop pests and their destructive tendencies that compromise high yields. Soil nutrient depletion is considered as the biophysical root cause of declining per capita food production in sub-Saharan Africa. According to Drechsel et al. (2001) data collected in 37 countries in SSA confirm a significant relationship between population pressures, reduced fallow periods and soil nutrient depletion including erosion. Another factor that is placing immense pressure on agricultural lands and crop yields per hectare is the increasing human population in Sub Saharan Africa. According to the United Nations (2011) fertility in SSA stood at 5.1 births per woman between the periods of 2005–2010. This high fertility combined with declining mortality has resulted in rapid population growth of 2.5 % per year. The UN projects the sub-Saharan population to grow from 0.86 billion in 2010 to 1.96 billion in 2050 and 3.36 billion in 2100. Bongaarts and Casterline (2013) noted that most important step required to make progress in addressing high and unwanted childbearing and rapid population growth is for policymakers in Africa to realize that the current demographic trajectory is a major obstacle to their countries' development. With respect to increasing population densities, it is argued that more than proper soil management will be required to sustain food security (Drechsel et al. 2001). Since SSA maintains the highest proportion of malnourished populations in the world, with one in three people chronically hungry, it is believed by GM supporters that it is through the formalised implementation and cultivation of GM crops, that crop losses can be curtailed. GM crops are defined in this chapter as new varieties of crop species developed by molecular modification through the insertion of foreign genetic materials (Jacobsen et al. 2013). Although only South Africa and Burkina Faso are the countries in SSA to have formalised the implementation of GM crop cultivation, countries such as Kenya, Ghana, Nigeria, and Uganda are involved in developing GM crops that are drought resistant, pest resistant, efficient water use, nutrient rich and high yield than conventional crops. This chapter describes the critical cogitations of GM crop in Africa to assess progress in various countries with an objective of the prospective role that GM crops can play in achieving better food security in the sub region of the African continent.

2.2 What is Biotechnology?

Biotechnology is not new to mankind and has been around for thousands of years in which mankind has been cross breeding and manipulating living organisms to meet his own dietary and industrial needs. Food fermentation for example is evidence

of one of the oldest known uses of biotechnology (Campbell-Platt 1994). The baking of yeast-leavened and sour dough breads also represents one of the oldest biotechnical processes, together with the brewing of beer, wine, and the production of yoghurt and cheese (Fleet 2007). These forms of biotechnology are labelled as "traditional".

Current process of biotechnology in its widest sense makes use of the improvement of cereal grains and starter cultures by recombinant DNA technology, through the use of enzymes as processing aids, to application of the most advanced batch and continuous fermentation technologies (Linko et al. 1997). Modern methods of biotechnology for the purpose of altering or modifying the genes of organisms include; Red biotechnology, which involves the medical processes, white Biotechnology which is also known as Gray Biotechnology, which is used for the industrial processes, Green Biotechnology which involves the processes and development of pest-resistant crops and disease resistant animals, and finally, Blue Biotechnology which is used for marine and aquatic processes (DaSilva 2004). Biotechnology also includes the application of a wide variety of biological, biochemical, bioengineering, genetic, microbiological and control techniques. Undeniable evidence in the form of vast bodies of scientifically proven literature demonstrates that biotechnological tools such as tissue culture, genetic engineering and molecular breeding (marker-assisted selection) continue to provide promising opportunities for achieving greater food security while improving the quality of life (ISAAA 2009). Crop genetic engineering process shown in Fig. 2.1

2.3 Benefits and Concerns in SSA

The use of genetically modified (GM) crop technology in tackling food security problems and poverty reduction in Africa (south of Sahel) has been debated upon countless occasions. Although policy makers from developing countries have increasingly considered GM crops as a potential tool for increasing agricultural productivity, contentious debates over both the benefits and concerns of implementing GM crops have hindered its implementation. There are currently 29 countries in the world that are cultivating GM crops. Out of these 29 countries 19 are developing countries (James 2011). Out of the 19 developing countries, only three come from the entire African continent. In Sub Saharan Africa only two countries have approved commercial cultivation of GM crops namely South Africa and Burkina Faso (Racovita et al. 2013). The development of GM crop varieties in Africa has raised a wide range of new legal, ethical and economic questions in agriculture (Azadi and Ho 2010). GM crops are promoted as the solution to the prevalent issues of food security and low agricultural productivity in sub Saharan Africa and other parts of the developing world. The promotion is however not restricted to the developing countries but also the first world. On the one hand, Sub Saharan African farmers are encouraged to accept and implement GM crops because of their higher productivity, while organic farming is encouraged because of socio-economic and

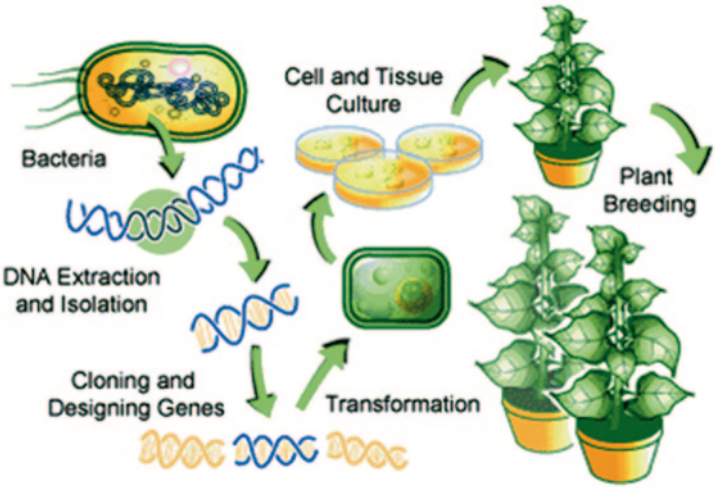

Fig. 2.1 Crop genetic engineering includes: *1* DNA isolation, *2* gene cloning, *3* gene design, *4* transformation, and *5* plant breeding. (Source: http://oregonstate.edu/orb/terms/genetic-engineering)

environmental considerations (Azadi and Ho 2010). The types of concerns that accompany GM crops are from the time they are planted right through to the time that they are consumed. Concerns of GM crops are present in the divisions pertaining to both environmental health and human health. They have been labelled countless times as a potential risk to animal and human health because of their potential toxicity and allergenicity (Racovita et al. 2013).

The following according to Malarkey (2003) are four concerns within food and feed safety issues that GM crop cultivation bring about:

1. The inherent toxicity of the novel genes and their products.
2. The potential to express novel antigenic proteins or alter levels of existing protein allergens.
3. The potential for unintended effects resulting from alterations of host metabolic pathways or over expression of inherently toxic or pharmacologically active substances.
4. The potential for nutrient composition in the new food differing significantly from a conventional counterpart.

Other reasons opposing GM include public attitudes, Socio-economic factors and intellectual property rights have also been raised (Racovita et al. 2013). There have been instances where traditional beliefs and ethical concerns have played a role in making the implementation of GM crops abominable. Coe (2014) noted that beliefs, habits and rituals are attached to religion and culture and are so deeply rooted that there is instant approval or disapproval of agribiotic products. Since the functioning and the future of biotechnology rest on network of a setup, awareness and understanding of how biotechnology relates to these ‘affiliations’ are imperative.

2.3.1 Potential Environmental Risks

Although the risks of genetically modified crops have sometimes been exaggerated or misrepresented, they do have the potential to cause a variety of health problems and environmental impacts (UCSUSA 2012). For instance, they may produce new allergens and toxins, spread harmful traits to weeds and non-GE crops, or harm animals that consume them (UCSUSA 2012). At least one major environmental impact of genetic engineering has already reached critical proportions: overuse of herbicide-tolerant GE crops has spurred an increase in herbicide use and an epidemic of herbicide-resistant "superweeds," (UCSUSA 2012).

With the evidence of escalating crop pests that suppress staple crop yields both on a subsistence and commercial scales, Sub Saharan Africa need crops that are disease-resistant, can fend off insect predators, and can withstand severe environmental conditions to produce larger crop yields (Pinstrup-Andersen and Schiøler 2001). It must be acknowledged that the implementation of GM crops alone will not solve the world's food problem, but they may be a useful element towards the fight against hunger. The contentious debates surrounding GM crops are a bottleneck to the implementation and probably the main reason that SSA is still lagging behind in accruing the benefits of this technology. Generally, people in developing countries should have ready access to information about both the benefits and the risks of the implementation of GM crops (Pinstrup-Andersen and Schiøler 2001). There have been many debates raising the concern as to whether the implementations of GM crops are feasible from both environmental and health perspective. Anti-GM activists argued that, due to monopoly power, GM crops would result in input costs and decrease diversity of seed choice, thereby forcing poorer farmers out and allowing a form of uniform, corporate-capitalist agriculture to dominate. These risks would be compounded, by potential threats to biodiversity from the spread of GM genetic material, and consumers could be at risk from potentially unsafe foods. Pro-GM advocates argued, by contrast, that GM seeds would reduce costs for farmers in a way, allowing rich and poor alike to benefit. By removing farmers from the burden of purchasing pesticides, for example, both health and economic benefits would result. No known health or environmental risks existed, they claimed, and, if governed by a streamlined regulatory system, all would be well, and the benefits of a 'gene revolution' would be realized.

Some commentators have dismissed anti-GM mobilizations as merely copycat responses by elite activists, using links with farmers' organizations as a way of raising funds (Paarlberg 2001).

2.3.2 Biosafety

Biotechnology is revolutionizing industrial and agricultural practice as the number of commercial biotechnology products is increasing each year. Simultaneously, several regulatory approaches are put in place to allow technological

advancement while preserving public health and the environment. Developing and/or emerging countries often face major barriers to access biotechnologies and biotechnology derived products as they frequently lack the institutional capacities and professional competence in exercising regulatory oversight. To address this need, intensive biosafety capacity building is required. Different training approaches can be used to train individuals in biosafety ranging from long-term leading to a postgraduate certificate or a Master's degree, to short term courses. The UNIDO e-Biosafety program annually organized at the Marche Polytechnic University (MPU) in Italy and Ghent University (UGhent) in Belgium since 2006 has identified that proper institutional capacities need to be in place for countries to deal with the complex issues related to the adoption of GM-technology. It is therefore important to continuously bring to the attention of governments, developmental agencies and international organizations, the value of biosafety capacity development including training through formal degrees to encourage them to mobilize resources for these projects. From October 2006 to 2012, 100 students from 37 different countries participated in the course at the UGent and MPU network nodes. More than half of the students came from Africa (58 %), followed by Europeans (23 %). Only a minority came from Asia, Russia and Middle-East (10 %), Central and South America (7 %) and North-America (2 %). East African countries have been well represented and more than one fifth of the participants were Kenyans (Pertry et al. 2014).

Biosafety capacity building is a complex task and requires a multidisciplinary approach, the main components being human resource development, institutional and policy development for regulatory bodies and relevant research institutions, to enable them efficiently and effectively use biotechnology products particularly GM crops, microbes and/or their processed products. In the last decade, various developmental agencies and donors, notably the Food and Agricultural Organization of the United Nations (FAO), the Global Environment Facility (GEF) and the United Nations Environment Programme (UNEP), have been supporting the biosafety capacity building needs of developing/emerging countries through their technical assistance programs (FAO 2009; Hull et al. 2010). The range of activities include: (i) the development of national policies and formulation of regulations; (ii) GMO detection and monitoring including equipping of laboratories and harmonizing protocols among countries; (iii) facilitating effective communication and public awareness and (iv) human resource development in biosafety (Pertry et al. 2014). Figure 2.2 shows the various biosafety is stages in African countries.

2.3.3 Socio Economic Concerns

In October 2002 relief effort took an unexpected twist, as the governments of Malawi, Mozambique, Zambia and Zimbabwe rejected US food aid because of concerns

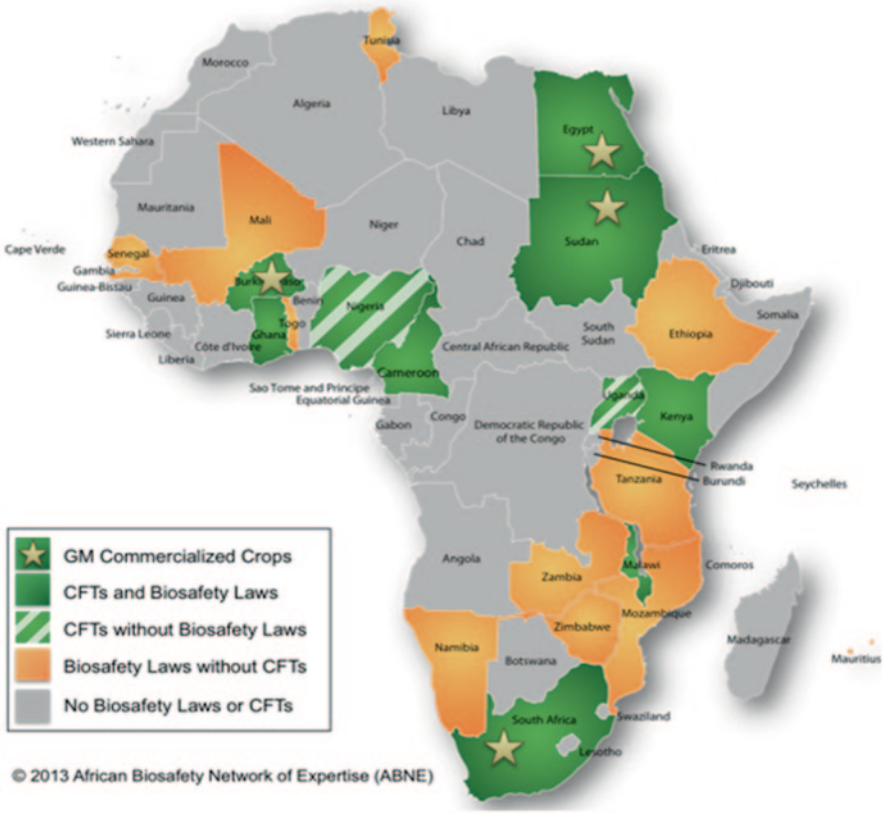

Fig. 2.2 Biosafety and confined field trials (CFTs) for GM crops at various stages in Africa

over the inclusion of genetically modified maize. Zerbe (2004) argues that genetically modified maize transported to Southern Africa in the form of aid, is not an initiative to end hunger in the region, but rather it was an initiative to expand market access and control of transnational corporations. In South Africa herbicide tolerant maize has been grown commercially since 2003, and in 2011, about 1 million ha out of total plantings of 2.71 million ha used this trait (Brookes and Barfoot 2012). From an economic perspective Sub-Saharan African farmers' opposition to the implementation of the cultivation of GM crops on their farm lands is understandable. Implementing the cultivation of GM crops would also disqualify their participation in certain European markets, or restrict them to providing only animal feed. Another problem with the adoption of GM crops seeds is that the farmers' ability to tap into the potential benefits of GM seeds can be limited by institutional issues (Falck-Zepeda et al. 2013).

2.4 Decision Making Tools to Aid Rejection or Implementation of GM Crops in SSA

Contentious nature of debates surrounding GM products have brought forth many suggested strategies that could help in the decision making process of whether to implement the cultivation of GM crops or not. Johnson et al. (2007) suggested that because the debate illustrates confusion between the role of scientists and that of the wider community in regulatory decision making it is important to reinforce the scientific results with non-scientific concerns to achieve an all-inclusive participatory approach on the continent. This is where decision making tools will help to address the issue of whether to implement GM crop cultivation in other Sub Saharan African Countries or not. Johnson et al. (2007) suggests two decision making tools that can help policy makers reach a consensus. Scientific risk assessment and Risk analysis methods will prove useful in regulatory decision making concerning the implementation of GM crops. In a nut shell Risk assessment forms the foundation for regulatory decisions on whether to authorize the environmental release of GM organisms (Keese et al. 2013). It is a structured and a reasoned approach that has the potential to assist in the identification or the discovery of a genetically modified organisms potential to cause adverse harm and to characterize the seriousness and the likelihood of potential harm (Keese et al. 2007). These methods will aid in assessing the risks that may accompany the implementation of GM crops. The result of the scientific risk assessment is not the decision whether or not to permit the cultivation of a GM crop and it is also not the only factor on which a decision is made. A decision will be made based on the amount of risk that is acceptable (the threshold value) if the crop is permitted to be cultivated, and, just as importantly, the risks of not permitting cultivation (Johnson et al. 2007). Acceptable risk cannot be determined solely on scientific based assessment although science is capable of predicting the likelihood of certain effects. Non-scientific criteria (risk analysis) must be included in the process of judging their acceptability so that results that are obtained outside of scientific assessments can be included in the decision making process (Johnson et al. 2007). This is what is referred to as public participation where communities that are both interested in and affected by the implementation of GM crops have the opportunity to voice their views and opinions. In Sub-Saharan Africa the main governmental agency in Burkina Faso responsible for the authorization of GMOs, l'Agence Nationale de Biosécurité, has provisions for involving the wider public in decision-making. The results of an all-inclusive decision making process has given the success of its insect resistant (*Bt*) cotton risk communication strategies in part to the involvement of varied stakeholders early in the adoption of the biosafety law, in the awareness-raising campaigns and also in undertaking confined field trials (Racovita et al. 2013). Scientific decision making tools such as environmental impact assessments, Life Cycle Assessments (LCA's) also known as "Cradle to the grave analysis" if implemented in Sub-Saharan African countries can help to evaluate the impacts of GM crops on its surrounding environment throughout its life cycle (Bennett et al. 2004).

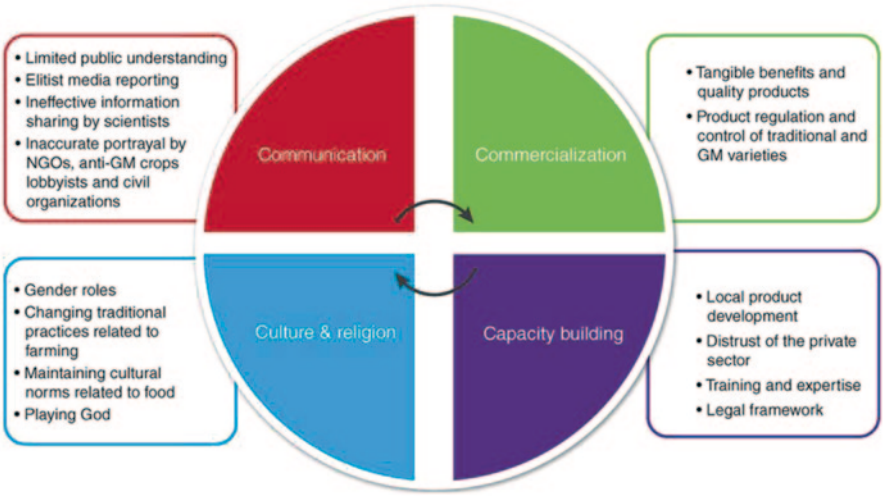

Fig. 2.3 Factors influencing the adoption and development of agbiotech in sub-Saharan Africa (Ezezika et al. 2012)

In brief Ezezika et al. (2012) spelt out the factors influencing agbiotech adoption and development in sub-Saharan Africa as in Fig. 2.3.

2.5 Progress of GM Crop Cultivation in SSA Countries

There are a myriad of concerns that surround the commercialization of GMOs in Sub Saharan African countries with the exception of South Africa and Burkina Faso. One of the concerns is that modern agricultural biotechnology or agbiotech, may have negative impacts on traditional seed systems such as seed selection, seed breeding, seed sharing and seed storage (Ezezika et al. 2012). Such impacts on these traditional seed systems can lead to a loss of indigenous varieties of seeds. This concern is one that has been of priority amongst NGO's and farmers associations. There are fears that the technology would lead to the (further) corporatization of agriculture, and that it is simply unethical to manipulate life in the laboratory. GM crops have been part of the agricultural landscape for more than 15 years and have now been adopted on more than 170 million hectares (ha) in both developed countries (48 %) and developing countries (52 %). On the basis of this substantial history and data spanning many years, the economic and environmental impacts of GM crops can now be summarized with some certainty, and the analysis indicates that, on balance, many benefits have accrued from the adoption of GM crops (Table 2.1). There are many ethical issues that are continuously being debated with many being resolved through institutional interventions. The future of agricultural productivity

Table 2.1 Status and trends in plant biotechnology in Africa (Brink et al. 1998)

Region	Country	Area of research
North Africa	Egypt	Genetic engineering of potatoes, maize and tomatoes
	Morocco	Micropropagation of forest trees, date palms
		Development of disease-free and stress tolerant plants
		Molecular biology of date palms and cereals
		Molecular markers
		Field tests for transgenic tomato
	Tunisia	Abiotic stress tolerance and disease resistance
		Genetic engineering of potatoes
		Tissue culture of date palms, *Prunus* rootstocks and citrus
		DNA markers for disease resistance
West Africa	Burkina Faso	Biological nitrogen fixation, production of legume inoculants, fermented foods, medicinal plants
	Cameroon	Plant tissue culture of *Theobroma cacao* (cocoa tree), *Hevea brasiliensis* (rubber tree), *Coffea arabica* (coffee tree), *Dioscorea spp* (yam) and *Xanthosoma mafutta* (cocoyam)
		Use of *in vitro* culture for propagation of banana, oil-palm, pineapple, cotton and tea
	Cote d'Ivoire	*In vitro* production of coconut palm (*Cocos nucifera*) and yam
		Virus-free micropropagation of egg-plant (*Solanum spp*)
		Production of rhizobial-based biofertilizers
	Gabon	Large-scale production of virus-free banana, plantain and cassava plantlets
	Ghana	Micropropagation of cassava, banana/plantain, yam, pineapple and cocoa
		Polymerase Chain Reaction (PCR) facility for virus diagnostics
	Nigeria	Micropropagation cassava, yam and banana, ginger
		Long term conservation of cassava, yam and banana, and medicinal plants
		Embryo rescue for yam
		Transformation and regeneration of cowpea, yam, cassava and Banana
		Genetic engineering of cowpea for virus and insect resistance
		Marker assisted selection of maize and cassava
		DNA fingerprinting of cassava, yams, banana, pests, and microbial pathogens
		Genome linkage maps for cowpeas, cassava, yams and banana
		Human resource development through group training, degree related training, fellowships and networking

Table 2.1 (continued)

Region	Country	Area of research
	Senegal	Well established MIRCEN programme that serves the region of West Africa in microbial-plant interaction
		Production of rhizobial and mycorhizal-based biofertilizers for rural markets
		Well established in vitro propagation of *Faidherbia albida*, *Eucalyptus canaldulensis*, *Sesbania rostrate*, *Acacia senegal*, in co-operation with several international agencies
East & Central Africa	Burundi	*In vitro* production of ornamental plants—orchids, tissue culture of medicinal plants, micropropagation of potato, banana, cassava and yam
		Supply of disease-free *in vitro* plants
	Congo	*In vitro* culture of spinach (*Basella alba*)
		Plant pathology—studies in controlling tomato rot due to *Pseudomonas solanacearum* Bioprospecting of nitrogen-fixing species
	Congo, Democratic Republic	*In vitro* propagation of potato, soybean, maize, rice and multipurpose trees, e.g. *Acacia auriculiforius* and *Leucaena leucocefhala*
		Production of rhizobial-based biofertilizers in experimental stage
		Tissue culture of medical plants, e.g. *Nuclea latifolia*, *Phyllanthus niruroides*
	Ethiopia	Tissue culture research applied to tef
		Micropropagation of forest trees
	Gabon	Large-scale production of virus-free banana and plantain (*Musa spp*) and cassava plantlets (*Manihot esculenta*)
	Kenya	Production of disease free plants and micropropagation of pyrethrum, bananas, potatoes, strawberries, sweet potato, citrus, sugar cane
		Micropropagation of ornamentals (carnation, alstromeria, gerbera, anthurium, leopard orchids) and forest trees
		In vitro selection for salt tolerance in finger millet
		Transformation of tobacco, tomato and beans
		Transformation of sweet potato with proteinase inhibitor gene
		Transformation of sweet potato with Feathery Mottle Virus, Coat protein gene (Monsanto, ISAAA, USAID, ABSP, KARI)
		Tissue culture regeneration of papaya
		In vitro long term storage of potato and sweet potato
		Marker assisted selection in maize for drought tolerance and insect resistance
		Well-established MIRCEN providing microbial biofertilizers in the East African region

Table 2.1 (continued)

Region	Country	Area of research
	Rwanda	Production of rhizobial-based biofertilizers, and *Azolla* for rice cultivation
		Tissue culture of medical plants and micropropagation of disease-free potato, banana and cassava
	Uganda	Micropropagation of banana, coffee, cassava, citrus, granadella, pineapple, sweet potato and potato
		In vitro screening for disease resistance in banana
		Production of disease free plants of potato, sweet potato and banana
Southern Africa	Madagascar	Tissue culture programme supporting conventional production of disease-free rice and maize plantlets, and Medicinal plants
		Production of biofertilizers to boost production of Groundnut (*Arachis hypogea*), bambara groundnut (*Vigna subterranea*)
	Malawi	Micropropagation of banana, trees (*Uapaca*), tropical woody species, tea
	South Africa	*Genetic engineering*
		Cereals: maize, wheat, barley, sorghum, millet, soybean, lupins, sunflowers, sugarcane
		Vegetables and ornamentals: potato, tomato, cucurbits, ornamental bulbs, cassava and sweet potato
		Fruits: apricot, strawberry, peach, apple, table grapes, banana
		Molecular marker applications
		Diagnostics for pathogen detection
		Cultivar identification—potatoes, sweet potato, ornamentals, cereals, cassava
		Seed-lot purity testing—cereals
		Marker assisted selection in maize, tomato
		Markers for disease resistance in wheat, forestry crops
		Tissue culture
		Production of disease free plants—potato, sweet potato, cassava, dry beans, banana, ornamental bulbs
		Micropropagation of potato, ornamental bulbs, rose rootstocks
		chrysanthemum, strawberry, apple rootstocks, endangered species, coffee, banana, avocado, blueberry, date palm
		Embryo rescue of table grapes, sunflower and dry beans
		In vitro selection for disease resistance—tomato nematodes, guava wilting disease
		Long term storage—potato, sweet potato, cassava, ornamental bulbs
		In vitro gene bank collections—potato, sweet potato, cassava, ornamentals
		Forest trees, medicinal plants, indigenous ornamental plants

Table 2.1 (continued)

Region	Country	Area of research
	Zimbabwe	Genetic engineering of maize, sorghum and tobacco
		Micropropagation of potato, cassava, tobacco, sweet potato, ornamental plants, coffee
		Marker assisted selection
	Zambia	Micropropagation of cassava, potato, trees (*Uapaca*), Banana
		Hosts SADC Nordic-funded gene bank of plant genetic resources

would be better served if the genetic modification debates were less polarized and were focused on the potential for complementarity of GM technologies within a diversified farming system framework (Bennett et al. 2013).

2.6 Advantages of Biotechnology

Although there are controversies and opposition surrounding GM crops in sub-Saharan Africa, the main advantages of these crops cannot be overlooked. GM food crops boast the promise of future food security, especially for small-scale agriculture in developing regions like sub Saharan Africa (Azadi and Ho 2010). In these countries, approximately 800 million people remain seriously malnourished, including at least 250 million children. The main arguments of GM supporters are safe food security, improved food quality, and extended shelf-life as the reasons why they believe in GM crops which will benefit not only consumers and farmers, but also the environment (Wisniewski et al. 2002). Moreover, there are claims that GM technology are healthier, cheaper, more stable, having nutrient of superior quality and better taste (Azadi and Ho 2010). Genetic engineering often complements rather than supplants conventional breeding (Whitty et al. 2013).

In developing countries especially, new cultivars will be key in addressing the challenge of feeding rising populations in the face of climate change along with better use of water and fertilizers, improved soil and crop management, and better storage and transport infrastructure (Whitty et al. 2013). Many alterations to crop varieties will prove beneficial in boosting yields, resistance to disease and pests, nutritional value and tolerance of droughts (Varshney et al. 2011). AATF (2012) noted that Africa is a drought-prone continent, making farming risky for millions of smallholder farmers who rely on rainfall to water their crops. Maize being the most widely grown staple crop in Africa has prompted the development of Water Efficient Maize for Africa (WEMA), a GMO technology. Ledermann (2012) stated that it is being done by a non-profit consortium in order to develop drought-tolerant maize varieties which will be made available to small-scale farmers in Sub-Saharan Africa which will initially be royalty free. The WEMA project is funded by the Bill & Melinda Gates Foundation and the Howard G. Buffett Foundation. The project

Fig. 2.4 Countries participating in WEMA

is run in partnership with the African Agricultural Technology Foundation (AATF), the national agricultural research systems in Kenya, Mozambique, South Africa, Tanzania and Uganda, the International Maize and Wheat Improvement Centre (CIMMYT, a member of CGIAR), and Monsanto Corporation. WEMA is a large scale and multi-pronged effort to provide drought-tolerant maize varieties specifically to Kenya, Mozambique, South Africa, Tanzania and Uganda (Fig. 2.4). The consortium is utilizing conventional (classical) breeding, marker-assisted breeding, and biotechnology approaches.

One of the most important general concerns of GM crops revolves around the issue of containment. To date, no GM crop under cultivation has reproductive characteristics which are any different than its non GMO counterpart. That is to say that the transgenes incorporated into GM crop varieties, once inserted, become just like every other gene in the organism. They can be passed down from one generation to the next and moved through the environment by any of the same mechanisms which can bring non GM crop genes into the environment (Ledermann 2012). Corn grain, whole plant (maize stover), corn silage, corn field residue, soybeans and/or soybean meal from the genetically enhanced plants have been fed to chickens, sheep, beef cattle and/or dairy cows and compared with feeds produced from isolines of non-genetically enhanced plants (Malarkey 2003). The results from 23 research trials indicate that genetically enhanced corn and soybeans that are available in the marketplace are substantially equivalent in composition, are similar in digestibility and have a similar feeding value for livestock (Malarkey 2003). Whitty et al. (2013) discusses three genetically modified field trial crops that could help transform the

quality of life for millions of people in Sub Saharan Africa, in Africa as a whole, and also in developing countries collectively. In Africa preschool children that are affected by vitamin A deficiency is estimated to be 250 million. More than 1 million deaths in children under the age of 5 years old can be prevented through vitamin A rich crops such as Pro-vitamin-A- enriched Golden Rice (Whitty et al. 2013). Furthermore genetic engineering can help to increase the yields of staple crops in Sub Saharan Africa. Cow pea is one of those crops and there are 200 million consumers of cowpea on the African continent. The Maroca Pod Bearer Resistant Cowpea has the potential of increasing yields by 70% while the use of insecticide spray will experience a 67% reduction use. Another Staple diet of Sub Saharan dwellers is maize which 300 million Africans depend on. They suffer yield loss ranging between 10–25% due to drought. Genetically modified "Water efficient maize" has capabilities to increase maize yields by 20–30% (Whitty et al. 2013).

Another staple crop for millions in Sub-Saharan Africa is the cassava crop. Cassava (*Manihot esculenta* Crantz) is an important source of calories for more than a billion people in developing countries, and its potential industrial use for starch and bioethanol in the tropics is increasingly being recognized (Chetty et al. 2013). Global production of cassava is about 256 million t, out of which 146 million t are produced in Africa (FAOSTAT 2012) Despite the importance of cassava there are traits that need improvement, such as pest and disease susceptibilities, accumulation of cyanogens, and post-harvest physiological deterioration (Ceballos et al. 2004; Sayre et al. 2011). Genetic transformation of cassava offers great potential for cassava improvement because it could help to address two foremost problematic viral diseases that are known to attack cassava crops, namely the cassava mosaic disease and the brown streak disease (Liu et al. 2011; Whitty et al. 2013). The Cassava mosaic disease (CMD), caused by several circular ss DNA begomoviruses (belonging to the Family: *Geminiviridae*), is the most important disease affecting cassava production on the African continent (Chetty et al. 2013). The cassava mosaic disease stunts the growth of these crops while the brown streak disease attacks the roots of the cassava causing it to rot. These diseases are known to affect the cassava crops especially in the East African region of the continent (Whitty et al. 2013). In Uganda and Kenya, an international team of researchers are currently investigating the possibilities of addressing this issues using GM technology. The importance of food composition in safety assessments of GM food is described for cassava that naturally contains significantly high levels of cyanogenic glycoside toxicants in roots and leaves. The assessment of the safety of GM cassava would logically require comparison with a non-GM crop with a proven history of safe use. Although acute and chronic toxicity benchmark values for human have been determined, intake data are scarce. In considering the nutritional values for cassava cyanogen glycosides residues in food should be a priority topic for research. Successful application of transgenic technologies in cassava will depend not only on technical advances, but also on successful transfer of knowledge, tools and expertise to the countries in which cassava has an important socioeconomic role (Nyaboga et al. 2013). In Mozambique and Uganda orange sweet potatoes being rich in vitamin A has been introduced into some sectors of the populations (Whitty et al. 2013). Apart from the

concerns surrounding genetic engineering in Sub Saharan Africa, low availability of human and financial resources in the region limits the overall innovative capacity to conduct Research and Development and to develop indigenous Genetically Engineered crops (Falck-Zepeda et al. 2013).

2.6.1 South Africa

According to the Department of Agriculture (2005) on the African continent, South Africa is very unique in terms of growing commercial GMO's because resistant and herbicide tolerant maize is available in the country. According to James (2004) biotech crops are estimated to account for 24 % of yellow maize, 10 % of white maize and 50 % of soybean and 8 % of cotton production in South Africa. In South Africa, the first Bt cotton trials were initiated by Monsanto in 1992 under the apartheid regime, with transgenic cotton released for commercial use in 1997 (Scoones 2008). The South Africa GMO Act was passed in 1997, but did not come into force until the end of 1999. In 1998 yellow Bt maize was also commercialized, with white Bt maize two years (Scoones 2008). *Transgenic Bt cotton, engineered to continuously produce activated Δ-endotoxins from the soil bacteria Bacillus thuringiensis, holds great promise in controlling Helicoverpa armigera and other lepidopteran pests* (Zhang et al. 2008).

Burkina Faso's experience over the past decade provides an excellent example of the processes and procedures required for a biotechnology product to be successfully introduced into a developing country. The commercial release of 12,500 ha of Bt cotton in 2009 was the result of nearly a decade of coordinated efforts on behalf of various Burkina Faso cotton stakeholders to satisfy a series of technical, legal, and business requirements. With input from many sources, the Burkina Faso legislature researched, developed, and passed biosafety legislation to formalize regulatory oversight for the research and commercialization of agricultural biotech products (Vitale et al. 2010).

2.6.2 Burkina Faso

Regulatory and institutional constraints, influenced to some degree by opposition groups, have delayed the introduction of bioengineered crops while commercial release and adoption of biotech crops have proceeded on most other continents (Cohen and Paarlberg 2002). Burkina Faso, however, has been most progressive and proactive than other countries in the West African region since its implementation of biotechnology (Vitale et al. 2008). Burkina Faso's interest in Bt cotton has been a result of the growing ineffectiveness of the conventional spray-based control methods for lepidopteron pests in cotton, and the growing costs of pest control. Burkina Faso spends over 60 million for chemically based pest control Products.

In a report " Second-Generation Bt Cotton Field Trials in Burkina Faso: Analyzing the Potential Benefits to West African Farmers" Vitale et al. (2008) shows results from an assessment of the success of GMO field trials in Burkina Faso, namely the Bollgard II field trials. According to Vitale et al. (2008) The purposes of the article was to test whether Bollgard II provided enhanced crop protection from Lepidoptera pests, and generated higher yields than conventional pest management. The report also evaluated the economic profitability of Bolgard II in Burkina Faso, and assessed the sensitivity of Bollgard II cotton profitability across a range of technology costs. The results indicated that Bollgard cotton provided a significant yield advantage of 14.7% of overall conventional cotton (Vitale et al. 2008). At the end of the analysis it was concluded that existing pest management practices in Burkina Faso only provide partial protection. In 3 years of confined field tests on INERA Experiment Stations, Bollgard II cotton has proven to be more effective than current standard practices of controlling lepidopteran pests. The data derived from the 3 year long Bollgard field trials suggests that transgenic cotton would significantly increase cotton income across a plausible range of Bollgard II cotton technology prices. The 3 year long field trial also indicated that the observed pest densities were lower than that typically found under actual farming conditions (Vitale et al. 2008); observations suggest that Bollgard II cotton plants were in better health and better able to withstand the late season attacks of the piercing/sucking insect (Vitale et al. 2011). They also indicated that Bt cotton had potential to increase cotton yields by an average of 21.3% and raised income by $ 106.14 per ha. using an energy balance model, the introduction of Bt cotton would also result in a 6.6% saving in energy use (Vitale et al. 2011). Hence, the introduction of Bt cotton in Burkina Faso could become a pivotal ev*ent that may have a substantial impact on the future use of biotechnology*. The significant increase in productivity and economic returns could be the catalyst for Burkina Faso, and other African countries, to emerge from years of stagnation and regain their competitive stance in world cotton markets while providing environmental and social benefits. In Burkina Faso cotton is perhaps the single most important commercial economic activity (Baffes 2007).

2.7 Conclusion

Africa is a continent that is plagued with sad cases of hunger and poverty. The severity of threat to environmental and food security is becoming more apparent. These forms of security are closely intertwined since food production is highly sensitive to environmental conditions and conversion of natural land for agriculture is a major cause of the deterioration of earth's life support systems. Some developing countries are already benefiting and should continue to benefit significantly from advances in plant biotechnology. Insect-protected cotton containing a natural insecticide protein from *Bacillus thuringiensis* (Bt cotton) is providing millions of farmers with increased yields, reduced insecticide costs and fewer health risks. Other orphan crops such as rice, tropical maize, wheat, sorghum, millet, banana, potato,

sweet potato and oil seed can also benefit from GM technology in developing countries (Adendle 2011). Genetic engineering has made it possible to produce crops that are improved in terms of their yield traits and their qualities (Uzogara 2000). The African continent, more than any other, urgently needs agricultural biotechnology, including transgenic crops, to improve food production (Wambugu 1999). This is because the priority of Africa is to feed her people with safe foods and to sustain agricultural production and the environment. SSA through the formalisation of the cultivation of GM crops can make up for the green revolution it lost out on; the same green revolution that helped in making Asia and Latin America self-sufficient in food production. The nature of the GM debate with a large opposition from SSA population indicates that there is a need to engage the community with researchers, policy makers and local communities in this discourse. In April 2007, biosafety and biotechnology scientists, regulators, educators, and communicators from Kenya, Tanzania, and Uganda, met to examine the status and needs of biosafety training and educational programs in East Africa. Workshop participants emphasized the importance of developing biosafety capacity within their countries and regions. Key recommendations included identification of key biosafety curricular components for university students; collaboration among institutions and countries; development of informational materials for non-academic stakeholders and media; and organization of study tours for decision makers. It was emphasized that biosafety knowledge is important for all aspects of environmental health, food safety, human and animal hygiene. Thus, development of biosafety expertise, policies and procedures can be a stepping stone to facilitate improved biosafety for all aspects of society and the environment. Basic biotechnology principles integrated into secondary and primary school curricula with the latter involving cartoons. If children could be made to appreciate astronautical events when they have not been to space, then biotechnology of the crops they handle and eat daily will not be a problem to appreciate. Frequent workshops on biotechnology and biodiversity career options involving professionals in the field should be organised. Database of Biotechnology professionals and Network list of professional societies and organizations should be made available.

Environmentalists and stakeholders from anti-GM crops groups express their concerns about the commercialization of GMOs, stating that the introduction of agbiotech will indeed pose a threat to the survival of indigenous crops and affect biodiversity (Ezezika et al. 2012). Food insecurity exacerbated by high and unaffordable food prices is a formidable challenge to which biotech crops can contribute but are not a panacea (James 2013) Despite public concerns, it is regarded that the main increase in agricultural productivity will be achieved through the direct use of genetic improvement and biotechnology (Villalabos 1995). If biosafety measures are rigorously adhered to and trans-boundary movements of GE products are monitored, Africa will reduce hunger and boost the health of its people.

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Chapter 3
Agriculture and Environmental Impacts of Glyphosate-Tolerant Soybean Cultivation in Romania

Elena Marcela Badea and Ion Păun Otiman

Abstract Glyphosate-resistant soybeans (GRS) were grown commercially in Romania beginning with1999 and accounted for 68 % (or 137,000 ha) of the entire soybean area planted in 2006. Since 2007, after its accession to the European Union (EU), the Romanian farmers' access to the technology was banned. As a result, in only 2 years, the area planted to soybeans shrunk by 70 %, while Romania became a net importer of plant protein. In 2006 alone, the 137,300 ha planted to GRS received 176,388 kg of herbicides less than on the 53,500 ha planted to conventional soybeans. The environmental impact coefficient (EIQ) was about 70 % lower—both per hectare and for the entire GM cultivated area. If soybeans were planted on 500,000 ha—almost half of the area that lends itself well to this highly-important economic crop in Romania—the total amounts of herbicide ingredients applied would be 2,100,100 kg in case only conventional varieties were grown and 765,000 kg in case only RR varieties were grown. Which means that in case GRS were grown, the environment would be spared 1,335,000 kg of herbicide active ingredients, and the EIQ would be 67 % lower than for conventional crops.

Keywords Status of soybean production · Glyphosate-resistant soybean (GRS) · Environmental impact of herbicide regimes · Economic and environmental impacts of discontinuing GRS cultivation

3.1 Introduction

Romania adopted its initial legislation on biotech products in 2000 (Government Ordinance/GO No. 49/2000). All activities involving genetically-modified organisms (GMOs) were regulated, including: the confined use of genetically-modified

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micro-organisms; their deliberate release into the environment for purposes other than being launched on the market; their placement on the market either as GMOs or in products derived there from; and imports/exports of GMOs as or in products.

Among the first biotech crops approved for commercial cultivation under the biotech GO 49/2000 were glyphosate-resistant soybeans (GRS) and the Superior New Leaf Potato (Badea and Pamfil 2009).

To date, the Romanian biotech legislation is harmonized with the EU legislation. Romania has transposed Directive 2001/18 into its regulatory framework by adopting Law No. 247/2009 regulating activities involving the deliberate release and placement on the market of GMOs. The Cartagena Protocol on Biosafety came into force on September 28, 2003.

Romania is one of the few European countries with favourable conditions for soybean production. GRS were commercially-grown in this country beginning with 1999, accounting for 68 % of all the soybean area planted in 2006. Farmers using GRS indicated that the plant was the most profitable arable crop grown in Romania, with gains derived from higher yields and improved quality of seeds, combined with lower production costs (Brookes 2005). In 2006, Romania was among the eight countries that grew the crop worldwide. In 2007, as a Member State of the EU, it banned the cultivation of the crop, despite the fact that growing HT soybeans had generated a substantially higher net farm income per hectare in this country than in any other country using the technology (Brookes 2005). As a result, in only 2 years, the area planted to soybeans shrunk by 70 % and Romania became a net importer of plant protein, just like the European Union (Dinu et al. 2011; Balaj et al. 2012a).

According to the Romanian biotech law GO No. 49/2000, post-marketing monitoring aims to confirm the conclusions of the environmental risk assessment and to identify unpredictable adverse effects. In Romania, monitoring of GRS was carried out by universities and research institutes, in close cooperation with the private sectors having received licenses in that respect. In order to confirm some conclusions on the environmental risk assessments submitted by the applicants, field experiments (case-specific monitoring) were undertaken to evaluate the impact of RR versus conventional technology on soil microorganisms, the arthropod fauna, and weed populations. Case-specific monitoring activities were carried out in 2004 on a monoculture (2002–2004) soybean experimental field at Moara Domnească Didactic Experimental Station, focusing on the structure and make-up of the weed population, the invertebrate fauna population, and the heterotrophic bacteria and microscopic fungi in the RR and conventional plant rhizospheres (Badea et al. 2006).

An overall monitoring took place in 2002–2004, where farmers cultivating RR soybeans were asked about the plant behaviour in the new agro-ecosystems. Responses were regarded as indicators for soybean behaviour in terms of the plant's invasiveness, persistence, rate and/or way of reproduction, dissemination, survivability, etc. (Badea et al. 2004).

The present paper presents soybean data in general as well as data regarding the agronomic and environmental impact of glyphosate-tolerant soybean cultivation in

Table 3.1 The 20 highest soybean-producing countries in 2012. (Source: FAOSTAT 2012)

	Country	Area harvested (ha)	Production (tons)
1.	United States of America	30,798,530	82,054,800
2.	Brazil	24,937,814	65,700,605
3.	Argentina	19,350,000	51,500,000
4.	India	10,800,000	11,500,000
5.	China	6,750,080	12,800,145
6.	Paraguay	3,000,000	8,350,000
7.	Canada	1,668,400	4,870,160
8.	Ukraine	1,412,400	2,410,200
9.	Russian Federation	1,375,200	1,806,203
10.	Uruguay	1,130,000	3,000,000
11.	Bolivia	1,090,000	2,400,000
12.	Indonesia	567,871	851,647
13.	South Africa	500,000	850,000
14.	Nigeria	440,000	450,000
15.	Democratic People's Republic of Korea	300,000	350,000
16.	Serbia	162,714	280,638
17.	Italy	153,000	422,100
18.	Vietnam	120,751	175,295
19.	Thailand	100,000	180,000
20.	Romania	77,927	104,330

Romania. We also aim to evaluate the impact that the potential shift from conventional to GRS crops would bring to farming systems, through a comparison between the environmental impacts of herbicide treatments used in GM and non-GM crops, respectively. For a study case, we have looked at the technology used for soybeans on one of the largest farms in Europe, located on the Great Brăila Island (Buzdugan 2011; Balaj et al. 2012b).

3.2 Status of Soybean Production

3.2.1 Global Perspective

Soybeans are one of the world's most important and fastest expanding crops, which contribute considerably to worldwide human nutrition. The main world soybean producers are: the U.S., Brazil, Argentina, India, and China, countries where the harvested area exceeded 10 million ha in 2012 (Table 3.1).

Since 1996—the first year of global marketing for the biotech crops—GRS has been the most grown engineered crop. In 2012, global GRS area stood at 80.7 million ha, representing 81 % of the world soybean area (James 2012). The crop has been commercially grown in the U.S., Argentina, Brazil, Paraguay, Canada, Uruguay, Boliv-

Table 3.2 Countries in which commercial cultivation of GRS is/was approved. (Source: James 2012)

Country	1996	1997, 1998	1999, 2000	2001, 2002	2003, 2004	2005, 2006	2007, 2008	2009–2012
USA	x	x	x	x	x	x	x	x
Canada		x	x	x	x	x	x	x
Brazil					x	x	x	x
Argentina	x	x	x	x	x	x	x	x
Mexico			x	x	x	x	x	x
South Africa				x	x	x	x	x
Romania			x	x	x	x	–	–
Paraguay						x	x	x
Uruguay				x	x	x	x	x
Costa Rica								x
Chile								x
Bolivia								x

ia, South Africa, Mexico, Chile, and Costa Rica (Table 3.2). With glyphosate as the primary herbicide for their soybean crops, growers achieved greater flexibility in timing herbicide applications, as well as simplicity, with less confusion of herbicide mixes and rates, an effective control of perennial, and other problem, weeds, excellent crop safety, and economical weed control. For these reasons, GRS has been adopted faster than any other new technology in the history of agriculture (Sankula et al. 2005). Since 2011, new soybean events have been approved for cultivation (Table 3.4).

The data in Table 3.2 indicate that Romania is the only country where cultivation of GM soybeans has been prohibited. The event took place after several years of steady increase in its adoption rate (Otiman et al. 2008).

3.2.2 Europe

In Europe, the main soybean producers are Ukraine and the Russian Federation (Table 3.3). In Eastern Europe, soybeans are grown in northern Serbia (Vojvodina) and various regions of Romania: west (the Banat), south (the Danube Plain), and northeast (FAOSTAT 2012).

In the EU, soybean production is fairly limited, mainly because of the less favourable climate. In 2006 and 2012, the major EU soybean growers were Romania, Italy, and Serbia, and Serbia and Italy, respectively (Table 3.3).

With a large protein deficit, Europe is highly dependent on soybean imports. In 2012, the EU imported an overall 32 million MT of soybeans and soybean meal ($ 16.5 billion), of which soybean meal represented 61%. Europe's main suppliers are the large biotech-producing countries: Brazil (15 million MT), Argentina (8.6 million MT), and the United States (2.8 million MT)—(Eurostat 2012).

Table 3.3 Soybean producers in Europe. (Source: FAOSTAT 2012)

Item	Countries					
	Romania	France	Republic of Serbia	Italy	Ukraine	Russian Federation
2006						
Area harvested (ha)	190,800	45,263	156,680	177,909	725,000	810,130
Production (tons)	344,900	122,995	429,639	551,292	889,000	806,570
Yield (kg/ha)	18,070	271,734	274,214	309,873	122,621	99,561
2012						
Area harvested (ha)	77,927	37,517	162,714	153,000	1,412,400	1,375,200
Production (tons)	104,330	104,327	280,638	422,100	2,410,200	1,806,203
Yield (kg/ha)	13,388	27,808	17,247	27,588	17,065	13,134

Roundup Ready soybeans (the 40-3-2 event) are approved for marketing in the EU (Commission Decision 96/281/EC of April 3, 1996, amended in 2012). The decision allows for the imports of seed into the EU for industrial processing into non-viable products, including animal feeds, food, and any other products using soybean fractions. In the EU, new soybean events have been approved for food and feed for direct use, or processing (Table 3.4).

Except for RR soybeans—which Romania grew between 1999 and 2006—no other herbicide-tolerant plant (sugar beet, maize, cotton, rapeseed) has been included among the European commercial crops. This is why most of the studies done so far have focused on potential changes in herbicide applications and the economic and environmental impacts of the possible adoption of herbicide-tolerant plants (Kleter et al. 2008; Devos et al. 2008; Dewar 2009).

3.2.3 Romania

In 1955, Romania produced 14,000 t of soybeans; however, the level declined steadily, reaching a low of 1000 t in 1965. Starting with 1966, Eastern Europe witnessed a renewed interest in soybean production, with Romania leading the way. Production took a leap from 20,000 t that year to 298,000 t in 1974, later rising to 448,000 t in 1980 (Shurtleff and Aoyagi 2007). In 1989, reported production was 303,900 t (Table 3.5)—FAOSTAT 2012.

Just as in the other EU Member States, Romania's soybean production is far from meeting its domestic demand; therefore, the country imports every year significant amounts of soybeans and soybean meal in order to cover its demand in the livestock sector. Romania's total soybean and soy meal imports rose by 11.5% in 2012 from the previous year, reaching 545,000 MT ($ 276 million), of which soy meal totalled 482,000 MT ($ 237 million). Its major suppliers were large biotech producing countries such as Brazil (339,000 MT), Argentina (52,000 MT), and the United States (47,000 MT; Eurostat 2012).

Table 3.4 Herbicide-tolerant soybean events and types of approvals. (Source: EU approval database)

	Event	Event code/ trade name	GM Traits	Type of approval	
				EU	Other countries
1.	GTS 40-3-2 (40-3-2)	MON-Ø4Ø32-6 Roundup Ready™	Tolerance to glyphosate	Food and feed: direct use or processing (2005, amended in 2012)	Cultivation (Table 3.2)
2.	MON87705	MON-877Ø5-6 Vistive Gold™	Tolerance to glyphosate; modified oil/ fatty acid	–	Cultivation U.S., Canada (2011)
3.	MON87701 x MON89788	MON-877Ø1-2 x MON-89788-1 Intacta™ Roundup Ready™ 2 Pro	Tolerance to glyphosate; resistance to Lepidopteran insects	Food and feed: direct use or processing (2012)	Cultivation Brazil (2010); Argentina, Uruguay (2012); Paraguay (2013)
4.	MON87708	MON-877Ø8-9 Genuity® Roundup Ready™ 2 Xtend™	Tolerance to glyphosate and Dicamba	–	Cultivation: Canada (2012)
5.	MON89788	MON-89788-1 Genuity® Roundup Ready 2 Yield™	Tolerance to glyphosate	Food and feed: direct use or processing (2008)	Cultivation Canada, USA (2007); Costa Rica (2008)
6.	A2704-12	ACS-GMØØ5-3 Liberty Link™	Tolerance to glyphosate	Food and feed: direct use or processing (2008)	USA 1996, Canada 1999, Brazil 2010, Argentina 2011, Uruguay 2012
7.	A5547-127	ACS-GMØØ6-4 Liberty Link™	Tolerance to glyphosate	Food and feed: direct use or processing (2012)	
8.	DP356043	DP-356Ø43-5 Optimum GAT™	Tolerance to glyphosate; tolerance to Sulfonylurea	Food and feed: direct use or processing (2012)	

GRS Cultivation in Romania Romania is one of the few European countries that has favourable conditions for soybean production. Commercial cultivation of GR soybeans was approved in 1999. In 2006, Romania represented one of the nine countries in the world growing this GM crop (James 2007).

Beginning with 2000, the country's RR area expanded constantly, reaching a peak in 2006 (the eighth year of using the technology) of 137,000 ha (Table 3.6)—(Otiman et al. 2008). The same year witnessed large GR soybean areas (Fig. 3.1),

Table 3.5 Soybean production in Romania. (Source: FAOSTAT 2012)

Year	Harvested area (ha)	Production (tons)	Yield (kg/ha)
1989	512,000	303,900	59,332
1990	190,228	141,173	74,213
1999	99,800	183,400	18,380
2000	117,000	69,500	99,402
2001	44,800	72,700	16,230
2002	71,800	145,900	20,330
2003	128,800	224,900	184,006
2004	122,400	298,506	24,520
2005	143,100	312,800	21,860
2006	177,481	344,909	19,430
2007	109,314	136,094	12,450
2008	46,135	90,579	19,633
2009	48,249	84,268	17,865
2010	63,424	149,940	23,641
2011	71,861	142,636	19,849
2012	77,927	104,330	13,388

Fig. 3.1 RR soybean areas in Romania. (Evidence of Ministry of Agriculture)

particularly in regions most favourable to the crop: the Danube Plain (the counties of Călăraşi, Ialomiţa, Brăila, and Galaţi), Dobrogea (Constanţa county), and Banat (Timiş and Arad counties).

Fourteen varieties were registered in the Romanian Official Catalogue (of which three by Pioneer and eleven by Monsanto). Six varieties were marketed in 2006 (one by Pioneer and five by Monsanto). Romania's variable soil and climate conditions—going from average temperate temperatures and heavy rainfalls inside the Carpathian arch to low rainfalls in the south—along with the country's specific seasonal pattern, with significant differences between the seasons meant growing different maturity group varieties. Low temperatures in winter destroy potential

Table 3.6 RR soybean area and production, 1999–2006

Item	1999	2000	2001	2002	2003	2004	2005	2006
Total soybean areas (ha)	99.8	117.0	44.8	71.8	128.8	121.3	143.9	199.2
GM soybean areas (ha)	15.5	32.2	17.3	31.1	39.6	61.6	86.1	137.3
Total production (MT)	183.4	69.5	72.7	145.9	224.9	298.5	312.8	340.7
GM soybean production	–	–	–	–	77.3	152.5	177.5	249.9

volunteer plants. The most favourable climate conditions for soybeans are in the Danube Plain.

3.3 Agronomic Impact of Growing RR Soybeans in Romania

The specific agronomic/management/harvesting practices used for RR soybeans are identical to those used for non-GM soybeans, except for herbicide treatments (technical file).

In a representative sample of cash crop farms, the profit margin per hectare ranged from € 100 and € 187, corresponding to a yield range of 3–3.5 t/ha, while in the same market year (2006), conventional soybean growers incurred losses. The income increment resulted from the herbicide cost cuts (on average, 1.9 treatments applied to GRS versus 4.3 treatments on conventional crops) and higher yields (3–3.5 t/ha for GR versus 2 t/ha for conventional soybeans; Otiman et al. 2008).

According to Buzdugan (2011), certain producers' reticence in growing conventional soybeans is fully justified. No farmer will agree to incur economic losses, practice energy-intensive farming, and infest their soils with weed seeds or noxious chemicals.

As for RR soybeans, they brought numerous benefits to the Romanian farmers (Buzdugan 2011), such as:

- A production growth of up to 30%;
- Protection against difficult weed species specific to soybeans, such as common reed (*Phragmites communis*), Johnson grass (Sorghum halepense), and black nightshade (*Solanum nigrum*)—for which no selective herbicides exist for soybeans (the species is highly present in soybeans and causes important losses through crop deterioration);
- Lower costs of weed control, both for the main crop and the subsequent crops;
- Lower fuel use for the overall rotation, made possible by minimum tillage;
- Increased nitrogen stock in the soil, by about 30–40 kg of active ingredient, as against any other crop, due to symbiotic bacteria activity;
- Higher revenues, due to production growth and higher kernel quality, enabling the producers to sell for higher prices.

3.3.1 Effects of Glyphosate Use

Based on the herbicide use data for 2000–2003, Brookes and Barfoot (2005) calculated that Romania's adoption of RR soybeans resulted in a small net increase in the amount of active ingredients applied, but a net reduction in the EIQ/ha. However, the authors do not deem the results conclusive, because the rates of herbicides applied to conventional crops during the same interval were below optimal levels. More specifically: during the analysed interval, recommended herbicide programs were often applied in part, if not completely ignored. Not least, information sources were not always reliable.

The results of a survey conducted at the end of 2006 on a sample of 160 soybean growers (running commercial farms equipped with an adequate input and technology mix) in 14 key counties indicated that conventional soybeans received on average 2.3 herbicide treatments/crop year, with about 10% of growers applying four treatments. GRS received on average 1.63 treatments/no more than two (Otiman et al. 2008).

The most efficient and used weed management scheme for GRS was the two-step treatment of 2 L of Roundup per hectare each time, after emergence, depending on the stage of development of the first weed generation and the evolution of the second weed generation (Buzdugan 2011).

3.3.2 Glyphosate-Resistant (GR) Weeds

Weed resistance is probably the highest risk related to growing GRS. GR weeds have already made their appearance in various U.S. states (totalling 13 weeds in mid-2011), as well as in more than a dozen countries throughout the world (totalling 21 weeds in mid-2011; Bonny 2011).

In Brazil, five weed species: hairy fleabane (*Conyza bonariensis*), Canadian fleabane (*Conyza canadensis*), Italian ryegrass (*Lolium multiflorum*), wild poinsettia (*Euphorbia heterophylla*), and sourgrass (*Digitaria insularis*) have developed resistance to glyphosate in GRS and are potentially major problems. Glyphosate-resistant biotypes of Johnson grass (*Sorghum halepense*) L. and Italian ryegrass (*Lolium multiflorum*) have developed in GRS crops in Argentina, as well as one of sourgrass (*Digitaris insularis*), in Paraguay (Via-Aiub et al. 2008).

Currently, some of the above weed species can be found in Europe, all with widespread distribution (bindweed/*Convolvulus arvensis,* ryegrass/*Lolium spp.,* amaranth/*Amaranthus spp.,* Canadian fleabane/*Conyza canadensis,* Italian ryegrass/*Lolium multiflorum,* morning glory/*Ipomoea spp*., and fat hen/*Chenopodium album*). The North American weed species *Conyza canadensis* was introduced to Europe in the seventeenth century. Almost nothing exists in the literature about naturally glyphosate-resistant European weed biotypes or about European weed biotypes that can potentially develop glyphosate resistance (Sandermann 2006).

No GR weeds were reported in Romania during the 8 years in which the country used the RR technology.

3.3.3 Impact of Discontinuing GRS Cultivation

Most Romanian farmers indicated that their economic efficiency was mainly due to their having adopted the GR technology (Badea and Otiman 2006; Brookes and Barfoot 2005, 2010).

In an attempt to intensify the pace of bringing Romania's biotech regulatory capacity in line with the *acquis communautaire*, the Romanian authorities were, as of January 2006, already highly committed to discourage biotech plantings. Therefore, they went on to announce a subsidy program for conventional soybeans for the year. Despite the announcement, the hectarage of transgenic soybeans went up to 137,000 (of a total 199,000). Thus for a second year in a row, with a production of almost 350,000 t, Romania started shipping its exportable surplus of soybeans, while its imports of soybean meal went down substantially (Dinu et al. 2011).

With no more access to RR technology, soybean area started to decline in 2007, dropping to 109,000 ha, and further down to no more than 46,000 in 2008 (FAOSTAT 2012). This was the equivalent of a 70 % reduction over the recent years.

In 2006, the national level of revenues obtained by farmers who grew glyphosate-tolerant soybeans totalled $ 7.6 million. During 1999–2006, nominal farm income rose by $ 44.6 million. In 2006, GM soybean production growth equalled a 9 % increase at the national level. During the 8 years in which the technology was used, producers recorded an annual average production increase of 10.1 %. In 2006, the combined impact of the higher crops, improved quality, and lower production costs upon farm incomes meant a production increase of 9.3 % (33,230 t).

As against 2006, in 2007, Romania had to make additional hard currency efforts—amounting to € 60.5 million—in order to compensate its deficit of soy beans (worth over € 30 million) and meals (almost € 20 million), as well as for the soybean oil it never exported (Dinu et al. 2011).

In 2008, the country's trade balance value deficit for the three commodities increased. The difference between the 2008 and 2006 trade balances reached € 117.353 million, of which € 58.084 million were due to the country's additional imports of soybean meal, € 39.322 million to the imports of soybeans, and € 19.947 million to the soybean oil (Dinu et al. 2011).

In real terms, the higher trade deficit meant an indirect loss to soybean farmers, particularly to those who had to renounce GM soybeans. As a result of the ban, most Romanian farmers gave up growing soybeans in general, viewing the newly-established subsidy program not remunerative enough to compensate for the lack of competitiveness of conventional varieties.

Dinu et al. (2011) appreciate that the ban on GM soybean cultivation had the following effects:

- A dramatic fall in the soybean areas and implicitly of soybean production, leading to problems in the raw material supply chain to processors;
- A significant increase in the country's soybean imports, as Romania re-became a net importer of soybeans, having undergone an additional hard currency effort of € 60.5 million in 2007 and of € 117.353 million in 2008;

Table 3.7 Romania's imports of soybean seeds and soy meal

Year	Seed		Meal	
	Quantity (MT)	Price (USD)	Quantity (MT)	Price (USD)
2006	11,945	4,244,413	81,544	21,851,061
2007	68,600	34,022,346	217,039	74,500,459
2008	94,361	55,512,308	315,012	155,403,943
2012	63,000	40,000,000	482,000	237,000,000

- Farmers' losing out on a potential profit of € 11.1 million in 2007 and of € 19.85 million, in the second year;
- The government paid out direct aids for conventional soybeans, totalling € 9.7 million in 2007 and € 8.3 million in 2008, which however proved insufficient in terms of compensating the lack of competitiveness of conventional varieties and rendering them attractive to farmers;
- Indirect losses to farmers, of € 3.4 million in 2007 and € 5.865 million in 2008 (let alone their additional efforts to control problematic weeds).

As a result of implementing the legislation that regulates GM plant cultivation in the EU, Romania has become an importer of this product, from Argentina, Brazil, and the United States, either directly or indirectly (via EU Member States)—Table 3.7.

Romania has become a net importer of plant protein, just like the European Union (Stein and Rodríguez-Cerezo 2010). The EU imports about 90 % of its total amount of soybeans, its main suppliers being the U.S., Argentina, and Brazil—countries that grow mainly RR soybeans. To the Romanian national economy, hard currency losses caused by the higher imports are estimated to exceed millions of Euros every year (Otiman et al. 2008; Dinu et al. 2011).

Compared to the potential that soybean crop has in Romania, Europe's total soybean area is small. Romania's soybean area could be as high as 500,000 ha, which at regular yields would bring an export surplus of 800,000 to 1 million t of beans, meal, and cakes. Another opportunity generated by the current dynamics of the world market is the use of soybean oil for biodiesel production. Yet in spite of all these possibilities, Romania is increasingly dependent on soybean imports (Dinu et al. 2011).

3.4 Environmental Impact of Growing GRS in Romania

3.4.1 Risk Analysis Elements

The risk analysis of GRS cultivation in Romania is based on the following main assertions:

- Soybeans are not sexually-compatible with any native or introduced wild plant species present in Europe (OECD 2001);
- Soybeans are a self-pollinated species, which is commercially propagated by seed, cross-pollination incidence representing less than 1 %, as a rule (OECD 2001);

- Soybeans cannot survive without human assistance and are unable to survive as a weed;
- Soybeans have few weedy tendencies, since they share few of the traits that are specific to genuine weeds (Baker 1974);
- GM soybeans will be commercially grown in pre-existing agro-ecological environments and the direct/indirect environmental effects of RR technology would likely be largely similar to those resulting from conventional chemical spraying;
- In Romania, *Glycine max* is not found outside cultivation and so far, no hybridization between soybeans and other spontaneous/cultivated pulses has been known to occur;
- The biology of soybeans has been studied and it is well known in Romania (Ciocîrlan 1990; Popescu and Sanda 1998).

Wild soybean species are native to China, Korea, Japan, Taiwan, and former USSR. In Romania, there are no wild relatives of soybeans, nor any other plants that are sexually-compatible with soybeans. Therefore, the potential of gene flow from cultivated soybeans to other plants is virtually impossible.

Soybeans are almost entirely self-pollinated, out-crossing levels averaging 1 % (OECD 2001). As result, implementing field coexistence measures can be relatively easy. According to Yoshimura et al. (2006), the greatest distances between the receptors (conventional soybeans—*Glycine max* (L.) Merr.) and an adjacent pollen source (namely a GM glyphosate-tolerant soybean crop) at which out-crossing was observed were 7 m in 2001, 2.8 m in 2002, and 3.5 m in 2004. Results regarding airborne pollen density measured during the flowering period indicated that the possibility of out-crossing by wind was minimal. In field conditions in Brazil, out-crossing of conventional crops by adjacent GM soybean fields is minimal if the fields are situated more than 10 m apart (Bindraban et al 2009).

The soybean plant is not weedy in character. Soybeans possess few of the characteristics of plants that are weeds (Baker 1974). Soybeans are not an overwintering crop: they are not frost-tolerant and do not survive freezing winter conditions (OECD 2001). In Romania, *Glycine max* is not found outside cultivation (Ciocîrlan 1990; Popescu and Sanda 1998). Monitoring activities undertaken in Romania since 1999 during the first years of cultivation confirmed the conclusion of Environmental Risk Assessment submitted by the producer: genetic modification did not enhance the weediness and invasiveness of herbicide-tolerant soybeans (Badea et al. 2004, 2006).

3.4.2 Monitoring Results

Under the Romanian biotech legislation, postmarketing monitoring aims to confirm the conclusions reached during the environmental risk assessment and to identify unpredictable adverse effects. In Romania, GRS is commercially grown in preexisting agroecological environments and the direct and indirect environmental impacts of RR technology are likely to be largely similar to those resulting from conven-

tional chemical spraying. In order to confirm some of the conclusions drawn in the environmental risk assessment submitted by the applicants, field experiments (case-specific monitoring) were undertaken to evaluate the impact of RR *versus* conventional technology on soil microorganisms, arthropod fauna, and weed populations. The results of these monitoring activities, carried out on RR soybean crops in Romania have been published (Badea et al. 2004, 2006).

A general surveillance program was conducted between 2002 and 2004, in which farmers growing RR soybeans were asked questions about plant behaviour in the new agroecosystems. The answers were viewed as indicators for soybean behaviour in terms of the plant's invasiveness, persistence, rate and/or way of reproduction, dissemination, survivability, etc.

A case-specific monitoring program was carried out in 2004 in a monoculture (2002–2004) soybean experimental field located at the Didactic Experimental Station in Moara Domnească, focusing on the structure and composition of the weed population, the invertebrate fauna population, and the heterotrophic bacteria and microscopic fungi in both RR and conventional plant rhizospheres. The experimental protocol included several variants of conventional cropping and one RR technology applied on two RR soybean cultivars (Badea et al. 2006).

The monitoring conclusion was that RR soybeans are not persistent in agricultural habitats and their invasiveness into natural habitats is not altered, compared to conventional soybeans. Annual and perennial weed species are drastically eliminated from soybean crops through herbicide applications. After the treatment, some annual species germinate from remaining seeds, develop, flower, and replenish seed banks in the soil. All weed species proliferate on field edges and along access pathways and produce a large quantity of seeds. Their existence is not jeopardized. The results of a field study aimed at assessing the impact of RR versus conventional on the arthropod fauna showed no significant differences among epigeal and beneficial insects in soybean crops (in terms of population size and/or make-up). No obvious adverse effects on the soil biota (heterotrophic bacteria and microscopic fungi) were identified during RR soybean cultivation. The environmental risk of using RR soybean technology may therefore be regarded as negligible (Badea et al. 2004).

3.4.3 Environmental Impact of Weed Management on Soybean Crops

Soybean weed control relies on products containing active ingredients in various amounts and combinations. For instance, in the U.S., conventional soybean weed control uses Trifluralin-, Pendimethalin-, Chlorimuron Ethyl-, Imazaquin-, and Imazethapyr-, based herbicides (Bonny 2008). In Argentina, the same operation relies on Haloxyfop-, 2,4-D-, Imazethapyr-, Metsulfuron-, Dicamba-, Metribuzin-, and (pre-emergent) Glyphosate-, based herbicides (Franke et al. 2011). Conventional soybean crops in Brazil use Imezethapyr-, Chlorimuron Ethyl-, Haloxyfop-, 2,4-D-, Fluazifop-P-Butyl-, and Lactofen-, based herbicides (Franke et al. 2011). Lastly, in

Table 3.8 Environmental impact of some active ingredients used in weed management in soybean crops

Active ingredient	Total EIQ (kg a.i./ha)	Farm worker EI	Consumer EI	Environmental EI
Bentazon	18.7	16	9	31
Fluazihop-P butyl	28.7	10.6	3.3	72.1
Glyphosate	15.3	8	3	30
Imazamox	19.5	8	8	42.5
Metribuzin	28.4	8	8	69.1
Metsulfuron-metyl	16.7	8	8	33
Quizalofop-p-tefuril	13.3	18	10	12
Quizalofop-p-etyl	22.1	10.6	3.3	52.4
Trifluralin	18.8	9	5.5	42
Tifensulphuron metyl	7.3	6	4	12

Romania, conventional soybean weed crops use Glyphosate-, Acetochlor- (banned as of June 2012), Metribuzin-, Tifensulfuron methyl-, Imazamox-, Bentazon-, or Quizalofop-P-Tefuryl-, based herbicides (Buzdugan 2011). Both the impact coefficients of these herbicide agents and the weights of the substances in the various commercial formulations vary largely (Tables 3.8 and 3.9).

Table 3.10 lists the main herbicide active ingredients used on soybean crops and the values of their impact coefficients (Kovach et al. 1992).

The data regarding the use of herbicides in RR and conventional soybean crops in the main producing countries indicate that a larger amount of products in this category is used for the former than for the latter, because of the amounts of glyphosate used (Bonny 2008; Franke et al. 2011). The environmental impact coefficient of glyphosate has, however, a low value (Table 3.11).

Herbicide Quantities in the Environment In 2006, Romania's soybean crops already totalled 190,800 ha, of which 137,300 were declared by the 1214 farmers registered with the National Registry of GM Crop Growers as having been planted to GM soybeans (Dinu et al. 2011).

Assuming that conventional soybean crops were treated to 4.97 kg of active ingredients per hectare and GM soybeans with only 1.62 kg/ha (Table 3.9), in 2006, on the 137,300 ha planted to glyphosate-tolerant soybeans the amount of active ingredients applied was lower by 43,469 kg compared to the amount applied on the 53,500 ha of conventional soybeans (Table 3.10).

If the entire area allocated to soybeans had been planted to conventional varieties and the crops had been treated according to the programs described in Table 3.10 (Buzdugan 2011), the environment would have received a residual amount of 948,276 kg of active ingredients from the herbicides (Table 3.10). If, however, the entire soybean area had been planted only to GM varieties and the crops had been treated only according to the GM-specific program, the environment would have received only 309,096 kg of herbicide active ingredients. Consequently, in 2006, if the soybean area had been planted only to glyphosate-tolerant cultivars, the environment would have been spared 639,180 kg of herbicide active ingredients.

Table 3.9 Herbicide active ingredients and the amounts used in weed management in GM or conventional soybean crops. (Source: Buzdugan 2011)

Product name	Active ingredient (a.i.)	Dosage g/kg or g/L	a.i./ha L/ha; kg/ha	Total a.i. (kg/ha)
Conventional soybeans (2006)				
Roundup	Glyphosate	360 g/L	4.5	1.620
Basagran F	Bentazon	480 g/L	2.5	1.200
Treflan 24 CE	Trifluralin	240 g/L	2.0	0.480
Surdone	Metribuzin	700 g/kg	0.7 kg/ha	0.490
Galaxy	Bentazon Acifluorfen	360 g/L 80 g/L	2.0	0.720 + 0.160
Fusilade Super	Fluazihop-P butyl	150 g/L	2.0	0.300
Total				4.970
RR soybean (2006)				
Roundup	Glyphosate	360 g/L	4.5 L/ha	1.620
Total				1.620

Table 3.10 Environmental impact of herbicide application in GM vs. conventional soybeans, Romania

Product name	Active ingredient (a.i.)	Dosage g/kg or g/L	Rate (L, kg/ha)	Active ingredient amounts (kg/ha)	EIQ/ha
Conventional soybeans[a]					
Roundup	Glyphosate	360 g/L	4.5	1.620	24,834
Basagran F	Bentazon	480 g/L	2.5	1.200	22,404
Treflan 24 CE	Trifluralin	240 g/L	2.0	0.480	9.038
Surdone	Metribuzin	700 g/kg	0.7 kg/ha	0.490	11,700
Galaxy	Bentazon Acifluorfen	360 g/L 80 g/L	2.0	0.720 + 0.160	17,213
Fusilade Super	Fluazihop-P butil	150 g/L	2.0	0.300	8.613
Total/ha				4.970	93,802
53,500 ha				265,895	5,018,407
190,800 ha				948,276	17,897,421
500,000 ha				2,485,000	46,901,000
RR soybeans					
Roundup	Glyphosate	360	2 × 2.5	1.620	24,834
Total/ha				1.620	24,834
137,300 ha				222,426	3,409,708
190,800 ha				309,096	7,676,090
500,000 ha				810,000	12,417,000

[a] Calculations based on data published by Buzdugan (2011)

In the herbicide treatment programs used for GM soybeans, not only the amount of active ingredients is lower, but also the impact upon health and the environment (Table 3.11). The values of the three impact components and of the total impact are considerably lower than in the herbicide treatment programs used for conventional

Table 3.11 Environmental impact of herbicide use in GR *versus* conventional soybeans

Item	Non-GM	GM—herbicide-resistant	Difference	Difference (%)
Pesticide use (kg AI ha^{-1})	4970	1620	−1847	−53.27
Total impact (EI ha^{-1})	93,802	24,834	−6896	−73.50
Farm worker impact (EI ha^{-1})	57,675	12,960	−4472	−77.53
Consumer impact (EI^{-1})	3112	4860	−2626	−84.38
Ecological impact (EI ha^{-1})	191,125	48,600	−14,252	−74.40
Pesticide use (kg AI on total area in 2006)	265,895	222,426	−43,469	−16.34
Total impact (EI ha^{-1})	5,018,407	3,409,708	−1,172,456	−32.05
Farm worker impact (EI ha^{-1})	3,085,612	1,779,408	−1,306,204	−42.33
Consumer impact (EI^{-1})	1,664,920	668,250	−996,670	−60.00
Ecological impact (EI ha^{-1})	10,225,187	6,672,780	−3,552,407	−35.00
Pesticide use (kg AI on the 500,000 ha)	1,797,775	737,568	−1,060,207	−59.00
Total impact (EI ha^{-1})	34,201,600	11,299,840	−22,901,760	−67,00
Farm worker impact (EI ha^{-1})	28,837,500	6,480,000	−22,357,500	−77.53
Consumer impact (EI^{-1})	15,560,000	2,430,000	−13,130,000	−84.00
Ecological impact (EI ha^{-1})	95,562,500	24,300,000	−71,262,500	−74.57

soybeans. For instance, the impact upon farm workers is 77% lower, whereas the impact upon consumers is 84% lower when RR technology is used, compared to conventional weed control methods.

If conventional soybean crops were extended on 500,000 ha and they were treated with the herbicides mentioned in Table 3.10, the environment would receive 1,797,775 kg of active ingredients. If the same area were planted to GM cultivars and the associated herbicide treatments were applied, the environment would receive only 737,568 kg of active ingredients, that is 59% less than for conventional crops, while the environmental impact coefficient would be 67% lower. Regardless of the size of the GM soybean area, the associated herbicide treatment program has a significantly lower impact upon human health compared to the herbicide program used in conventional soybeans (Table 3.11).

According to Brookes and Barfoot (2005), who used the same methodology to calculate the amounts of herbicides used in conventional as well as GM soybean crops in 1999–2006, RR technology led to a small increase in the amount of active ingredients used and a net reduction in the EIQ compared to the data recorded and calculated in exclusively conventional crops.

Kleter et al. (2007) calculated the EIQ using data collected by the NCFAP (National Centre for Food and Agricultural Policy) regarding the use of herbicides in GM and conventional soybean crops in 2000, 2003, and 2004, in the United States. In 2004, collected data and calculation results revealed a 25% reduction in the active herbicide ingredients used as well as a lower impact thereof on the environment—down by 59% for GM soybeans, compared to their conventional counterparts. The

Table 3.12 Environmental impact of herbicides used in herbicide-resistant GM crops in the U.S.—2004. (Pesticide use data, 2004, Sankula et al. 2005)

Item	Non-GM	Herbicide-resistant GM crops	Difference	Difference (%)
Pesticide use (kg AI ha^{-1})	1.5	1.2	−0.4	−25
Total impact (EI ha^{-1})	42.8	17.7	−25.1	−59
Farm worker impact (EI ha^{-1})	29.3	9.2	−20.1	−68
Consumer impact (EI^{-1})	14.1	5.8	−8.4	−59
Ecological impact (EI ha^{-1})	85.0	38.1	−46.9	−55

herbicide program applied on GM soybeans had a smaller impact upon farm workers (down by 68%), consumers (down by 59%), and ecosystems (down by 55%) compared to the herbicide program used for conventional soybeans (Table 3.12).

The environmental impact of herbicide treatments used as a result of GM crop adoption becomes even more evident if one considers the "behaviour" of the herbicides in the environment. For instance, in Argentina, once soybean RR technology was adopted, the use of Toxicity Classes II and III herbicides was reduced by 83–100%, while the use of less toxic herbicides in Class IV rose by 248%. In North Carolina, U.S., GM cotton crops use herbicides whose potential of leakage is 25% lower than herbicides applied on conventional cotton (Kleter et al. 2007).

Stewart et al. (2011) studied the efficiency, environmental impact, and profitability of various herbicide treatments used on glyphosate-resistant soybeans, for 3 years, in three different locations in the United States. The study also determined the herbicide treatment with the lowest selection pressure in terms of glyphosate-resistant weed species. In order to reduce the selection pressure related to the glyphosate-resistant weed biotypes, to reduce the environmental impact, and increase profitability, the authors recommend using a mix of herbicide agents with two modes of action. The results indicated that the best alternative method to the two-step glyphosate treatment was a combination between glyphosate and Imazethapyr.

Dill et al. (2008) recommend herbicide programs that include a preemergent herbicide—either combined with a glyphosate-base product, or followed by a treatment with glyphosate. Using agents with different modes of action is the proper strategy of preventing resistance to herbicides in weed populations. The risk of weeds developing glyphosate resistance is much smaller in Romania than in other countries growing RR soybeans. Taking into account the agronomic practices, the social demand, and the need to ensure maximum efficiency, the best rotation is 4 years, using six crops: wheat/rapeseed + barley/corn + sunflower/soybeans (Buzdugan 2011).

Conclusion: both the amount and the impact of herbicide agents applied to GM plants would go down, compared to the alternative programs used for conventional crops.

Soil Management and Conservation Tillage Biotech herbicide-tolerant soybeans have enabled the adoption of conservation tillage in most countries that have

adopted the technology (Cerdeira and Duke 2010). In Romania, farmers have not adopted the low-till or no-till systems.

Worldwide, several studies have shown that both previous and potential effects of glyphosate—in terms of soil, water, and air, contamination—are minimal, compared to the impact of herbicides that would be otherwise used and which glyphosate herbicides replace when GRS are adopted. In the U.S. and Argentina, the advent of glyphosate-resistant soybeans has led to a significant shift to low-, and no-, tillage practices, thereby significantly reducing environmental degradation by agriculture (Cerdeira et al. 2010; Cerdeira and Duke 2010). According to Cerdeira and Duke (2010), both glyphosate and aminomethylphosphonate (AMPA)—its degradation product—are considered to be much more toxicologically and environmentally benign than most herbicides replaced by glyphosate.

3.5 Conclusion

Romania is one of the few European countries with favourable conditions for soybean production. GRS were grown commercially in this country beginning with 1999 and accounted for 68 % (or 137,000 ha) of the entire soybean area planted in 2006. In 2006, Romania stood among the eight countries that cultivated the crop worldwide. Since 2007, after its accession to the European Union—which had approved the imports, but not the cultivation of RR soybeans on its territory—the Romanian farmers' access to the technology was banned, at a time where it had started being used on increasing areas, because of its efficiency in controlling weeds at low costs. Glyphosate-tolerant soybeans have been regarded by farmers as their most profitable crop, since it enabled them to obtain higher yields, at lower costs. As a result, in only 2 years, the area planted to soybeans shrunk by 70 %, while Romania became a net importer of plant protein, just like the European Union.

The results of a field case-specific monitoring conducted in order to evaluate the impact of GR *versus* conventional technology on the arthropod fauna showed no significant differences among the epigeal and beneficial insects typical of soybeans (in terms of population size and/or composition). No obvious adverse effects on the soil biota (heterotrophic bacteria and microscopic fungi) were identified upon growing RR soybeans. No GR weeds were reported during the 8 years in which the RR technology was used in Romania in two-steps treatments of 2 L/ha each, after emergence.

Romania was the only country in Europe that grew a GM, herbicide-tolerant plant—glyphosate-resistant soybeans—for commercial purposes. In 2006 alone, the amount of herbicide agents applied per hectare for conventional soybeans was considerably higher than what was used for GM soybeans. In 2006 alone, the 137,300 ha planted to glyphosate-tolerant soybeans received 176,388 kg of herbicides less than on the 53,500 ha planted to conventional soybeans. The environmental impact coefficient was about 70 % lower—both per hectare and for the entire GM cultivar area (which represented 72 % of the total soybean area that year).

If soybeans were planted on 500,000 ha—almost half of the area that lends itself well to this highly-important economic crop in Romania—the total amounts of herbicide ingredients applied would be 2,100,100 kg in case only conventional varieties were grown and 765,000 kg in case only RR varieties were grown. Which means that in case glyphosate-resistant, GM, soybeans were grown, the environment would be spared 1,335,000 kg of herbicide active ingredients, and the herbicide impact coefficient would be 67 % lower than for conventional crops.

A legal framework is a necessary, but not sufficient condition to make the right decisions in a certain field and at a certain time. Equally important is the enforcement—on a scientifically sound basis and in good will—of existing laws, enabling their use by a certain social group and, at the end of the day, by the whole society. At the same time, an excessive legal framework, enforced without responsibility, may trigger dramatic socioeconomic consequences.

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Chapter 4
The Effects of Transgenic Crops on Non-target Organisms

Chandrakanth Emani

Abstract A non-monitored cultivation of transgenic crops can potentially have adverse effects on animal biodiversity when the transgenic plants or their expressed products negatively impact the organisms that are not intended to be the targets that need to be controlled. Agro-ecosystems house a diverse array of species above and below the cultivated ground that can come in contact with the cultivated plants and their metabolites. When a transgenic crop intended for pest control is planted in the field, the resulting effect on the agro-ecosystem cannot exclude the rest of the species in the habitat non-intended to be harmed by the transgenics, and these are defined as the non-target species. The present review summarizes the possible effects of transgenic plants on non-target species in agro-ecosystems with a focus on possible strategies to minimize the unintended effects of transgenic crop cultivation on animal biodiversity, while complementing the efforts of integrated pest management.

Keywords Transgenic crops · Non-target species · *Bt* maize · Monarch butterfly · Pollinator

4.1 Introduction

Transgenic crops are the fruits of biotechnological research that enable plant genetic engineers to ensure the stable integration, desired level of expression, and predictable inheritance of numerous agriculturally important genes. The present agricultural revolution sometimes referred to as gene revolution, which continues the green revolution of the 1960s resulted in the cultivation of transgenic crops expressing herbicide tolerant and insect resistant genes. Herbicide resistant transgenics, especially glyphosate-resistant soybean, cotton and corn have contributed to effective weed management strategies in the respective crops (Green and

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Owen 2011). Unlike the herbicide-resistant transgenics, the insect-resistant transgenics involve effect of the expressed *cry* genes derived from the soil bacterium *Bacillus thuringenesis* on living organisms in the biosphere such as insects of the species Lepidoptera and Coleoptera (Arpaia 2012). Since the insect species including insect pests make up a significant proportion of an agro-ecosystem, legitimate concerns exist of the commercial transgenics affecting the non-target insect and soil microorganism populations. Monitoring agencies such as the USDA and the European Union provide guidelines for the non-target organism testing prior to transgenic commercialization, but few if any of the guidelines relate to risks at the ecological level (Arpaia 2012). An agro-ecosystem also depends on physiological field functions such as microbial decomposition, nutrient cycling, crop pollination by animals and biological pest control and these in turn involve diverse animal species other than insects (Curtis et al. 2002) and hence, a thorough examination of transgenic cultivation effects should include all these in the non-target populations. Some of the recent reviews on effects of transgenics on non-target organisms dealt with studies focused on arthropods at field and laboratory level (O'Callaghan et al. 2005; Lövei and Arpaia 2005; Romeis et al. 2006), sometimes utilizing meta-analysis (Marvier et al. 2007; Lövei et al. 2009). Reviews also exist of the effects on soil microorganisms (Widmer 2007; Filion 2008) and soil-associated meso and macro-fauna (Icoz and Stotzky 2008). The present review aims to present an overview of various studies on the effect of transgenic crops on non-target organisms focusing on sound environmental assessments with emphasis on agro-ecosystems.

4.2 The Study that Started it All—The *Bt* Maize-Monarch Butterfly Controversy

The much discussed and dissected study that started a serious discussion on the effect of transgenic crops on non-target insect populations was the Losey et al. (1999) report that showed the *Bacillus thuringenesis* (Bt) corn plants' pollen on monarch butterflies. In a laboratory assay, it was demonstrated that larvae of monarch butterfly, *Danaus plexippus*, reared on milkweed leaves, dusted with pollen from *Bt* corn, ate less, had slow growth and high mortality compared to those reared on normal corn pollen (Losey et al. 1999). It was argued that the dispersal of the corn pollen by wind to almost 60 m and its deposition on other plants might affect non-target insect populations. Subsequent studies examined various flaws in the experimental set up (Hodgson 1999) such as non-relatable field conditions in terms of pollen availability and existence of milk weed plants in the real world, inappropriate experimental controls and the absence of stringent quantification of the amount of pollen used in the experiments. However, environmental groups and media seized the opportunity to sensationalize the issue of transgenic crops being a threat to non-target insect populations.

4.3 Effects of Transgenic Crops on Natural Pests

The negative effects of transgenic crops on non-target organisms can be functionally categorized at the levels of ecological, agricultural and other anthropocentric values (Arpaia 2012). If looked at under these broad perspectives, a more realistic picture about the effect of transgenics on the non-target organisms can be elucidated. A starting point for this kind of examination would be with natural pests and the natural pest control due to predators and parasitoids in agriculture that does account for about 95% control of potential pests (Debach and Rosen 1991). Transgenic plants with insect resistant genes potentially introduce novel metabolites into an agro-ecosystem such as *cry* toxins (Naranjo 2009), proteinase inhibitors (Malone et al. 2000) and lectins. The expressed insecticidal toxins are selectively and specifically toxic to various insect orders, and this specificity by itself can be considered as an important characteristic that limits any effects on non-target insect populations (Arpaia 2012). Nevertheless, after examining many lab studies related to effects of the transgenic insecticidal toxins using meta-analysis, it was concluded that the few studies that showed significant negative effects of transgenics on non-target species were replete with limitations in terms of sample size, statistical insufficiency and the duration of toxicity tests (Lövei et al. 2009). An extensive meta-analysis of extant literature on the invertebrate non-target effects that revealed hazards identified in laboratory tests may not always manifest in field, and the minor negative effects exhibited by transgenic *Bt* plants were insignificant when compared to insecticide-based pest suppression (Marvier et al. 2007; Naranjo 2009). Studies with transgenic *Bt* potatoes also examined the natural pests (Wolfenbarger et al. 2008) as well as the tangential effects on sucking herbivores that maintain the toxic products upon ingestion (Obrist et al. 2006) showed no adverse effects of the expressed Bt toxin on non-target species such as the lacewings (Andow et al. 2006), ladybirds (Dhillon and Sharma 2009), ground beetles (Haughton et al. 2003; De la Poza et al. 2005) and honeybees (Duan et al. 2008). Varying to insignificant effects were seen on parasitoids, but these indirect effects need to be considered in an ecological contexts such as abundance and diversity of the parasitoids for both pest and non-pest species (Arpaia 2012).

4.4 Effects of Transgenic Crops on Pollinators

Since transgenic plants, just like the regular crop species depend on pollinators for their optimal reproduction, it is imperative to consider the effects of the expressed transgenic products on the various pollinator insect species that are non-target population. Recent studies showed no deleterious effects of transgenic herbicide-tolerant or insect-tolerant on pollinators (Malone and Burgess 2009). The one *Bt* toxin that was shown to have a potent effect on Hymenopteran insects was Cry5 (Garcia-Robles et al. 2001), but no Cry5-exprssing plants have been approved for commercial cultivation. The more popular Cry1 toxin expressing plants have no effect on pollinators such as honeybees (Ramirez-Romero et al. 2005; Rose et al.

2007), a fact confirmed by extensive meta-analysis of laboratory tests assessing honeybee survival on commercial *Bt* crops (Duan et al. 2008). Other transgenes such as serine protease inhibitors were shown to affect honeybees and bumblebees at very high concentrations (Malone and Burgess 2009). Herbicide tolerant transgenic crops may have an indirect effect on pollinators (Haughton et al. 2003) in an agro-ecosystem due to reduced flowering.

4.5 Effects of Transgenic Crops on Soil Fertility Inducers

The availability of beneficial nutrient-rich fertile soils is dependent on effective microbial functioning in the soil and the presence of soil-dwelling invertebrates involved in nutrient recycling and decomposition of organic matter is a significant parameter of an efficient agro-ecosystem (Moore et al. 1988). In an agro-ecosystem, plants themselves contribute to the thriving of beneficial soil fertility inducers through the release of useful soil exudates (Brussaard et al. 2007; Arpaia 2012). Cry1 toxins expressed in *Bt* corn did not show any adverse effects on one of the most efficient soil fertility inducer, earthworm (Saxena and Stotzky 2001; Schrader et al. 2008). Though some laboratory studies showed effect of Bt corn on mean fresh weight of earthworms after a 160 day exposure, the same was not observed in the field (Zwahlen et al. 2003). Cry toxins were proposed to be hazardous to the nitrogen fixing nematode population of *C. elegans* (Höss et al. 2008), but this was later dubbed an indirect effect as the Cry toxin in *Bt* maize samples was not sufficiently high to produce the same toxic effects in growth chamber studies (Saxena and Stotzky 2001), a fact further corroborated by both glasshouse studies (Griffiths et al. 2007b) and field studies (Griffiths et al. 2007a). Apart from *Bt* maize, nematodes being effected was studied with *Bt* oilseed rape and a direct correlation was observed between transgenic oilseed rape and the abundance of fungal feeding nematodes (Manachini and Lozzia 2002). Studies on a model decomposer, the woodlouse with *Bt* maize and purified cry toxins did not show any adverse effects (Escher et al. 2000; Clark et al. 2006). Another important group of soil fertility inducers and indicators of soil health that live in root zones of plants, the collembolan were exposed to purified cry toxins and did not show any adverse survival, growth or reproduction impacts (Sims and Martin 1997). Other decomposers such as diplopods that regularly occur in corn fields showed no adverse effects when fed with *Bt* maize (Weber and Nentwig 2006).

4.6 Effects of Transgenic Crops on Soil Microorganisms

Soil microorganisms are involved in fundamental processes such as decomposition of organic matter, mineralization, chemical decomposition and improvement of soil structure (Gupta and Yeates 1997) and root exudates released by field crops

selectively regulate these organisms in an agro-ecosystem (Lynch 1994). A majority of recent studies (Icoz and Stotzky 2008) indicate *Bt* transgenics having no adverse effects on soil microorganism populations. Xue et al. (2005) found a lower ratio between gram-positive and gram-negative bacteria in fields of *Bt* maize, but a reverse of the same in a *Bt* potato field. Root exudates of *Bt* corn were shown to result in genetic modification specific to a changed physiology and composition of root exudates that in turn affect symbiotic and rhizosphere microorganisms (Castaldini et al. 2005), but this might be disadvantageous to the individual crop and would not affect the agro-ecosystem as a whole (Widmer 2007).

4.7 Revisiting the Monarch Butterfly Controversy—Assessing Risks in Field

The study of Losey et al. (1999) combined with the charismatic and iconic christening by over enthusiastic environmentalists and media of the lepidopteran monarch butterfly triggered numerous studies on the effect of transgenic corn on monarch butterfly populations. Later field studies showed that the risk to monarch butterfly in terms of toxic levels of transgenic pollen is minimal simply due to the limited spatial distribution of pollen (Pleasants et al. 2001) and the insignificant exposure of larva during the pollen shed (Oberhauser et al. 2001). Studies in USA transgenic *Bt* corn fields specific to effects on monarch butterfly larvae continuously exposed to the transgenic crop during anthesis showed insignificant effects on mortality (Dively et al. 2004), though laboratory studies continued to show reduction in feeding and weight gain (Anderson et al. 2004; Prasifka et al. 2007). The differences in field results was later attributed to the fact that early larval instars are less exposed to *Bt* pollen drift as they feed on the upper third of milkweed plants that have lesser densities of anthers and the larva tending to move on the underside of leaves avoiding contact with anthers (Pleasants et al. 2001; Anderson et al. 2004; Jesse and Obrycki 2003).

4.8 Conclusion

After the rich dividends reaped by the agricultural community through green revolution, the commercial cultivation of transgenic crops is being seen as its successor and this important scientific event is being christened as the gene revolution (Birch and Wheatley 2005). Though the adoption of transgenic technology was one of the fastest across the world (James 2009), the backlash in Europe as compared to USA can be attributed to societal and political differences (Marshall 2009). An important factor when examining issues such as the subject of this review puts the onus on the scientific community to properly educate the general public and the political decision makers, and when disseminating their findings take into consideration the larger perspective of the agro-ecosystem instead of jumping to unwarranted conclusions based on individual laboratory studies.

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Chapter 5
Agricultural Biotechnology for Health and the Environment

Sven Ove Hansson

Abstract Due to the social circumstances surrounding agricultural biotechnology, its potential to help achieving environmental improvement and more healthy foodstuffs has not been actualized. With respect to health, biotechnology can improve the micronutrient contents of staple food. It can provide crops with a more balanced amino acid composition and a more healthy fatty acid composition. Toxic and allergenic substances can be removed, and energy density can be reduced in order to lessen the risk of obesity and diabetes. With respect to the environment, cultivars can be developed that require less tilling, thereby bringing down soil erosion and nitrogen leakage. More drought tolerant cultivars will decrease the need for irrigation that is a major cause of environmental problems. Plants with improved nitrogen efficiency will diminish the use of fertilizers, and pesticide resistant crops the use of pesticides. Although by no means a panacea, genetic technology facilitates breeding and widens the scope of what it can achieve, not least in terms of more healthy products and a more environmentally friendly production.

Keywords Agricultural biotechnology · Genetic modification · Recombinant DNA · Agricultural ethics · Food ethics

5.1 Introduction

In discussions on the social consequences of a technology it is important to distinguish between its potential and actual consequences. Many technologies can be used in a large number of different ways, some of which may be beneficial and others harmful. If current uses of a technology have adverse effects, the solution may either be to stop using the technology or to ensure that it is used in better ways. These distinctions should be self-evident, but in the discussion about biotechnology they are often blurred. In this chapter I will focus on what I consider to be important beneficial uses of agricultural biotechnology for human health and the environment

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that as yet remain largely unrealized. (I will not discuss the potential negative effects or detrimental uses.)

Biotechnology is a broad and somewhat vague term (IAASTD 2008). The Oxford English Dictionary defines it as "[t]he application of science and technology to the utilization and improvement of living organisms for industrial and agricultural production and (in later use) other biomedical applications". In what follows, the focus will be what is usually called "genetic engineering", i.e. the introduction of exogenous genes in an organism.

If the beneficial potentials of biotechnology had been fully unfolded then there would have been no need for a text like this. Due to the social circumstances surrounding this technology, some of its major positive capacities have not yet been realized. Many governments have withdrawn from plant breeding, leaving the field to breeding companies who cannot be expected to have the same breeding goals as publicly funded research. In most of the world GM crops are subject to regulations that on the one hand make the introduction of new crops very expensive but on the other hand provide innovators with strong intellectual property rights to their products.

The current legal structure surrounding plant breeding is counterproductive in the sense of thwarting the aims that it was set up to achieve. Due to the high entrance costs for most of the more innovative new cultivars, breeding activities are focused on very few crops that have a large market in the industrialized economies, and much too little work is being done on crops that are suitable for subsistence farming in the third world. The same mechanism has led to a focus of breeding on too few cultivars for each crop. This may lead to a loss of traditional cultivars that could (if improved for example with resistance properties) have contributed significantly to biodiversity. Agribusiness companies have strong incentives to make it necessary for farmers to buy new seed every year. Current incentives also lead to a one-sided focus on yield at the expense of other breeding goals that would help solving environmental problems or developing more healthy food products.

It should be acknowledged that the current regulatory system was constructed to deal with legitimate worries that the new technology might have unforeseen negative effects. However, in the four decades that have passed since the short voluntary moratorium on recombinant DNA research, knowledge about the effects of genetic modification has increased dramatically (Berg et al. 1974; Berg and Singer 1995). Furthermore, since the first field trials with transgenic plants in 1986 (James and Krattiger 1996) knowledge about the potential risks of crop biotechnology has risen to a new and much higher level (Magaña-Gómez and Calderón de la Barca 2009). The original worries concerning health and the environment were rather vague and often referred to the possibility of unknown effects (Price 1978). They can now be replaced by much more specified issues concerning potential hazards that can be assessed and regulated. But current regulations are still based on an outdated view of the uncertainties in plant breeding technologies. Today, with a dramatically increased scientific understanding of genetics, and with GM crops being grown on about 11 % of the soil used for agriculture (James 2011) it is no longer tenable to treat biotechnology as a step into the completely unknown or as a gamble that can

lead to the construction of a monster that will destroy us. (But that is how the technology is still often portrayed, see for instance Smith 2007.) We need a risk policy that deals efficiently with today's uncertainties, rather than with those of the past. Instead we have counterproductive policies that prevent the realization of some of the major positive potentials of agricultural biotechnology to which I will now turn.

5.2 Biotechnology for Better Health

Diet is one of the major environmental factors that have influence on health. This can be seen for instance from the Global Burden of Disease Study 2010 that summarizes the health effects of a large number of risk factors on a global scale in terms of the calculated global number of excess deaths that they give rise to (Lim et al. 2012).

- *Obesity or overweight:* 3.37 million deaths/year. Obesity is a diet-related disease that is associated with substantially increased risk of a large number of diseases, including diabetes, ischaemic heart disease, and several types of cancer. Obesity is a growing problem not only in rich countries but also in countries such as India where at the same time malnutrition and food shortage is still a problem for significant parts of the population (Sarkar et al. 2012).
- *Childhood underweight*: 0.86 million deaths/year. The number of children dying from starvation has decreased substantially in the last two decades but is still high in many countries, in particular in Subsaharan Africa.
- *Too much processed meat:* 0.84 million deaths/year. By processed meat is meant smoked, cured and salted meat and meat with added chemical preservatives.
- *Diet high in red meat:* 0.04 million deaths/year.
- *Diet high in sugar-sweetened beverages:* 0.30 million deaths/year.
- *Diet high in trans fatty acids*: 0.52 million deaths/year. Trans fat is primarily found in fast food and bakery products.
- *Diet high in salt:* 3.10 million deaths/year.
- *Insufficient breastfeeding:* 0.55 million deaths/year.
- *Maternal and infant iron deficiency:* 0.12 million deaths/year.
- *Vitamin A deficiency in children*: 0.12 million deaths/year. Lack of vitamin A is also a major cause of blindness.
- *Zinc deficien*cy: 0.10 million deaths/year.
- *Diet low in fruits:* 4.90 million deaths/year.
- *Diet low in vegetables*: 1.80 million deaths/year.
- *Diet low in whole grains:* 1.73 million deaths/year.
- *Diet low in fibre*: 0.74 million deaths/year.
- *Diet low in nuts and seeds*: 2.47 million deaths/year.
- *Diet low in milk:* 0.10 million deaths/year.
- *Diet low in calcium*: 0.13 million deaths/year.
- *Diet low in seafood omega-3 fatty acids*: 1.39 million deaths/year.
- *Diet low in polyunsaturated fatty acids:* 0.53 million deaths/year.

To what extent can agricultural biotechnology contribute to solving these problems? For some of them biotechnology cannot be of much help. One example is excessive intake of salt. It is added salt rather than salt in the agricultural products themselves that cause the problem. But on closer inspection it will be seen that many of these problems can, at least from a technical point of view, be attacked with biotechnology. The following are some of the major examples of what can be done:

Micronutrients Lack of minerals such as iron, calcium, selenium and iodine, and vitamins such as folate and vitamins E, B_6 and A is a worldwide problem but most prominent in developing countries. Enriching staple food with essential minerals and vitamins is an efficient means to improve health, in particular in developing countries. This can be done either by postharvest addition to the food product or by modification of the crop itself. The former method is usually impracticable in subsistence farming and local small-scale distribution (Hirschi 2009; Best et al. 2011).

The most advanced project to improve the micronutrient content of food aims at enriching rice to prevent vitamin A deficiency. Golden Rice includes a maize gene that raises the β-carotene level in the product (Paine et al. 2005). Current estimates indicate that in terms of cost-efficiency and coverage it will be superior to alternative methods to reduce vitamin A deficiency (Mayer 2007). However, its introduction into agriculture has been delayed by regulatory hurdles (Potrykus 2010; Bhullar and Gruissem 2013).

Experimental work has also been done to increase the iron content of rice (Lee et al. 2009) and the content of vitamin E in soybean, maize and canola (Chassy 2004; Hirschi 2009).

Amino Acids The balance of amino acids in major staple foods is often deficient. Cereals tend to be low in lysine and legumes in methionine and cysteine. Maize, canola, and soybeans with improved amino acid composition have been developed (Chassy 2004). This is particularly important for developing countries where protein malnutrition is common.

Fat Composition The nutritional value of dietary fat depends on the chain length and the saturation of fatty acids. Studies have shown that the type of fat is more important than the amount. This applies to the risk of coronary heart disease and probably also to the risk of other diseases such as type 2 diabetes (Risérus et al. 2009). A recent Cochrane review concludes that the incidence of cardiovascular disease can be diminished by modifications in fatty acid composition; saturated fat should be reduced and partially replaced by unsaturated fat (Hooper et al. 2012). It is recommended that about two thirds of the total fat intake should be unsaturated and that trans fat should be eliminated as far as possible. The health relevant parameters of fatty acids, chain length and saturation, can relatively straightforwardly be genetically modified in many of the major crops that provide us with vegetable fats. Examples include low- and zero-saturated fat soybean and rape oils, high-oleic acid soybean oil, and rape oil without trans fatty acids (Chassy 2004).

Toxic Substances Some staple foods contain components that are toxic or cause food intolerance or allergy. Biotechnology can often eliminate or reduce the contents of the harmful substances. The content in potatoes of solanine has been substantially reduced in this way (Chassy 2004), and so have allergenic components in wheat, rice and peanuts (Lemaux 2008). The toxic cyanogenic glucosides in cassava (a staple food for 250 million sub-Saharan Africans) are another important target of genetic change (Powell 2007).

Energy Density Reduction in total energy intake is essential to reduce the incidence of obesity, type 2 diabetes, and concomitant diseases. Food intake is regulated by satiety, and foodstuffs with low energy density produce satiety at a lower level of energy intake than foodstuffs with high energy density. This is one of the reasons why intake of food with high contents of water and fibres, such as vegetables and fruit, are important components of diets for the treatment and prevention of overweight and obesity (Ello-Martin et al. 2007; Ledikwe et al. 2006; Rolls et al. 2004). It is a plausible hypothesis that reducing the energy density of traditional major foodstuffs with high energy density might potentially contribute to the prevention of obesity and related diseases.

Not much would be gained by producing healthy food that few consumers actually eat. Therefore the contributions of agricultural biotechnology to improved diets have to be parts of a larger strategy that leads to changes in what people actually eat. Such a strategy will have to take agricultural and agroeconomical considerations as well as consumer behaviour into account. New, healthier foodstuffs can either be introduced universally or optionally. Universal introduction is in practice only possible if there is no consumer demand for the previous, less healthy alternative. There does not seem to be any demand for bakery products with trans fats, and presumably there would not be much demand for poisonous cassava if a non-poisonous alternative became available.

Optional introduction, i.e. introduction as an alternative alongside the older variant, can have significant health effects if there is a consumer demand for the healthier product. One example might be low-energy but highly satiating foodstuffs that can be chosen by consumers wishing to prevent or reduce overweight.

5.3 Biotechnology for the Environment

In the period from 1700 to 1990, the global area of cropland is estimated to have increased 5.5-fold, and the area of pasture land 6.6-fold. In 1990, 29% of the world's forest areas and 49% of its grasslands, steppe and savannas had been transformed into agricultural land (Goldewijk 2001). Currently, agriculture is responsible for about 80% of the world's deforestation (UNFCCC 2007, p. 81) This dramatic reduction in wildland has a large part in the ongoing loss in species that threatens to substantially decrease the planet's biodiversity (Pimm et al. 1995; Butchart et al. 2010). In addition, agriculture gives rise to a number of well-known

environmental problems such as eutrophication due to fertilizers, erosion and soil degradation due to tilling and overgrazing, hydrological changes due to irrigation, and damage to wildlife due to pesticides. Furthermore, farming is a major contributor to the greenhouse effect. Deforestation reduces the removal of carbon dioxide from the atmosphere via photosynthesis, and farms release greenhouse gases: carbon dioxide (from tractors and machinery using fossil fuel), methane (from ruminants), and nitrous oxide (from fertilizers).

A wide range of measures are needed to reduce the negative effects of agriculture on the environment and the climate (Hansson and Joelsson 2013). Biotechnology can potentially contribute in several important ways, such as the following:

Reduced Tillage In spite of being an age-old agricultural practice that is taken to be a "natural" part of farming, tillage has severe environmental disadvantages (Yao et al. 2010). It gives rise to soil erosion and to the runoff of surface waters containing sediments, nutrients and pesticides. The loss of nutrients increases the need for fertilizers and causes eutrophication of lakes and coastal waters. Furthermore, modern methods of tillage make use of fossil fuels, thereby contributing to greenhouse gas emissions. Therefore, cultivars that need less tilling than those currently grown would be an important contribution to a more environmentally friendly agriculture. Since tilling is largely performed in order to control weeds, the need for tilling is reduced if weeds can be controlled by other means. Glyphosate resistant crops that make it possible to control weeds with this herbicide have facilitated the adoption of reduced or no-tillage agriculture (Cerdeira and Duke 2006). However, increased dependence on herbicides is not a desirable development, and other means to decrease tillage should therefore be pursued. A particularly interesting option is to replace annual cereal crops and legumes by perennial variants. Many annual cereals have perennial wild relatives with a genetic material that can be used for this purpose (Downes 2000).

Increased Drought Tolerance In many countries, irrigation has a large part in the environmental problems that agriculture gives rise to. Therefore the environmental benefits of crops that require less irrigation would be substantial. A private-public sector partnership called WEMA (Water Efficient Maize program for Africa) hopes to release a royalty-free drought tolerant maize by 2017 in Sub-Saharan Africa. Promising results have already been obtained (Thomson et al. 2010; Izge and Dugje 2011).

Less Need for Fertilizers Most of the major agricultural plants such as rice and wheat take nitrogen from the soil. Nitrogen fertilizers increase the yield of these crops by increasing the availability of nitrogen. However, fertilizers have substantial negative environmental effects, contributing both to eutrophication and to the anthropogenic greenhouse effect. Several traits are under investigation that can potentially reduce the need for fertilization, such as improved nitrogen efficiency (Foyer et al. 2011), uptake of nitrogen as amino acids (Näsholm et al. 2009) and—

more radically—transferring the ability of legumes to take up nitrogen from the atmosphere (in symbiosis with rhizobia) to cereals and other nonlegume crops (Cocking 2009; Bhattacharjee et al. 2008).

Reduced Need for Pesticides Pesticide use has negative effects on non-target animals, not least pollinators such as honey bees that have essential roles both in agriculture and in natural ecological systems (Krupke et al. 2012). Pesticides also cause serious health problems in farm workers, in particular in third world countries. Pest-resistant crops can potentially improve the situation by reducing the use of pesticides. The use of genes from *Bacillus thuringiensis* (Bt) has had substantial such affects. In Spain the adoption of Bt maize led to a large reduction in insecticide use, at the same time as yields have increased (Qaim and Zilberman 2003; Qaim 2009). In India, Bt cotton has reduced the use of pesticides by 50%, with even larger reductions (70%) for the pesticides that are most hazardous to human health (Kouser and Qaim 2011).

Pesticide resistance cannot be achieved once and for all. Pests evolve in response to new resistance mechanisms (whether they are the result of evolution or breeding), and therefore new forms of resistance have to be developed continuously in order to keep down the pests. Just like the pesticides that are applied externally for instance by seed treatment or spraying, substances synthesized by the plant itself can be toxic and have to be carefully evaluated for their effects on humans and other non-target organisms. However, substances produced by the plant itself have the advantages of less collateral spreading and (often) of only being produced when the plant is under actual attack.

Increased Yield The relationship between agriculture's yield and its environmental impact is complex. On the one hand, high yields are often associated with intensive land use that has negative environmental effects. On the other hand, high yields diminish the total area that has to be cultivated in order to produce a given amount of food, thereby making it possible to preserve more natural habitats. The potential environmental benefits of increased yields may is particularly important in developing countries (Wolfenbarger and Phifer 2000; Beddington 2010). Current breeding activities (both with and without biotechnological methods) have a strong, often virtually exclusive, focus on increasing yields. Although the major message of this chapter is that other breeding goals than yield should have a larger role, it must be conceded that increases in yield can contribute substantially to reducing the threat to the environment of an expanding agriculture that devours more and more wilderness every year. However, positive environmental effects of yield increases should not be taken for granted. Especially since World War II, yield increasing technologies have had substantial harmful effects on the surrounding environment (Pimentel and Patzek 2005; Heathcote and Downing 2012; Mitra et al. 2011). It should be a central concern in plant breeding to find ways to increase yields without negative impact on the environment.

5.4 Conclusion

Hopefully, this rundown of potential biotechnological developments is sufficient to show that plant breeders can make important contributions to the attainment of both environmental and health-related goals in agriculture. In addition to these concerns, close attention should be paid to the social effects of agricultural technology, in particular to the situation of subsistence farmers in the third world. Their situation depends to a large extent on legislation and other social conditions surrounding the technology, but the design of the technology itself is also important. As one example of this, new cultivars can be constructed either to increase or decrease farmers' dependence on seed companies. Decreased dependence can be achieved with apomictic cultivars that make it possible to use part of the harvest as seeds rather than buying new seed every year (Reeves 1997).

There can hardly be any doubt that breeding will continue to be one of the most important tools for agricultural improvement, as it has been since the beginnings of farming about 10,000 years ago. It is equally obvious that genetic technology, although by no means a panacea, facilitates breeding and widens the scope of what it can achieve, not least in terms of more healthy products and a more environmentally friendly production. Therefore it is unfortunate that many of those who are most worried about health and the environment have chosen to reject all uses of the most efficient tools that we have to improve our crops, instead of calling for their prudent use to solve the difficult environmental and health-related challenges that today's agriculture is facing. We need to refocus agricultural biotechnology towards health-related and environmental breeding goals, and in these endeavours the constructive voices of all friends of the environment are urgently needed.

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Chapter 6
Next Generation Plant Biotechnology

M.R. Ahuja

Abstract Modern plant biotechnology began with the transfer of foreign chimeric genes into plants. Initially recombinant genes were derived from bacteria, animals and plants for gene transfer. Gene transfer was accomplished by *Agrobacterium*-mediated or biolistic methods into the plant genome. The first wave of transgenic plants that were monitored for transgene integration and expression, the second wave transgenic plants carried economically important genes for herbicide tolerance, pest resistance, drought and salt tolerance, growth traits, and flowering control. Subsequently, a number of genetically modified crops with several useful traits have been commercialized. Although relatively stable transgene expression has been observed in a number of plant species, there were also unintended unstable events in transgenic plants. This is due to the fact that transgene integration achieved by the two traditional methods (*Agrobacterium* or biolistic) of gene transfer in the plant genome is random, and one to several copies of the transgenes may be integrated at one or several locations in the genome. In order to overcome the problem of randomness of transgene integration, site-specific transgene integration strategies have been experimentally tested in plants, and offer prospects of stable gene integration and expression in transgenic plants. In order to broaden the scope of transgenic plants, biotechnologists started looking for other useful avenues for their utility. With finite reserves of fossil fuels and climate change, and growing demands for fuels, plastics, and pharmaceuticals, transgenic plants have been also explored as production platforms for these commodities. This paper is an overview of next generation transgenic plants that can serve as bioreactors or biofactories for the cost-effective production of biofuels, biopharmaceuticals, bioplastics, and as a resource for nutritional supplements to meet human demands in the future. New developments in nanobiotechnology offer prospects for improved production of crop plants.

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6.1 Introduction

The world population has been increasing at an accelerated rate during the last century. The world population was around 3.25 billion in the 1960s. By the year 2000 the world population reached the 6 billion mark. During the 40 years (1960–2000) it almost doubled. By the year 2010 it increased by another 1 billion to reach 7 billion. It is expected to reach the 9 billion mark by the year 2050 (http://www.fao.org/index-en.htm 2013). That is an increase of 2 billion from the current world population of 7 billion over the next 40 years. The bulk of the population increase has occurred in the developing countries; by the year 2050 the world population would be around 8 billion in the developing countries, and only 1 billion in the developed countries. How to feed the world by the year 2050 remains a daunting challenge for the food security. In order to feed this larger world population food production would necessarily have to increase by 70 % (Godfray et al. 2010; Bogdanski 2012). This explosion in world population requires an enormous increase in food production, improvement of nutritional quality of the staple food (biofortification), production of safe and natural pharmaceutical proteins (molecular farming), and increase in energy and plastics by alternatives routes (biofuels and bioplastics) in the future to meet the human demands. In addition to conventional breeding, plant biotechnology can play a significant role for the improvement of human resources in the future.

Modern plant biotechnology began with the transfer of chimeric genes, constituted by the DNA recombinant technology, into the plant genome in the1980s, and the first transgenic plants were produced by *Agrobacterium*-mediated gene transfer (Bevan et al. 1983; Herrera-Estrella et al. 1983; Fraley et al. 1983). Subsequently, transgenic plants from a number of different plant species, including agricultural crops and tress, were produced with novel genotypes by *Agrobacterium*-mediated and particle bombardment gene transfers (Peña and Séguin 2001; Sharma et al. 2002; Boerjan 2005; Jauhar 2006; Herdt 2006; Dunwell 2010). The first wave of transgenic crops carried transgenes for resistance to fruit rotting, herbicide tolerance, and pest resistance.

Genetically engineered FlavrSavr tomatoes, developed by Calgene (Kramer and Radenbaugh 1994), carrying an antisense polygalacturonase (*PG*) transgene which makes tomatoes resistant to rotting, was released in the market in 1994. Under normal conditions, the enzyme polygalacturonase which degrades the pectin in the cells wall results in softening of the fruit and consequently makes it susceptible to fungal infection. FlavrSavr tomatoes, on the other hand, have a relatively longer shelf-life, and also can be allowed to ripen on the tomato vine. However, the FlavrSavr tomatoes turned out to be a disappointment in that the antisense *PG* gene,

Table 6.1 Global area of biotech crops in 2013. (Data from James (2013))

Rank	Country	Area (millions of hectares)	GM crops
1.	USA	70.1	Maize, soybean, cotton, canola, sugar beet, alfalfa, papaya, squash
2.	Brazil	40.3	Soybean, maize, cotton
3.	Argentina	24.4	Soybean, maize, cotton
4.	India	11.0	Cotton
5.	Canada	10.8	Canola, maize, soybean, sugar beet
6.	China	4.2	Cotton, papaya, poplar, tomato, sweet pepper
7.	Paraguay	3.6	Soybean, maize, cotton
8.	South Africa	2.9	Maize, soybean, cotton
9.	Pakistan	2.8	Cotton
10.	Uruguay	1.5	Soybean, maize
11.	Bolivia	1.0	Soybean
12.	Philippines	0.8	Maize
13.	Australia	0.6	Cotton, canola
14.	Burkina Faso	0.5	Cotton
15.	Myanmar	0.3	Cotton
16.	Spain	0.1	Maize
17.	Mexico	0.1	Cotton, soybean
18.	Columbia	0.1	Cotton, maize
19.	Sudan	0.1	Cotton
20.	Chile	<0.1	Maize, soybean, canola
21.	Honduras	<0.1	Maize
22.	Portugal	<0.1	Maize
23.	Cuba	<0.1	Maize
24.	Czech Republic	<0.1	Maize
25.	Costa Rica	<0.1	Cotton, soybean
26.	Romania	<0.1	Maize
27.	Slovakia	<0.1	Maize
Total area		175.2	

1 ha = 2.47 acres

which had a positive effect on the shelf-life, resulted in a negative effect on fruit firmness, and also had a very bland taste. Consequently, Calgene halted the production of FlavrSavr tomatoes in 1997.

In the next decade a number of genetically modified crops that were resistant to herbicide tolerance and insect and disease resistance were commercially released in the marketplace in many countries (Table 6.1). These included soybean, maize, cotton, canola, sugar beet, papaya, squash, tomato, poplar, and sweet pepper. Initially, transgenic crops were engineered for either herbicide or pest resistance. However, later on herbicide and insect resistant transgenes were stacked in some of the transgenic crops (James 2013). In spite of the commercially profitable transgenic crops, there are questions regarding the genetic stability of and mode of inheritance of transgenes in the subsequent generations, transgene containment to effectively

prevent escape of transgenic pollen and seed, and impact of genetically modified crops on the ecosystem and human nutrition. In order to address some of these concerns and, at the same time, improve human nutrition and other utilities of biotech plants, new areas of biotechnology are being explored for the next generation of genetically modified plants. These include: (a) gene targeting and genome editing; (b) nanobiotechnology; (c) biofortification; (d) molecular farming; (e) biofuels; and (f) bioplastics.

6.2 Gene Targeting and Genome Editing

The established methods of gene transfer have so far led to unpredictable insertion and integration of transgenes in plants, including trees (Finnegan and McElroy 1994; Buteye et al. 2005; Filipecki and Malepszy 2006; Ahuja 1997, 2009, 2011). Gene transfer has been accomplished by *Agrobacterium*-mediated and particle bombardment methods in plants. Transgene integration in the plant genome is a complex process. Generally, *Agrobacterium*-mediated genetic transformation produces transgenic lines with a relatively low (1–3) transgene copy number (Kohli et al. 2003; Olhoft et al. 2004; Oltamanns et al. 2010; Fladung et al. 2013). Particle bombardment transformation method, on the other hand, typically integrates on average higher (1–10 or even up to 100) transgene copy number and complex integration in the genome (Svitashev and Somers 2001; Makarevitch et al. 2003; Kohli et al. 2003). A large number of diverse recombinant genes have been transferred in the genomes of agricultural crops and trees. Transgene integration occurs in plants by illegitimate recombination (Gheysen et al. 1991; Mayerhofer et al. 1991) between T-DNA and host genome. Integration of transgene is a random process, and transgenes may be integrated at one location or dispersed on different chromosomes in plants (Gelvin and Kim 2007; Kohli et al. 2010). One to a number of copies of a transgene may be generally integrated at one or several sites in the host genome. Transgene integration can occur throughout the plant genome (Alonso et al. 2003). Depending on the site of integration of a transgene in the genome, transgene expression may be fairly stable, or there may be variation in transgene expression, or instability/silencing of the transgene.

In order to overcome possible problems of variable transgene expression arising from randomness and multicopy insertions of a transgene in the plant genome, gene targeting systems have been developed in the past decades for directing a single copy of a transgene, or its multiple copies thereof, in a predefined site in the host genome (Pazskowski et al. 1998; Lyznik et al. 2003; Kumar et al. 2006; Poczai et al. 2013; Puchta and Fauser 2013; Ahuja and Fladung 2014). Site-specific recombination systems developed from viruses, bacteria and yeast has been proposed as tools for gene targeting (Liu et al. 2000; Kumar and Fladung 2001; Srivastava and Ow 2004). Two components are needed for site-specific recombination: (1) a site-specific recombinase, and (2) its recognition site (that is a defined sequence). The recombinase systems include, the Cre-*lox* system of bacteriophage P1 (Sauer

and Henderson 1990), the FLP-*FRT* (Golic and Lindquist 1989), the R-*RS* (Onouchi et al. 1991) system of yeast, and the Gin/*gix* system of the bacteriophage Mu (Odell and Russell 1994). Site-specific recombination takes place at a recognition site or a specific DNA sequence and involves cleavage, and reunion leading to integration of a recombinant gene, or deletion or inversion of a DNA fragment (Wang et al. 2011). Site-specific recombination systems, experimentally used for *in vivo* excision of donor DNA sequence, have been suggested as strategy to remove the antibiotic marker genes or even the whole transgene cassette from the genome of transgenic plants (De Buck et al. 2007; Luo et al. 2007; Gidoni et al. 2008; Wang et al. 2011). However, the same system, but in the reverse reaction, can be used for targeted integration of DNA (Kumar and Fladung 2001; Lyznik et al. 2003; Tzfira and White 2005). As a prerequisite, one copy of the recognition site must be present in the targeted region, and a second one is located in the DNA to be inserted (Fladung and Becker 2010). If the respective site-specific recombinase is temporally expressed, the desired DNA fragment can exactly be inserted in the targeted region.

A gene targeting approach routinely requires two rounds of transformation: in round one, the target site (e.g. *lox* or *FRT*), is randomly introduced into the plant genome, and in the second round, a *lox*- (or *FRT*) containing recombinant gene is inserted into the previously targeted genomic site (De Buck et al. 2007; Li et al. 2009). Cre-mediated site-specific gene integration has been demonstrated in rice (Srivastava et al. 2004; Srivastava 2013), *Arabidopsis* (Vergunst et al. 1998; Louwerse et al. 2007; De Buck et al. 2007), and hybrid aspen (Fladung and Becker 2010); while FLP-mediated site-specific gene insertion has been shown in soybean (Li et al. 2009), and hybrid aspen (Fladung et al. 2010). In addition, stacking multiple transgenes via repeated recombinase-mediated transformation, at selected genomic sites is experimentally feasible in plants (Li et al. 2010; Ow 2011). The advantage in stacking transgenes at the same site on a chromosome is that the linked-transgenes, following crossings, will most likely be transmitted as a single locus to the progeny. However, it still has to be demonstrated whether site-specific recombination is practically feasible for targeted transfer of numerous stacked transgenes to one genomic position.

A second gene targeting system utilizes synthetic recombinases or nucleases for site-specific insertion of transgenes in the plant genome (Carroll 2011; Curtin et al. 2012; Tzfira et al. 2012; Puchta and Fauser 2013). These nucleases are engineered proteins that are designed to break the double stranded DNA at a specific site, and then exploit homologous recombination to insert a gene at a predetermined location in the host genome. Three sequence-specific nuclease systems have been developed for site-specific integration of genes and mutagenesis in plants. These include: zinc finger nucleases (ZFNs), transcription activator-like nucleases (TALENs), and LAGLIDADG homing nucleases, also known as “Meganucleases” (Carroll 2011; Bogdanove and Voytas 2011; Curtin et al. 2012). Targeted integration of herbicide tolerance genes by site-directed homologous recombination using ZFNs has been reported in maize (Shukla et al. 2009) and tobacco (Cai et al. 2009), and by TALENs in tobacco (Townsend et al. 2009). Both recombinase- and nuclease-mediated gene insertions also require transformation systems that include either *Agrobacterium* or particle bombardment.

Although recombinases and nucleases are promising avenues for gene targeting, alternative methods for plant genome editing are being developed because of the complicated designs and laborious assembly of specific binding proteins for each specific target site (Belhaj et al. 2013). Recently, new methods of gene editing/genome engineering have emerged that involve clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases (Cong et al. 2013; Mali et al. 2013; Belhaj et al. 2013; Gaj et al. 2013). The CRISPR/Cas system based targeted cleavage of genomic DNA is guided by a small customized non-coding RNA in gene targeting by both non-homologous and homology-directed repair mechanism.

The real power of the engineered nucleases, recombinase, and CRISPR/Cas systems lies in their ability to precisely engineer not only foreign genes, but also native plant genes for the production of transgenic plants. Another utility of synthetic gene editing systems lies in their potential for activating/editing native plant genes for herbicide tolerance and disease resistance, drought resistance and other qualitative and quantitative traits, rather than engineering exogenous genes for these traits. Such innovations will pave the way for next generation biotech crops to be less regulated or not regulated by federal oversights, as these novel genotypes will be, more or less, substantially equivalent to genetically unmodified plants. Although, site-specific gene integration by recombinases and nucleases, and CRISPR/Cas systems is a promising avenue for stable integration of transgenes in plants, it is still in experimental stages and further research is necessary for their application to next generation crop plants and trees.

6.3 Nanobiotechnology

Nanobiotechnology is a promising field of interdisciplinary research in life sciences. It has enormous potential in the fields of medicine and agriculture. Although, nanobiotechnology is considered to become an important technology in the twenty-first century, it is still in experimental stages in plants. Nanoparticles are extremely small with dimension ranging between 1 and 100 nanometer (nm). Nanoparticles used in agricultural plants generally range in size-dimension from 5 to 200 nm (Ghormade et al. 2011). A wide variety of physical and chemical methods have been used to fabricate nanoparticles, including iron, silver, gold, silicates, and polymers (Mohanraj and Chen 2006; Hayashi et al. 2008; Barman et al. 2014). However, use of toxic compounds used in producing nanoparticles by these methods limit their application. More recently eco-friendly biological methods using plants as biofactories for the production of metallic nanoparticles are being used (Nair et al. 2010; Hasna et al. 2012; Burris et al. 2012; Kavitha et al. 2013; Rai and Yadav 2013; Marchiol et al. 2014; Vadlapudi and Kaladhar 2014; Barman et al. 2014). Potential applications of nanobiotechnology in agriculture include: (1) nano-encapsulated agrochemicals, including fertilizers, for controlled release in the soil; (2) nano-encapsulated herbicides and insecticides for weed and pest control; and (3) nanoparticle-mediated genetic material delivery in plants (Nair et al. 2010; Ghormade et al. 2011; Rai and

Ingle 2012). Mesoporous nanoparticle-mediated DNA delivery has been demonstrated in plants (Torney et al. 2007; Fu et al. 2012; Xia et al. 2013). More recently, mesoporous nanoparticle-mediated site-specific co-delivery of DNA and proteins also been demonstrated in plants (Martin-Ortigosa et al. 2012, 2014). These studies on gene and protein transfer mediated by nanoparticles open up new avenues for loading of nanoparticles with multiple DNA, protein, and chemical complexes for delivery in the plant cells (Martin-Ortigosa et al. 2014). Further experimental studies in nanobiotechnology offer promising prospects not only for the production of genetically targeted transgenic plants, but also for novel applications in improving production and management agricultural crops.

6.4 Biofortification

Recent research in biotechnology has also been focussed on the nutritional enhancement of micronutrients and vitamins in genetically modified crops. Genetic engineering has been employed for fortification of minerals, amino acids, anti-oxidants, vitamins for improving the nutritional quality of the staple crops. Biofortified crops can alleviate essential micronutrient malnutrition in the human population, particularly in the developing countries (Mayer et al. 2008; Hirschi 2009; Beyer 2010; Bashir et al. 2013; Murgia et al. 2013; Pérez-Masscot et al. 2013; Saltzman et al. 2013; Zhu et al. 2007, 2013). More than 50 % of the human population worldwide has little or no access to healthy staple fresh foods (Christou and Twyman 2004). Malnutrition of humans, particularly children, is rampant in underdeveloped countries. Strategies to develop genetically modified plants as a resource for nutritionally enhanced crop plants for food security have been developed by plant biotechnologists in the past decades. Of course, some of the transgenic crops for food security are still in experimental stages, while others have moved to field trials and may become available in the market place in the future. In this direction, the next generation biofortified transgenic crops include:

- Rice that produces β-carotene (provitamin A) in the endosperm (Golden Rice) (Ye et al. 2000; Beyer 2010), and has increased amounts of folate, and mineral (iron and zinc) in the seed (Beyer 2010; Lee et al. 2009, 2012; Yang et al. 2013);
- Wheat grain with enhanced levels of iron and zinc (Borg et al. 2012; Sui et al. 2012; Borrill et al. 2014);
- Potatoes that are protein-rich (Chakraborthy et al. 2010), have better aroma and less browning (Llorente et al. 2010), and exhibit reduced cold-induced sweetening and increased carotenoid content (Giuliano et al. 2006; Chen et al. 2008; Bhaskar et al. 2010; Barrell et al. 2013);
- Tomatoes with increased lycopene and β-carotene (Guo et al. 2012; Liu et al. 2014);
- Bananas that are resistant to fungal wilt (Panama wilt) and black leaf streak diseases, and exhibit increased β-carotene and iron (Aravanityonnis et al. 2008; Kovács et al. 2013; Cressey 2013);
- Corn with enhanced levels of multivitamins, including β-carotene (provitamin A), ascorbate (vitamin C), and folate (vitamin B9) (Naqvi et al. 2009);

- Soybean with lower levels of saturated fat and higher levels of unsaturated oleic acid, and higher levels of omega-3-fatty acids (Herschi 2009);
- Citrus with enhanced levels of β-carotene (provitamin A) (Cao et al. 2012), and antioxidants in the fruit (Pons et al. 2014);
- Cassava with improved nutritional quality, including starch (Ihemere et al. 2006; Zeeman et al. 2010), provitamin A, and other micronutrients (Montagnac et al. 2009; Welsch et al. 2010; Sayre et al. 2011; Adenle et al. 2012);
- Apple resistant to apple scab, fire blight, and improved growth (Malnoy et al. 2008; Borejsza-Wysocka et al. 2010; Joshi et al. 2011; Xu 2013; Schäfer et al. 2012; Krens et al. 2012); early flowering to reduce generation time for breeding to create new cultivars, and earlier yield (Flachowsky et al. 2011; Yamagishi et al. 2014);
- Crop plants with improved nutrition (McGloughlin 2010; Winkler 2011; Murgia et al. 2013; Pérez-Masscot et al. 2013; Saltzman et al. 2013), fortified micronutrients (Mayer et al. 2008; White and Broadley 2009), antioxidants (Zhu et al. 2013), vitamin A (Giuliano et al. 2008), vitamin B1 (Pourcel et al. 2013),vitamin C (Locato et al. 2013), vitamin E (Yabuta et al. 2013), amino acid lysine (Galili and Amir 2013), polyunsaturated fatty acids (omega-3-fatty acid) (Rogalski and Carrer 2011; Petrie et al. 2012; Haslam et al. 2013;), and multivitamins (Hirschi 2009; Fitzpatrick et al. 2012);
- Microalgae as a resource for fatty acids, such as omega-3-fatty acid (Adame-Vega et al. 2012; Vaezi et al. 2013; Martins et al. 2013).

6.5 Molecular Farming

Plants have been used for medicinal purposes for thousands of years by mankind. Molecular farming, or biopharming, is a recent development using transgenic plants, including algae, for the production of high-value pharmaceuticals, including recombinant proteins (vaccines, cytokines, growth hormones) and other secondary metabolites (Daniell et al. 2001; Fischer et al. 2004, 2009; Karg and Kallio 2009; Obeme et al. 2011). Plants offer great potential as production platforms for important pharmaceuticals for safe and effective use by consuming edible plant tissues and seed (Hefferon 2013). Although biopharmaceuticals are predominantly produced in transgenic animal and microbial bioreactor systems, transgenic plants also offer alternatives to large scale biopharmaceutical production in plant tissues and plant cell bioreactors. Plants cells are capable of full post-translational modification of recombinant proteins to fold properly and maintain their structural and functional integrity, with simple growth factor requirement, minerals and light, and essentially unlimited biosynthetic capacity and scalability of biopharmaceuticals in leaves, stems, tubers, seeds, or whole plant, whether grown in the field or in bioreactors. Besides, plants do not harbour human or animal pathogens, including prions, human viruses and oncogenes, making them as safe hosts for the production of biopharmaceuticals (Ma et al. 2003; Davies 2010). Although in earlier works,

Table 6.2 A short list of biopharmaceuticals expressed in transgenic plant systems

Plant	Product	Disease	Reference
Algae	Human papilloma virus Type 16	Human papilloma virus	Giorgi et al. 2010
Tobacco	VLP	Influenza	D'Aoust et al. 2010
Tomato	Hepatitis B surface antigen	Hepatitis B	Gao et al. 2003
Lettuce	Hepatitis B antigen	Hepatitis B	Stearfield 2006
Tomato	Cholera Clox A and B	Cholera	Sharma et al. 2008
Algae	*Plasmodium falciparum* specific protein	Malaria	Jones et al. 2013
Tobacco	CTB-EsAT6 fusion protein	Tuberculosis	Lakshmi et al. 2013
Tobacco	Cytokines	Cancer, immune disorders	Sirko et al. 2011
Tobacco	E-glycoprotein	Dengue	Kim et al. 2009
Maize	Heat labile toxin B	Diarrhea	Tacket 2005

transgenic plants or their cell cultures were used for the expression of recombinant proteins, more recently transient expression systems, involving the agroinfiltration methods (Kapila et al. 1997; Pouge et al. 2010; Chen and Lai. 2013), the virus infection method (Porta and Lomonossoff 2002; Varsani et al. 2006; McCormick et al. 2008), and the magnifection technology (Gleba et al. 2005), have been developed in plants for the production of biopharmaceuticals. Transient expression platforms, perhaps the most convenient and efficient platforms, allows the cultivation of plants under stringent controlled conditions, without stable genetic transformation, for the rapid production of high grade pharmaceutical proteins on a large competitively commercial scale (Rybicki 2010; Komarova et al. 2010; Tremblay et al. 2010; Circelli et al. 2010).

The first recombinant proteins (human growth hormone) and with therapeutic potential was successfully expressed in transgenic plants (Barta et al. 1986). A few years later, the use of transgenic plants producing edible vaccines was reported (Mason et al. 1992). It was not until 1997 that a recombinant protein, avidin (an egg protein) was produced for commercial purposes in transgenic maize (Hood et al. 1997). Subsequently, it was shown that transgenic plants have the ability and capacity for the expression of a number of functional mammalian proteins with therapeutic value, such as human serum proteins, growth hormones, antibodies, vaccines, cytokines, and enzymes (Daniell et al. 2009; Karg and Kallio 2009; Obeme et al. 2011; Franconi et al. 2010; Penney et al. 2011; Sirko et al. 2011; Kumar et al. 2013; Rigano et al. 2013; Da Cunha et al. 2014; Specth and Mayfield 2014). Recombinant biopharmaceutical production is moving at a very fast pace since 2007 when it captured about 10% of the pharmaceutical market (Lowe and Jones 2007). By the year 2010, there were more than 200 bio-drugs approved biopharmaceuticals on the global market, generating more than $ 100 billion in the global pharmaceutical market (Walsh 2010). It is expected that the biopharmaceutical market will continue to expand in the future and the bio-drug sales may reach up to $ 240 billion by 2015 (Stewart 2010). Some of the biotech plants, including microalgae, used for the production platforms of a number of biopharmaceuticals are listed in Table 6.2. For more detailed listings of plant-based biopharmaceuticals see reviews by Daniell et al. (2009), Walsh (2010), Obeme et al. (2011), Da Cunha et al. (2014), and Specht and Mayfield (2014).

6.6 Biofuels

Finite reserves of petroleum-based fossil fuels, accompanied with global warming and depletion of fossil fuels at a rapid pace, have provided a strong impetus to search for alternative and sustainable sources of renewable energy. Plant cell wall carbohydrates are the most abundant renewable resource on our planet Earth. The major components of plant cell walls are cellulose, hemicellulose, and lignin that comprise up to 90% of its dry biomass (Harris and Stone 2008; Pauly and Keegstra 2008; Gibson 2012; Hadar 2013). The biofuels from renewable plant-based biomass could alleviate the world-wide dependence on the fossil fuels. The biofuel that would partially sustain the global energy demands is the liquid fuel that can be produced from abundant and freely accessible fatty acids and sucrose in plants. Biodiesel is presently produced from the fatty acids in palm oil, soybean oil, and oilseed rape. Bioethanol is currently produced mainly from sugars from sugar cane, and digested starch from the seeds of maize. However, there is an ongoing debate about the "food versus fuel" regarding the use of staple crop grains for biofuel production (Kullander 2010; Valentine et al. 2012; Zhang 2013).

In order to secure food for the rapidly growing world population, food-plant residues and non-food plants are also being used to produce biofuels. These include agricultural crop residues, grasses, and forest trees. Lignocellulosic feedstock from wood, straw and grasses is currently the focus of biofuel production from plants. The abundant resource for lignocellulosic feedstock can be found in short-rotation fast-growing trees species as, poplars (*Populus* spp. and hybrids), salix (*Salix*), eucalypts (*Eucalyptus*), and grasses, switchgrass (*Panicum virgatum*) and miscanthus (*Miscanthus* x *giganteus*) (Yuan et al. 2008; Capita and McCann 2008; Jørgensen 2011; Mizrachi et al. 2012; Nieminen et al. 2012; Joyce and Stewart 2012). In spite of the abundant lignocellulosic biomass, the costs incurred for extraction of biofuelic ethanol by routinely used methods are at least two to threefolds higher than sugar or starch based ethanol production (Sticklen 2008). Although breeding and mutation induction in plants for reduction of lignin content in biomass feedstock remains a viable alternative (Bourton 2007), it will take a long time to achieve this goal in plants, particularly trees. Therefore, the current focus on these non-food plants is on the reduction of lignin content by genetic manipulation for cost-effective ethanol production from the lignocellulosic feedstock (Gressel 2008; Hisano et al. 2009; Simmons et al. 2010; Mansfield et al. 2012). Next generation biotech plants for increased content and improved quality of biofuel production include:

- Maize with altered lignin biosynthesis by suppression of CAD activity to improve bioethanol production (Fornale et al. 2012);
- Switchgrass with down-regulation of *CAD*, or *COMT*, or silencing of *4CL* genes in the biosynthetic pathway to reduce lignin content, or use of transcription factor PvMYB-4 as potential for developing lignocellulosic feedstock for improved fermentable sugar for biofuel production; (Fu et al. 2011; Xu et al. 2011; Ye et al. 2011, Yee et al. 2012; Shen et al. 2012, 2013);

- Poplar with down regulation of *CCR* gene to reduce lignin content that resulted in increased saccharification and high ethanol yield (Van Acker et al. 2014); or suppression of other genes (*CAD*; *4CL*. *C3H*,*COMT*) involved in the biosynthesis of lignin to enhance biofuel production (Coleman et al. 2008; Mansfield et al. 2012; Voelker et al. 2011; Ye et al. 2011)
- Microalgae as a resource for production ofbiodiesel and bioethanol (Schenk et al. 2008; Beer et al. 2009; Brennan and Owende 2010; Gouveia and Oliveira 2009; Mata et al. 2009; Bajhaiya et al. 2010; Dragone et al. 2010; Huang et al. 2010; Khola and Ghazala 2012; Wu et al. 20123; Harun et al. 2014);
- Industrial waste agricultural residue biomass as a potential source of biofuel (Mwithiga 2013);
- Forest trees (especially poplars, eucalypts, and salix) as a resource of lignocellulosic feedstock, and lignin modification for improved production of biofuels (Rockwood et al. 2008; Simmons et al. 2010; Seguin 2011; Mizrachi et al. 2012; Nieminen et al. 2012; Pilate et al. 2012).

6.7 Bioplastics

In addition to current focus on using plants for biofuels, plants also produce a large number of useful chemicals and biopolymers. Plants naturally produce a large number of biodegradable polymers, which include starch, cellulose, proteins and rubber (Kulkarni et al. 2012). Starch and cellulose play a major role in food and fibre production for mankind. However, plants do not produce bioplastics, but many bacteria including, *Ralstonia*, *Pseudomonas*, *Azobacter*, *and Rhizobium* do (Dalton et al. 2011). Bacteria (e.g. *Ralstonia*) accumulate the polyester polyhydroxyalkanoate (PHA) as a bioplastic for carbon and energy reserve in response to nutritional stress (Anderson and Dawes 1990). Polyhydroxybutyrate (PHB), a short side-chain polymer of PHA, in produced in bacteria from acetyl-coA via a three enzymes biosynthesis pathway. These enzymes are encoded by three genes, *phaA*, *phaB*, and *phaC* respectively (Slater et al. 1988). Currently, the PHA bioplastics are being commercially produced in bacterial fermentation systems, with renewable resources as sucrose, glucose, fatty acids, or plant oils, or waste effluents (molasses, whey), and glycerol as carbon substrates (Chee et al. 2010; Chen 2009, 2010; Du et al. 2012). Wild type bacterial strain of *Ralstonia eutropha* (formerly known as *Alcaligenes eutropha*) has been most commonly used for industrial production of bioplastic (Chen 2009). However, the bacterially produced biodegradable bioplastic polymers are not cost-competitive with non-biodegradable petroleum-based plastic polymers. Alterative platforms to bacterial fermentation are being explored for production of bioplastics using transgenic plants that might be more cost-effective than bacterial fermentation and petroleum-based plastics (van Beilen and Poirier 2008; Mooney 2009; Somleva et al. 2013; Petrasovits et al. 2013).

A publication entitled ‘In search of plastic potato’ by Pool (1989) generated great expectation in the scientific community that bioplastic PBA could be produced by

Table 6.3 Bioplastic production in some transgenic plants

Plant	Polymer	Tissue	Yield DW%	Reference
Arabidopsis	PHB	Leaves	13.2–40	Bohmert et al. 2000, 2002
Tobacco	PHB	Leaves	18.8	Bohmert-Tatarev et al. 2011
Switchgrass	PHB	Leaves	7.3	Somalev et al. 2013
Corn	PHB	Leaves	5.73	Poirier and Guys 2002
Sugar beet	PHB	Hairy root culture	5.5	Menzel et al. 2003
Sugarcane	PHB	Leaves	4.8	Petrasovits et al. 2012
Poplar	PHB	Leaves	1–2	Dalton et al. 2011

genetic engineering on plants. A few years later, PHB (0.01% fresh weight; FW) was first produced in the model plant *Arabidopsis thaliana* transgenics (Poirier et al. 1992), which initiated a continuing wave of research to optimize the production of PHB in transgenic plants (Somleva et al. 2013). Some improvement in the PHB yield (14% dry weight; DW) was reported in transgenic *Arabidopsis*, with no obvious effect on the growth and fertility of the transgenic plants (Nawrath et al. 1994). Subsequently, a later study on transgenic *Arabidopsis* showed efficient production of PHB (40% DW) in the chloroplasts of the leaves, but that was accompanied by severe growth reduction of the transgenic plants (Bohmert et al. 2000). Since then a large number of studies with many plant species have been conducted that show variable production levels of the bioplastic PHB (0.005–40% DW) in transgenic plants (van Beilen and Poirier 2008; Somleva et al. 2013). Different tissues, including whole plant, shoot, stem, leaves, and cell suspension were employed for PHB production, which mostly accumulated in the plastids, but also in the cytoplasm (Van Beilen and Poirier 2008; Somleva et al. 2013). In addition, transgenic microalgae, such as green algae *Chlamydomonas reinhardtii*, and diatoms*Phaeodactylum tricornutum* engineered with PHB pathway genes from *Ralstonia eutropha* have also been explored as bioreactors for bioplastic production (Chaogang et al. 2010; Hempel et al. 2011). A large number of transgenic plants, both crops and non-crops, have been employed, and show different amounts of biodegradable bioplastics (van Beilen and Poirier 2008; Someleva et al. 2013). Some of transgenic plants for bioplastic production are listed in Table 6.3.

6.8 Future Prospects

A lot of progress has been made in different areas plant biotechnology in the last two decades. Initially crop plants were engineered with foreign genes derived mostly from bacteria for herbicide and pest resistance to improve crop yields. Later on, other transgenes for lignin modification, early flowering, male sterility, and abiotic stresses were experimentally tested in crop plants. Earlier studies in plants used alien genes from bacteria, animals and plants for genetic engineering. Recent trend has been towards development of cisgenic and intragenic transgenic crop plants (Holme et al. 2013; Espinoza et al. 2013). Cisgenic plants are derived

from transformation with identical copy of a gene from sexually compatible pool, including promoter, intron and terminator regions that are derived from the donor plant. On the other hand, intragenic plants are derived by transformation with combinations of different genes from the same or sexually compatible species. While both cisgenic and intragenics plants are guided by their own genes, they both require genetic transformation by *Agrobacterium* or biolistic methods. Recent research in gene targeting and directed genome engineering promises site-specific integration of transgenes in predetermines regions of the host genome, or tinkering of the endogenous genes of economic importance for their stable transgene expression and inheritance in the next generation crop plants. Next generation genome sequencing is already providing useful information regarding gene discovery and molecular markers associated with a number of diverse economic traits (Edwards and Batley 2010; Hamilton and Buell 2012). DNA sequencing is providing insight information genes that would be useful for plant improvement through plant biotechnology.

While these promising investigations are progressing at a rapid pace for the commercialization transgenic/biotech crops, plant biotechnology has also harvesting other useful plant products. These include next generation transgenic plants as future production platforms for biopharmaceuticals, biofuels and bioplastics, and nutritional supplements. In a sense, plants are becoming biofactories/bioreactors for useful bioproducts for mankind. Nevertheless, transgenic plants are subject to regulatory oversights. Containment of transgenes must be in place to effectively prevent escape of transgenic pollen seed, and vegetative propagules from the transgenic plants. In addition, these new areas in plant biotechnology must take into account risk assessment and biosafety considerations (Shama and Peterson 2008; Breyer et al. 2009; Sparrow and Twyman 2009; Domingo and Bardonaba 2011; Snell et al. 2012; Buiatti et al. 2013; Jouzani and Tohidar 2013), including the impact of these new developments on human health (new allergies), ecosystem, plant biodiversity, and sustainable agriculture. New developments in nanobiotechnology offer prospects for precise delivery of genetic material and enhanced production of agricultural crops. It would be necessary to maintain genetic biodiversity in the next generation biotech plants (Sharma and Sharma 2013) used for the bioproduction platforms as well as for crop improvement.

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Part II
Section B: Biotechnology and conservation of Biodiversity

Chapter 7
Conservation of Forest Genetic Resources

Mirjana Šijačić-Nikolić, Jelena Milovanović and Marina Nonić

Abstract Forest genetic resources represent the genetic diversity contained in thousands of tree species on Earth, and can be defined as the genetic variability of tree species, which has a potential or real value for humans (FAO, Plant genetic resources: their conservation in situ for human use, 1989). The increasing demand for wood, as a raw material for various purposes, as well as general useful forest functions, has made the protection (conservation) and directed utilization of forest genetic resources became a priority task of forestry science and profession. Conservation of forest genetic resources could be defined as a set of activities and strategies that are being implemented with the aim of ensuring the continued existence, evolution and availability of these resources for present and future generations. Conservation of these resources should be considered as the efforts to preserve specific genotypes or populations and the combination of genes within them. Therefore, the aim of genetic resources management is to improve conditions for the continuous evolution of the species, which represents the defense mechanism of organisms in suppression the environmental changes. Genetic variability, which is the result of different genetic processes: mutation, recombination, gene flow, natural selection and genetic drift, presents the basis for conservation of forest genetic resources. The principles of genetic variability conservation can be regarded as identical for all living beings. However, the methods which are applied vary depending on the specificity of the conservation goals, distribution and biological nature of the material that is the object of conservation.

Keywords Forest genetic resources · Genetic variability · Conservation

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7.1 Biodiversity and Conservation of Forest Genetic Resources

Modern man, by its various activities, continually destroys and changes the nature which leads to irreversible loss of biodiversity through the disappearance of a large number of organic species or reducing their natural population to critical limits.

The destruction of species does not occur as planned and targeted human activity, but usually indirectly, by destroying habitats where species live. The causes of biodiversity loss are numerous, mutually interdependent and usually anthropogenic character (Šijačić-Nikolić and Milovanović 2007).

7.1.1 Biodiversity

Biodiversity presents a fundamental human resource, which, among others, high-returns man in the following areas: agriculture, medicine—pharmaceutics, forestry, horticulture and tourism. It can be defined as a set of diversity of wildlife, which includes a total diversity and variability of genes, species and ecosystems on Earth. It is the result of spatially and temporally continuous, evolutionary process, which is performed through three fundamental, mutually conditioned and biologically inseparable levels: genetic, species and ecological.

Genetic diversity includes the overall diversity of genes and genetic information contained in all individual species of plants, animals, fungi and micro-organisms. It is contained in the individuals and populations of individual species that are part of the *species diversity* and can be found in different ecological relationships (trophic and production cycles, cycles of matter circulation, etc.) in various ecosystems which belong to the *biological diversity* (Stevanović and Vasić 1995).

Preservation and protection of biological diversity (biodiversity), in addition to preserving the environment, represents the most important mission in the global environmental and nature protection on Earth. These strategic directions for nature conservation on a global scale are defined in June, 1992 in Rio de Janeiro (Brazil), when the Heads of State and Government of 168 countries adopted the Convention on Biological Diversity (Convention on Biological Diversity-CBD) with the main aim to prevent the disappearance of species and their habitats. Since then, awareness and conscience of the human population is increasingly turning towards the understanding and acceptance of basic ecological principles and biodiversity conservation. At the beginning of this millennium, the biodiversity science ranks among the most important disciplines, from the aspect of the survival of humanity. Biodiversity protection is based on determining the scientific basis, defining adequate legislation and the activities carried out in practice.

Forests are characterized by a wide range of production and multiple-use functions. Forest trees and other woody plants provide habitat to other organisms, building complex mechanisms of genetic diversity. Therefore, forests should be also seen in the context of the overall biodiversity. Genetic variability, both between and within species, has a multiple fundamental value. Thanks to it, trees and shrubs adapts to new environmental conditions, even if they are a consequence of the negative impacts of pests, diseases and/or climate change (Šijačić-Nikolić and Milovanović 2007).

7.1.2 Forest Genetic Resources and Their Conservation

Forest genetic resources represent the genetic diversity contained in thousands of tree species on Earth, and can be defined as the genetic variability of tree species, which has a potential or real value for humans (FAO 1989). The term *forest* refers to the habitats and populations of trees and other typical associations of woody plants. The term *genetic* means variability of the genetic (DNA) structure at different levels: variability between species, variability between the population within the species, and type of variability between the individuals within the population. The term *resources* relates to the use of genetic variability for the purpose of satisfying the human needs.

The increasing demand for wood, as a raw material for various purposes, as well as general useful forest functions, has made the protection (conservation) and directed utilization of forest genetic resources became a priority task of forestry science and profession (Šijačić-Nikolić and Milovanović 2010). Today we are recording a large number of various destructive activities, which significantly reduce areas under forests. This is especially dangerous in cases where the destruction is done in populations of forest trees with limited or disjunctive areal, in rare ecotypes within a limited habitat, as well as in cases of endemic-relict forest tree species.

However, not only the genes or gene complexes are threatened, but also the entire population, which in extreme cases may lead to the disappearance of entire species. As a result of these effects, some species or provenances of forest trees already were reduced to just a few hundred or tens of surviving trees (Isajev and Šijačić-Nikolić 2003).

Conservation of forest genetic resources could be defined as a set of activities and strategies that are being implemented with the aim of ensuring the continued existence, evolution and availability of these resources for present and future generations. Genetic resources and their conservation process are characterized by an expressive dynamics. Conservation of these resources should be considered as the efforts to preserve specific genotypes or populations and the combination of genes within them (Šijačić-Nikolić and Milovanović 2012). Therefore, the aim of genetic resources management is to improve conditions for the continuous evolution of

the species, which represents the defense mechanism of organisms in suppression the environmental changes. Forest management aimed at improving production and protection functions can and should be harmonized with the concept of conservation at various levels: local, national and regional. Conservation of forest biodiversity, which includes forest genetic resources is of essential importance for the sustainable use of forest values, for improvement the health condition and vitality of forest ecosystems and the promotion and development of their protective, aesthetic and cultural functions (Šijačić-Nikolić and Milovanović 2007).

The importance of conservation of forest genetic resources at the level of European countries was recognized in 1994 when The European Forest Genetic Resources Programme (EUFORGEN) was formed. The EUFORGEN program started its activities with pilot networks on a few model tree species; gradually, the program evolved into a collaborative platform focusing on broader groups of tree species. It has developed long-term gene conservation strategies for several tree species or groups of species.

During recent years, species-specific technical guidelines, targeted at practical forest managers, have also been developed based on available knowledge of the species and on widely accepted methods for the conservation of forest genetic resources. The on-going efforts focus on how to support practical implementation of gene conservation in the member countries; for this purpose, so-called "common action plans" are being developed for several pilot tree species. The common action plans aim at sharing responsibilities in conservation of forest genetic resources among the countries, and identifying gaps in these efforts at the pan-European level. This involves improving information management and obtaining geo-referenced data on the existing gene conservation units of forest trees throughout their entire distribution ranges in Europe for further analyses and strategy development (Koskela et al. 2004; Koskela 2007).

7.2 Forest Genetic Resources in International Initiatives

Timeline of major international initiatives and adopted documents (Milovanović et al. 2012), which, directly or indirectly, related to the conservation of forest genetic resources, can be represented as follows:

1990		*The First Ministerial Conference on the Protection of Forests in Europe, Strasbourg, Resolution S2 „Conservation of Forest Genetic Resources“*
1992	Rio Earth Summit 1992	*United Nations Conference on Environment and Development (UNCED), Rio de Janeiro, Convention on Biological Diversity (CBD)*
1993		*The Second Ministerial Conference on the Protection of Forests in Europe, Helsinki, Resolution H2 „General guidelines for the Conservation of the Biodiversity of European Forests“*
1995	EUFORGEN	*European Forest Genetic Resources Program (EUFORGEN) of the International Institute for Plant Genetic resources (Bioversity International) in cooperation with Food and Agriculture Organization of the United Nations (FAO)*
1998		*The Third Ministerial Conference on the Protection of Forests in Europe, Lisbon, Resolution L2 “Pan-European Criteria, Indicators and Operational Level Guidelines for Sustainable Forest Management“, including Appendix 1 and 2*
1998	Convention on Biological Diversity	*Conference of the Parties - 4 (COP-4) of the CBD, adoption of the Working program on Forest Genetic Resources (Decision 4/7)*
2003		*The Fourth Ministerial Conference on the Protection of Forests in Europe, Vienna, Resolution V4 “Conserving and enhancing forest biological diversity in Europe”*
2007	United Nations Forum on Forests	*The 7th United Nations Forum of Forests, adoption of „Non-legally binding instrument on all types of forests“*
2007	FORESTS FOR QUALITY OF LIFE	*The Fifth Ministerial Conference on the Protection of Forests in Europe, Warsaw, Warsaw Declaration (“...maintain, conserve, restore and enhance the biological diversity of forests, including their genetic resources through sustainable forest management...”)*
2008	Convention on Biological Diversity	*COP-9 of the CBD, adopted decisions on further implementation of the Working program on Forest Genetic resources*
2010	Convention on Biological Diversity	*COP-10 of the CBD, adopted Decision X/1 „Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization”; Decision X/36 on Forest biodiversity*
2011		*The Sixth Ministerial Conference on the Protection of Forests in Europe „Forest Europe“, Oslo, adopted European goals for 2020; Announcement of the International Year of Forests 2011*
2012	Convention on Biological Diversity	*COP-11 of the CBD, Hyderabad, India, Decision X/1 “Status of the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization and related developments”*

The First Ministerial Conference on the Protection of Forests in Europe The first Ministerial Conference on the Protection of Forests in Europe (MCPFE) was held on December 18th 1990 in Strasbourg, France (Anonymous 1990). The conference adopted six resolutions, but only the Resolution S2 "*Conservation of Forest Genetic Resources*" directly related to the conservation of genetic resources of forest trees. Parties and international organizations believe that in addition to conservation of forest species, the basic aim is to preserve the genetic diversity of these species, which is an essential part of the heritage of humanity. The Parties are committed to implement policies to conserve forest genetic resources using the most appropriate methods.
In order to provide and expand efforts at the national and international level, the Parties have agreed that it is necessary to immediately establish a functional, but on a voluntary basis set, instrument of international cooperation among existing relevant organizations, to promote and co-ordinate:

- *In situ* and *ex situ* conservation of genetic diversity of European forests;
- The exchange of reproductive material;
- Monitoring progress in this field.

United Nations Conference on Environment and Development (UNCED) The Convention on Biological Diversity (CBD) adopted at UNCED is the most important document adopted at the conference in terms of conservation and directed utilization of genetic resources. The Convention was the first ever signed document that is related to the conservation of biological diversity at all levels: genetic, species and ecosystem. Discussion on the conservation of forest biological diversity under the Convention began at the second meeting of the Conference of the Parties (COP-2), held in 1995.

The Second Ministerial Conference on the Protection of Forests in Europe Decisions of the United Nations Conference on Environment and Development (UNCED), led to the organization of the Second Ministerial Conference on June 16–17, 1993 in Helsinki (Anonymous 1993). The conference adopted four resolutions, but in terms of conservation of forest genetic resources, the most important is Resolution H2 "*General guidelines for the Conservation of the Biodiversity of European Forests*".

European Forest Genetic Resources Program (EUFORGEN) In order to implement Resolution S2 from Strasbourg 1995, the EUFORGEN was launched (Turok 1998), which has successfully identified common needs of European countries for research and conservation, to facilitate the exchange of information between countries and focused public attention on issues of genetic resources and their conservation. EUFORGEN is financed by the participating countries; the program is managed by the Bioversity International, in collaboration with the Forestry Department of the Food and Agriculture Organization of the United Nations (FAO). The main activities of the EUFORGEN include regular exchange of data and information, development of long-term conservation strategies, publication of technical guidelines, manuals and databases, preparation of proposals, exchange of genetic

material, the literature, the activities on informing public, cooperation with other regional programs, eg. Central Asia, Africa (Geburek and Turok 2005).

The Third Ministerial Conference on the Protection of Forests in Europe The Third Ministerial Conference on the Protection of Forests in Europe was held on June 2–4, 1998 in Lisbon, Portugal. Responsible for the European forestry adopted two resolutions, L1—People, Forests and Forestry—improving the socio-economic aspects of sustainable forest management and L2—Pan-European criteria, indicators and operational guidelines for sustainable forest management. As a result of this cooperation, a Pan-European project called "The Work Programme for the conservation and enhancement of biological and landscape diversity in forest ecosystems, 1997–2000" was launched and successfully implemented.

COP-4 of the CBD During COP-4, held in 1998, forest biological diversity has been identified as one of the five thematic areas of the Convention on Biological Diversity. At the same meeting, the working program for forest biological diversity focused mainly on the development of research cooperation and technology was approved, and the *Ad Hoc* Group of Experts tasked with further development of the Work Programme was established. Special emphasis was placed on *in situ* conservation. In addition to *in situ* conservation, it is necessary to measure the existence of complementary *ex situ* conservation. In all aspects of the Convention, it is necessary to ensure the participation of the public, especially when it comes to the assessment of the environmental impact of those that pose a threat to biodiversity.

The Fourth Ministerial Conference on the Protection of Forests in Europe The Fourth Ministerial Conference on the Protection of Forests in Europe under the name "Living Forest Summit" was held on April 28–30, 2003 in Vienna, Austria. Resolution 4 recognizes biological diversity as a key element in sustainable forest management. Annexes to the Resolution are specifically defined framework for cooperation and coordination between the MCPFE and The World Conservation Union, IUCN.

The Seventh United Nations Forum for Forests United Nations Forum for Forests at its seventh meeting, held in 2007, adopted the so-called "Non-legally binding instrument on all types of forests", which, once again, expressed concern about deforestation, forest degradation, low rates of afforestation, as well as feedback from these factors on the survival of biodiversity and genetic resources.

The Fifth Ministerial Conference on the Protection of Forests in Europe The Fifth MCPFE entitled "Forests for Quality of Life" was held on November 5–7, 2007 in Warsaw, Poland. Within the Warsaw Declaration, the Parties committed themselves to insisting on sustainable forest management, which contributes significantly to the ecological, economic, social and cultural dimension of sustainable development, in particular the achievement of adopted international goals, including the goals of the Convention on Biological Diversity.

COP-9 of the CBD At the Ninth Meeting of the Parties of the Convention on Biological Diversity, held in 2008 in Germany, the Decision 4/7 on Forest Biological

Diversity was adopted. This decision is supporting further implementation of the Work Programme on forest genetic resources and emphasis is given to the development of a precise methodology for the application of criteria and indicators for monitoring forest biodiversity.

COP-10 of the CBD On the tenth meeting of the Parties of the Convention on Biological Diversity, held in 2010 in Japan, the Decision X/1 "Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization" and the Decision X/36 on Forest Biodiversity was adopted. Emphasis is placed on the preparation of national reports on the state of forest genetic resources with the full participation of all stakeholders. National reports will contribute to the development of a complex report on the state of the world's stock of forest genetic resources.

The Sixth Ministerial Conference on the Protection of Forests in Europe The Sixth Ministerial Conference on the Protection of Forests in Europe "Forest Europe" was held in 2011 in Oslo, and the main adopted document was related to European targets and goals for 2020, and Year 2011 was declared for the International Year of Forests. Sixth European goal for 2020 is particularly related to forest biodiversity and foresees halving or reducing to zero the rate of loss of forest biodiversity at the habitat and taking measures to reduce fragmentation and forest degradation and restoration of degraded forests.

COP-11 of the CBD The 11th Conference of Parties of the CBD was held in 2012 in India. One of the most important decisions from this meeting is Decision X/1 "Status of the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization and related developments" which is recalling the mandate of the Open-ended *Ad Hoc* Intergovernmental Committee for the Nagoya Protocol to undertake the preparations necessary for the first meeting of the Conference of the Parties serving as the meeting of the Parties to the Nagoya Protocol.

According to the review of existing strategic frameworks related to the conservation of forest genetic resources at international level, it is possible to define several conclusions:

- The conservation of genetic diversity in forest ecosystems is understood and accepted as one of the important aspects of nature conservation and the environment at the beginning of the last two decades. Until then, the loss of genetic diversity in forests is not singled out as a problem that deserves attention, but is considered as a part of discussions on the protection of the gene pool of plant and animal species of importance to agriculture and food production;
- The term "forest genetic resources" is present in almost all current international processes, initiatives and strategic documents related to the sustainable management of forest ecosystems and biodiversity conservation in general;
- As opposed to non-binding international processes and initiatives, forest genetic resources are not treated as a separate issue in binding legislation. The problem of conservation of forest genetic diversity is considered through the prism of

nature conservation and natural resources, as well as the regulation of trade and market issues of forest reproductive material;
- Recognition of the concept and the problem of conservation of forest genetic resources in the strategic documents, which are intended to indicate the desired direction of development in the field of sustainable forest management, indicating the existence of intentions and desires of society for the prevention of adverse effects, and the lack of commitment to this category of natural value and capital.

Raising awareness of forest owners and users about the importance of conserving genetic diversity can significantly contribute to the improvement and implementation of strategic priorities. Forest owners can be very useful in the design and implementation of conservation programs, but they are not sufficiently aware of the biological value of their forests, or existing sources of financial support for conservation activities.

7.3 Forest Genetic Resources Conservation Methods

Genetic variability, which is the result of different genetic processes: mutation, recombination, gene flow, natural selection and genetic drift, presents the basis for conservation of forest genetic resources. The principles of conservation of genetic variability can be regarded as identical for all living beings. However, the methods which are applied vary depending on the specificity of the conservation goals, distribution and biological nature of the material that is the object of conservation (FAO 1989).

From the aspect of preserving genetic variability, there are different methods of conservation. The term "method" is used in the context of a certain concept of conservation of genetic resources: *in situ* or *ex situ*, dynamic or static, while the species, ecosystem, population, individual or part of an individual, present objects of conservation (Šijačić-Nikolić and Milovanović 2009; Šijačić-Nikolić and Milovanović 2010). Every process of conservation should start by clearly defining of its objectives. When the process of conservation allows adaptation and changes in gene frequency, in accordance with local selective influence, it is *dynamic* (evolutionary) conservation. If the process of conservation is planned with the aim of preserving the current gene frequencies of the original population, wherein lack the effects of the genetic process, we are talking about a *static* conservation (Guldager 1975). Forest genetic resources conservation model is presented in Fig. 7.1.

7.3.1 In Situ Conservation Methods

In situ (at the site) conservation means the conservation of forest genetic resources in:

- natural populations (seed stands, groups of trees or individual trees),

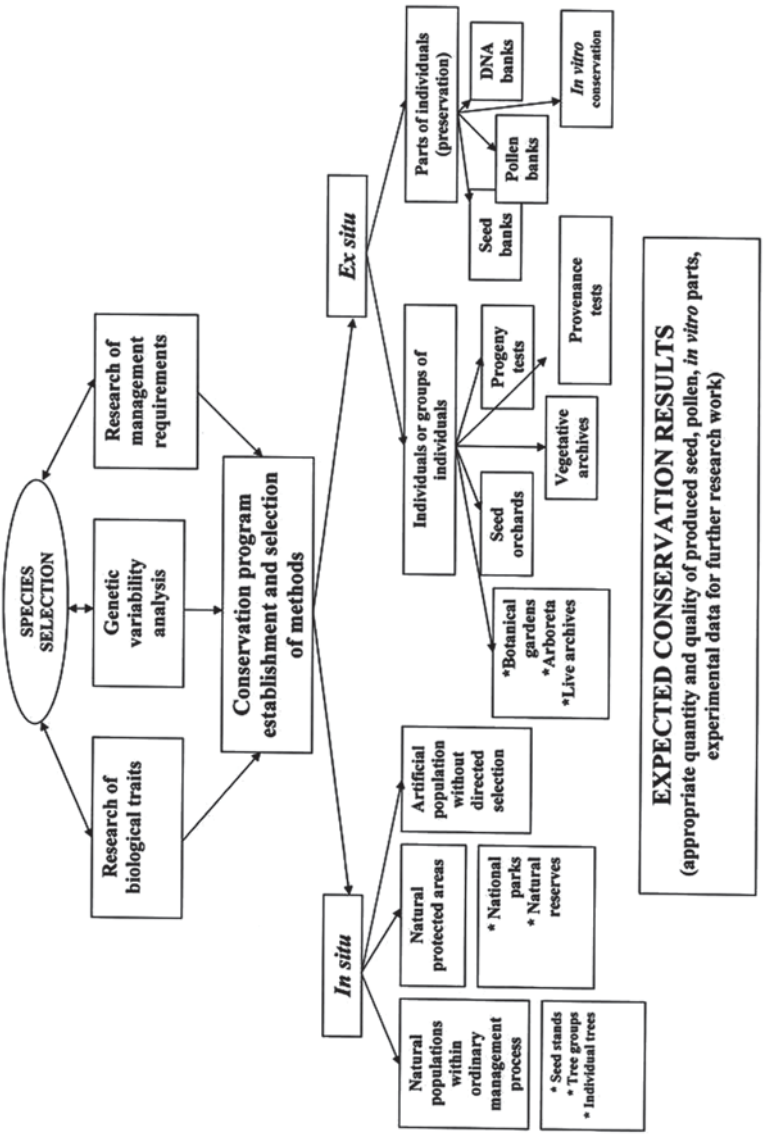

Fig. 7.1 Forest genetic resources conservation model (Šijačić-Nikolić and Milovanović 2007)

- protected areas (national parks, nature reserves), and
- artificially established populations without directed selection.

Seed stands means the stands characterized with high silvicultural form, good health condition and phenotypic traits, which are isolated and sufficiently distant from the stands of the same species, to prevent inter-pollination. By phenotypic and population characteristics, seed stands present the largest accumulation of genetic resources of tree species in which genetic variability is the most expressed.

Groups of trees or individual trees, which are selected as conservation units, should be in good health condition, exceptionally good morphological characteristics and adaptability to habitat conditions. Their selection includes: nomination, evaluation and registration, and often can be done in parallel with the selection of plus trees that are used primarily for qualitative improvement of plant production. Protected areas are selected in order to protect the best preserved parts of nature.

Presented conservation types are based on the concept of *status quo* protecting the natural conditions of the local habitats in which is achieved optimal allelic gene frequency that ensures survival and reproduction in the existing conditions. This is a dynamic conservation type that includes preservation of genetic composition of population, with continuous genetic-evolutionary processes, as a result of the interaction between genotypes and environmental factors. In this way, *in situ* conservation programs of selected target species often result in significant conservation of other, accompanying plant and animal species (Šijačić-Nikolić and Milovanović 2010).

In situ conservation is considerably more efficient in conservation of ecosystem functions rather than individual species. In most woody species, *ex situ* conservation methods cannot be applied due to a number of biological, technical, and resource constraints, which increases the importance of *in situ* methods. Since the conservation of genetic resources is a part of the integrated system of management of natural forests and protected areas, *in situ* conservation will, in most cases, represent the most economical option.

Application of *in situ* conservation of natural forests improves the function of the entire ecosystem and interspecific interactions. In addition, there are numerous woody and shrub species in forests that may not be of great importance to the forest management, but can be of great value in terms of genetic resources and their use in the future. However, the conservation of these species may require specific management measures, which will be implemented through the selection of areas for genetic conservation. From a theoretical point of view, network of areas for the conservation of forest genetic resources must be an effective way of conservation of genetic resources of target species, if it is in accordance with the spatial distribution of genetic variability (Eriksson and Ekberg 2001).

7.3.2 Ex Situ Conservation Methods

Ex situ conservation is a form of conservation of forest genetic resources outside their natural habitat (out of site). It may include conservation of single individuals, groups of individuals or just conserving their individual parts. Preserving individuals as individual conservation units is performed in the botanical gardens, arboreta or living archives. Conservation of the smaller or larger groups of individuals is performed by establishing seed orchards, clonal archives, progeny tests or provenance trials. Preservation, and conservation of certain parts of an individual, is realized in gene banks. As classical methods of static *ex situ* conservation can be considered only seed banks, pollen banks, DNA banks, in vitro explants, cryopreservation and clonal archives in which are, due to genetic uniformity of individuals and vegetative turnover of generations, almost eliminated the effects of natural selection. Any other method of *ex situ* conservation, primarily, can be regarded as dynamic.

7.3.2.1 Arboreta and Botanical Gardens

Botanical Garden represents a collection of living plants, which is mainly used for improvement and dissemination of botanical knowledge (Potočić 1980). As forerunner of today's botanical gardens can be considered private gardens where wealthy people, in being and nature lovers, collected and cultivated different plants. The first public botanical garden was founded by the Venetian Republic in 1545, in Padua as a scientific institution of the university of that time. Modeled on the botanical gardens in Padua, Pisa and Bologna, botanical gardens were founded throughout Europe. Nowdays, there are being kept unique specimens of various plant species, some of which a long time ago disappeared from the natural habitat. An arboretum (lat. *arbor*-tree) is a separate space or part of the botanical gardens, in which are grown trees and shrubs in the scientific, ornamental and breeding purposes (Potočić 1980). The oldest such collection of trees and shrubs was mentioned in botanical garden in Tokyo, which is reportedly over 800 years old.

7.3.2.2 Seed Orchads

Establishment of *ex situ* populations requires considerable financial resources. The economic justifiability of these populations appears only in cases when achieve double aim, such as simultaneously collecting seeds for commercial purposes and long-term conservation of genetic diversity of seed sources, which is best achieved by establishing seed orchards. Seed orchards and clonal archives are examples of static *ex situ* conservation units, because there are no dynamic changes in their genetic structures. However, those are important pilot objects in the program of testing the genetic potential of initial population, because effects of simultaneous natural and artificial selection processes may be observed. The purpose of establishing these

orchards is to establish populations that will maintain the original genetic variability to the maximum extent and allow long-term adaptation to local conditions, where planting was done. In addition to preserving the original genetic variability, seed orchards are used as sources of reproductive material for commercial forestry.

Seed orchard means specialized, artificial culture for long-lasting production of genetic quality seeds of economically significant tree species (Tucović 1990). Seed orchards are established from phenotypic and genotypic best individuals of one, or various, species, at area which is supposed to be spatially isolated from physically mature trees of the same species, in order to prevent uncontrolled pollination of individuals that come out of the seed orchard. According to the character of planting material, plantations can be vegetative, formed of clones or generative, formed of plants originating from the half-sib or full-sib lines of selected genotypes.

7.3.2.3 Progeny Tests

Progeny tests represent an opportunity for exploring the genetic potential of certain species, provenances, populations or the genotype. The basic principle of the establishment of such experiments is to create uniform conditions for the cultivation of plants that are being tested, so that mutually appeared differences are reflection of various genotypes, not various environmental conditions. Progeny tests may be established of *full-sib lines*, which represent offspring of controlled hybridization (both mother and father are known), or *half-sib lines,* where the mother is known, and the father is unknown (free pollination). Tests can be performed in the phytotrons, greenhouses, nursery or field conditions. According to duration of experiment, there are early tests, short-term and long-term tests. A particular form of genetic potential assessment, which are constantly gaining in importance are early tests. Research conducted in the earliest stages of ontogenesis in Serbian spruce have pointed to the importance of the same for exploring the variability of species, its taxonomy, genetic potential, cultivation, breeding and conservation direction (Šijačić-Nikolić and Milovanović 2010). The assessment of adaptive and production potentials of different lines of half-sibs in progeny tests is used for the selection of elite trees, which represent a basis for seed collection and seedling production, aimed at spreading the population of some species and preservation of genetic variability (Nonić et al. 2012).

The age variability is the result of natural selection, which is expressed at each stage of the life cycle. Therefore, the variability of plants in the juvenile development phase can be considered as products of the interaction of hereditary basis and selection. Genetic variability of the mature population is significantly reduced, compared to the variability of baseline levels seedlings, 1 year or 2 years old plants, considering that selection was the highest in the germination stage, after which it was reduced, but continuous. Wherein the larger dimensions of the cotyledons, hypocotyls and epicotyls characteristics, good root system characteristics of, and similar, indicate the potential superiority of adult individuals in comparison to the average. Every geneticist, breeder or nurseryman, who knows spontaneous variability,

already after the formation of cotyledons, can observe possible deviations from the usual form, in particular in the haploids, polyploids or polysomics, which provides high speciation (Tucović 1990). The results of the early tests should be considered approximate they need to be checked in the following years of research.

7.3.2.4 Provenance Trials

Provenance trials, within gene pool conservation, are applied as a method of assessing the degree of diversity and potential, both autochthonous and allochthonous trees species. Also, provenance trials may contribute to determining the potential and the degree of divergence of isolated populations, in terms of higher productivity and adaptability, respectively, can be used to determine the differences in genetic variability between and within different provenances.

7.3.2.5 Preservation

The gene banks are formed to preserve the total genetic variability on a scientific basis. The task of gene banks was to collect, determine, document, reproduce and preserve genetic variability at the long term, and made it available for use. A fully and easily accessible information are the primary requirements for the use of genetic variability that is stored in gene banks (Penčić et al. 1997). Plant gene banks are formed from the parts of plants that contain germplasm—genetic basis of species. The most commonly applied method is cryopreservation, which allows long-term storage of plant parts (Withers and Engelmann 1998). This term refers to seed storage at extremely low temperatures, typically with the use of liquid nitrogen (−196 °C). Applied together with the in vitro technique, cryopreservation is often the only reliable, safe and economically viable method of storage of some species. In cases where the seed is not suitable for cryopreservation, the extract of embryo or the core of the embryo should be applied, at the appropriate stage of development. Establishment of seed banks, where the seed is stored in refrigerators or under other appropriate conditions, is another form of static *ex situ* conservation. Seed banks can be used only for species whose seed storage is possible. Most species have seeds with high germination rates sustainable a few years, which is extremely short, compared to the long life span of trees, and therefore the seed supplies must be renewed at regular intervals. It involves germination, seedling production, tree growing to the beginning of fruiting, collection and storage of new seeds.

This “rejuvenilization” leads to the appearance of new genetic recombination and new selection pressures during the propagation and growth. For most species, seed banks have to be a short-term form of conservation. Seeds of endangered populations can be collected and stored in a bank for a fixed period, until the most suitable moment for the seeding and seedlings development for the establishment of *ex situ* conservation habitats.

In addition to seed banks, germplasm conservation can be also realized by establishing pollen banks in which the pollen, dryish to 5 % moisture, is stored on dry surfaces and temperature of 0 °C. However, some species produce pollen whose longer storage is not possible. There are experiences with species whose pollen loses viability and germination after 5 years of storage (Towill 1985). Certainly, pollen has a relatively short period of viability compared to the seed. For these reasons, despite the great importance of this technique for germplasm conservation of species with low seed storage capabilities using pollen banks is very limited.

Germplasm conservation can be realized by applying the method of DNA banks, which are increasingly gaining in importance. Nowadays, DNA, isolated from the nucleus, mitochondria, or chloroplast, can be easily immobilized in the nitrocellulose fibers, where it can be stored. Reproduction of a specific oligonucleotide, or the entire gene from genomic DNA has become a routine procedure by the development of PCR techniques. This method has enabled the creation of an international network of DNA storage (Adams 1997). The advantages of this technique are its efficiency, simplicity and small space requirement. The main disadvantage, besides demanding equipment and facilities, lies in the restrictions during the isolation, cloning and gene transfer, i.e. impossibility of regenerating of the whole plant (Maxted et al. 1997).

Another type of static *ex situ* conservation strategy is *in vitro* conservation. Depending on the species and techniques, some genetic changes (such as mutations) can occur during the *in vitro* growth and storage, and may sometimes be called somaclonal variability (Fourre et al. 1997). *In vitro* (in glass) method involves the manipulation of the explants in a sterile environment, without the pathogen. It is used for the conservation of species whose seeds cannot be stored for a longer time period, the species that do not provide seed or vegetative material obtained in order to maintain the target genotypes (Engelmann 1997).

Although the first studies on the *in vitro* technique were conducted only a few decades ago, this technique is already used for multiplication, storage and collection of germplasm of over 1000 species (Bigot 1987). *In vitro* technique can be effectively used for collection, multiplication and storage of particularly problematic species (Engelmann 1997). The technique was developed in order to produce plants from seed or vegetative material, directly from the field, under aseptic conditions (Withers 1995). Such an approach allows the creation of germplasm collections in the fields of restoration, or in situations where the seeds transport is economically unjustified. However, due to the very high costs of implementation, this method is generally less useful in the forest genetic resources conservation. The static conservation type includes also clonal archives, which represent a set of vegetatively propagated clones. Grafted or rooted trees will still be able to grow up to a certain age, if the grafting was successful. Naturally, for a certain time, grafting or rooting procedure must be repeated. This static conservation type requires continuous and intensive management by a man.

7.4 Linking Forest Genetic Resources with People

One of the main developments in forestry practice over the last three decades has been its evolution from a practical discipline with a primary, or even exclusive, focus on management of forests for timber, to a more holistic approach recognizing that forests provide a wide range of environmental and social services and that provision of these should form an objective of management. The development of concepts such as forest ecosystem management and multi-purpose forestry are symptomatic of this process.

The importance of forests to people has been increasingly recognized, as illustrated by the widespread implementation of forest management approaches explicitly aimed at or involving local communities, such as community forestry and social forestry. The importance of actively involving local communities and other stakeholders is consistently an element of approaches to sustainable forest management (Newton 2007).

Participation in forest conservation is often associated with the concept of community forestry. Community forestry basically means that a forest is managed or co-managed by people who live close to it (Wily et al. 2000). Local participation is important in almost all forest conservation, but there are situations where it is absolutely necessary, for instance in areas characterized by high population pressure and conflicts of resource use; in areas under communal ownership; and in smaller protected areas because of the vulnerability to surrounding human activities (FAO, FLD, IPGRI 2004).

The impact of any form of property on conservation depends on a variety of external and internal factors, such as the economic status of the owners and the structure of ownership. Small properties can be good for conservation, since whatever management decision a particular owner makes, it has a limited impact. Thus these holdings form a mosaic of habitats. Many small forest owners follow in fact the traditional silvicultural systems. Although looked down upon by forestry professionals, private forests are a reservoir of "unwanted" genotypes—irregular forms, forked or twisted trunks. Often old trees, of no commercial value, are left to live their natural life span. Woody debris is removed only if used as fuel. Larger private properties, that are regularly managed, are influenced by traditions of even-age silviculture. This, as in state forests, leads to simplification of ecosystems and loss of biodiversity. Management mistakes in large properties have greater impact and are more difficult to rectify.

The concept of conservation is poorly understood both in society at large, and within the group of forest owners. Appreciation of the role of elements of the ecosystem is missing—many owners do not see that forest is not just trees. Forest owners often understand a need to protect game, birds or some rare flowers. However, they do not understand very well importance of conservation of genetic variability (IUCN 2004).

In an effort to save genetic diversity, a number of approaches to conservation have been suggested. Some approaches focus on species' habitats, ecosystems, or

other area-based classifications such as hotspots and eco-regions. Such approaches seek to save nature in a place or region by ensuring that the ecosystem processes and structures which support nature are maintained. Although these approaches are critical to conservation of nature, they are insufficient on their own. Just as species need well functioning ecosystems in which to live, ecosystems depend on their species. An exclusively area-based approach can result in species being lost from the areas of concern. Conservationists have long appreciated that many species, and species groups, need particular attention, requiring species-focused conservation strategies. Furthermore, because many people have deep attachments to particular species, these can be used to catalyze conservation efforts. In other words, endangered species can serve as iconic ambassadors for the conservation of nature (IUCN/SSC 2008).

People's participation is essential in development projects as well as in conservation of natural resources including forest genetic resources. Conservation of forest resources requires that stakeholders trust one another and commit themselves to the task of sustainable forest use. Participatory Conservation Planning draws on some common participatory rural appraisal techniques, and is designed so that the resulting information can be recorded with minimum modification. Major assumptions in using this tool are that (Conservation Training and Resource Center 2004):

- Community support and acceptance is the most important factor in designing successful management strategies: the early identification of acceptable strategies, and the elimination of unacceptable ones, is a valuable management objective;
- The use of qualitative data in the analysis is strength, allowing discussions to be inclusive, involving a range of people with varying levels of technical skills, rather than the exclusive preserve of "experts". It also allows for issues to be resolved on the basis of existing information, and can easily be reviewed when more detailed information becomes available; and
- In general, major threats identified by expert conservationists will act on a wide range of systems across a protected area, so the same threats will also be acting on the systems identified by communities, and vice-versa.

No two participatory processes will ever be exactly identical because people, forests and other circumstances vary from place to place and from time to time. Even so, most participatory processes will involve a number of different phases or steps for conservation of forest genetic resources (Window 1, according to FAO, FLD, IPGRI 2004).

All these steps require full participation of all relevant interested parties. Participatory research is a collaborative learning process where local people and researchers are full partners in creating knowledge. This means that community members are involved in the formulation of the research question, methodology, data collection and analysis phases.

Participatory research requires constant self-reflection on the relationship of the researcher to the community and on the impact of that relationship on the research

(Thompson et al. 2005). Participation has been described as both a means and an end, a vehicle and a goal itself (Jennings 2000).

Participatory tools reflect the dual nature of participation. A practitioner might use participatory activities purely to elicit local knowledge and perspectives. Local people's input is limited to providing information, while the information that the tool generates is used by decision makers elsewhere.

Window 1 Steps for forest genetic resources conservation (according to FAO, FLD, IPGRI 2004)

STEP 1: Identification of FGR conservation objectives (species and areas to be protected);
STEP 2: Selection of suitable sites (if sites are initially selected by government, planners and scientists, it is crucial that other stakeholders are able to challenge or change this decision later in the process);
STEP 3: Stakeholder analysis (Who will be affected by conservation activities? What are their interests? Who has a right to participate? How do different stakeholders affect the conservation area?);
STEP 4: Collection of baseline data on target species, local communities, and government plans in selected sites;
STEP 5: Re-evaluation of FGR conservation objectives and formulation of activity plan done in co-operation by all involved interest holders (the most important step!)
STEP 6: Identifying the institution to be responsible for conservation activities (establishment of social institution on local level to take up responsibilities for implementing and monitoring conservation activities represents a case of full participation—planning by the community!);
STEP 7: Implementation of conservation activities (At any time, during the participatory process, stakeholders may realize that available baseline data need to be revised or supplemented by additional forms of information, project need to be designed with a high degree of flexibility to accommodate changes);
STEP 8: Monitoring of target species, other objectives as well as participatory process itself!

On the other hand, involving local people in decision making might be the objective for using a participatory tool. This participatory approach is called "collaborative management". There are several participatory tools that are particularly strong in collaborative management while selection of appropriate tool depends on the objective that researchers want to reach (Table 7.1).

Collaborative management actually brings the community into the decision making process, involving local people in discussion, negotiation and planning. This

Table 7.1 Some of the participatory tools which can be used in collaborative forest genetic resources conservation management

Participatory tool	Objectives
Participatory mapping/resource mapping	Elicit knowledge about local resources
Spidergrams	Stakeholder identification, quantitative values, elicit values about resources and social interactions
Venn diagrams	Identify stakeholders, stakeholder relationships, elicit values about resources
H diagram	Elicit knowledge and opinions about local resources and improvement of current situation
Brainstorming	Elicit knowledge and opinions about local resources and improvement of current situation

approach is the most appropriate for planning of forest genetic resources conservation, having in mind that people live with forests and vice-versa (Evans et al. 2006).

There is no real alternative to public involvement and collaboration among all stakeholder groups in the development and implementation of policies for the conservation of forest genetic resources. This is the only way of generating policies that meet the final and absolute test: to be sustainable, policies must be socially acceptable (Lindenmayer and Franklin 2002).

7.5 Global Climate Change and Conservation of Forest Genetic Resources

Rapidly changing climates represent considerable threats to the conservation of forest genetic resources, which are among the most essential natural resources. Climate change is recognized as one of the most important challenges faced globally by ecosystems. The forest trees are very sensitive to climate changes because they have a long life span and it takes considerable time to adapt to changing environmental conditions (Lindner 2007; Koskela et al. 2007)

According to the Intergovernmental Panel on Climate Change (IPCC), global average surface temperatures will rise about 1.8–4.0 °C during the twenty-first century (Intergovernmental Panel on Climate Change 2007). Climate change is unlikely to stabilize in the foreseeable future, and may become more frequent, contributing to losses of forest genetic resources. Therefore, conservation of forest genetic resources and the maintenance of both adaptive and neutral genetic diversity are essential for the survival of forest trees (Volis and Blecher 2010; Ahuja 2011; St. Clair and Howe 2011).

Genetic diversity is needed to maintain the vitality of forests and has a crucial role in maintaining forest biological diversity. It ensures that forest trees can survive and adapt to changing environmental conditions and makes it possible to continue and advance the adaptive processes on which evolutionary success depends. The probability that genetic diversity will be lost depends on natural processes, human

factors, and their interactions. Threats to genetic diversity are further complicated by interactions with deforestation, habitat loss and poor management (St. Clair and Howe 2011). It is essential that the genetic diversity in plant genetic resources be properly understood and efficiently conserved and used. If the level of genetic diversity in a species is greater, the chances for its survival are better (Ledig 1988; Ramanatha Rao and Hodgkin 2002; Koskela et al. 2007; Ahuja 2011). Climate change will have long-term and widespread consequences for many species, but it is important to give greater priority to those species and populations at greatest risk.

Considering the longevity and the long regeneration cycle of forest tree species, climate changes must be one of the key factors in creating conservation strategies at the individual and population levels. Forest trees define the essential characteristics of forests, and threats to forest genetic diversity include threats to species, populations, and genetic variation within populations (St. Clair and Howe 2007, 2011). Trees can respond in different ways in the face of global climate change. They may have high phenotypic plasticity and tolerance, which will play an increasing role in the adaptation of forest stands to changing environmental conditions (Mátyás 2006; Savolainen et al. 2007). The ability of forest tree species to respond to climate change is limited by their long life spans, long juvenile phases and long generation intervals. However, forest trees maintain high levels of gene flow and genetic variation, which should facilitate their ability to evolve in response to rapid changing climate (Hamrick et al. 1992; Erickson et al. 2012).

According to Loo et al. (2011), survival will depend on the capacity to quickly adapt genetically to new conditions at existing sites; high degree of phenotypic plasticity and/or migration to newly evolving environments that match basic physiological requirements (Loo et al. 2011).

In regions where climate change is expected to be rapid and extensive, many forest tree species will be exposed to stress. Changing climates will result in new species invasions, new pests and diseases, flooding, temperature extremes, altered patterns of gene flow and the hybridization of species and populations, competitive pressures, and wildfires are expected to be more frequent. Therefore, high mortality due to extreme climatic change, in combination with regeneration failure, will result in the loss of forest genetic resources and the local population extinction (Woods et al. 2005; Carroll et al. 2006; Westerling et al. 2006; Koskela et al. 2007; Loo et al. 2011; St. Clair and Howe 2011; Erickson et al. 2012). The specific effects of climate change will vary depending on the degree of sensitivity, exposure and the adaptive capacity of individual species and populations (Parry et al. 2007; Chimura et al. 2011; Erickson et al. 2012).

According to Mátyás (2006), there are various mechanisms (genetic and non-genetic) balancing changes in environmental conditions on different levels: individual, population, species and ecosystem level. On individual genotype level, environmentally induced phenotypic plasticity and "*carryover effects*" (Jablonka et al. 1995) provide the ability to survive in a wider range of environments without classic genetic change. On the level of populations, natural selection adjusts the adaptability of the population to changing conditions through genetic adaptation, and sufficiently large genetic diversity is precondition for fast and effective genetic

adaptation. The maintenance of long-term genetic adaptability is therefore directly depending on the conservation or even reconstruction of adaptive genetic variance. On species and ecosystem level, a possibility of responding to changes in the environment is migration through seed and pollen dispersal (Mátyás 2006).

The research exclusively based on qualitative properties (molecular markers) does not provide enough information for conservation planning process, with the aim of mitigating the effects of future climate change. Observation of adaptive quantitative traits within the field experiments is necessary to confirm the results of molecular research, but also for studying non-genetic mechanisms—phenotypic plasticity and ecological interactions (Mátyás 2006). It is necessary to improve existing field experiments and establish new comparative tests.

Genetic and environmental research and data from comparative tests represent the basis for adequate conservation planning process, which is a key to preserving vulnerable species and populations for the future. Climate change mitigation measures should be focused on the selection of appropriate populations in problematic areas and the establishment of new enriched populations in the less endangered areas.

In the case of extremely endangered, rare species and populations, it may be necessary to establish archives and plantations for the conservation and development of the offspring. Furthermore, it might be useful to deploy a mixture of seeds from widely divergent populations from different environments as a resource for seed orchards and planting material for potential future changed environmental conditions (Ledig and Kitzmiller 1992; Ahuja 2011). Particular attention should be paid to alternatives of genetic adaptability, such as phenotypic plasticity, which connects evolution, ecology and genetics, and should be the priority selection criteria in the process of conservation and breeding.

The understanding of the balance between genetic constraints, natural selection and phenotypic plasticity is essential for predicting responses and tolerance limits for dominant species in forest ecosystems (Mátyás 2006; Šijačić-Nikolić and Milovanović 2007). The urgency for gene conservation has become greater with increasing percent of the world's plant species that will be at increased risk of extinction. Establishment of forest tree species and populations at new locations where they are better adapted to future changed environments may be important for conserving genetic diversity. Synthesis of evolution, ecology, qualitative and quantitative genetics is crucial to keep the adaptability of populations of forest species in the process of global climate change.

7.6 Conclusions

The increasing demand for wood, as a raw material for various purposes, as well as general useful forest functions, have made the protection (conservation) and directed utilization of forest genetic resources, become a priority task of forestry science and profession.

Conservation of forest genetic resources could be defined as a set of activities and strategies that are being implemented with the aim of ensuring the continued existence, evolution and availability of these resources for present and future generations.

Genetic variability, which is the result of different genetic processes: mutation, recombination, gene flow, natural selection and genetic drift, presents the basis for conservation of forest genetic resources. The principles of conservation of genetic variability can be regarded as identical for all living beings. However, the methods which are applied vary depending on the specificity of the conservation goals, distribution and biological nature of the material that is the object of conservation.

From the aspect of preserving genetic variability, there are different methods of conservation. The term "method" is used in the context of a certain concept of conservation of genetic resources: *in situ* or *ex situ*, dynamic or static, while the species, ecosystem, population, individual or part of an individual, present objects of conservation (Šijačić-Nikolić and Milovanović 2010).

Forest management aimed at improving production and protection functions can and should be harmonized with the concept of conservation at various levels: local, national and regional. Conservation of forest biodiversity, which includes forest genetic resources is of essential importance for the sustainable use of forest values, for improvement the health condition and vitality of forest ecosystems and the promotion and development of their protective, aesthetic and cultural functions.

Forest genetic resources—genetic diversity contained in thousands of tree species on Earth—represent a source of social, economic and environmental values for numerous human generations. Conservation of forest genetic resources, therefore, can be defined as a set of activities and strategies that are being implemented with the aim of ensuring the continued existence, evolution and availability of these resources for present and future generations. Genetic resources and their conservation process are characterized by exceptional dynamics. Consequently, the conservation of these resources should be regarded as the efforts to preserve specific genotypes or populations and their characteristic gene combination.

The term "forest genetic resources" is present in almost all current international processes, initiatives and strategic documents related to the sustainable management of forest ecosystems and biodiversity conservation in general. As opposed to non-binding international processes and initiatives, forest genetic resources are not treated as a separate issue in binding legislation. The problem of conservation of forest genetic diversity is considered through the prism of nature conservation and natural resources, as well as the regulation of trade and market issues of forest reproductive material. The last decades, the activities on the conservation of forest genetic resources are becoming more intense and are realized within the European Forest Genetic Resources Programme (EUFORGEN), which is finalizing Phase IV (2010–2014).

Raising awareness of forest owners and users about the importance of conserving genetic diversity can significantly contribute to the improvement and implementation of strategic priorities. There is no real alternative to public involvement and collaboration among all stakeholder groups in the development of policies for

the forest genetic resources conservation. Conservation priorities set by the community represent the best strategic approach for forest genetic resources management especially when we have in mind various threats such as climate changes.

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Chapter 8
Advances in Cryogenic Techniques for the Long-Term Preservation of Plant Biodiversity

Maria Teresa Gonzalez-Arnao, Marcos E. Martinez-Montero, Carlos A. Cruz-Cruz and Florent Engelmann

Abstract This chapter presents different technical aspects related to the development and large-scale application of cryopreservation techniques, as a biotechnological approach for the long-term storage of plant biodiversity. The main cryogenic procedures and the key steps for their successful adaptation to diverse forms of germplasm are described. Some representative examples of cryopreservation of different plant species are presented to illustrate the significant progress achieved in the practical utilization of cryopreservation as a complementary alternative for germplasm conservation. In addition, other potential uses of this technology to support genetic breeding programs, and its recent utilization to eliminate systemic plant pathogens through cryotherapy are discussed.

Keywords Biodiversity · Cryopreservation · Storage · Breeding · Cryotherapy

8.1 Introduction

Biotechnology has an impressive impact on the characterization, utilization and conservation of plant biodiversity. Some biotechnological techniques such as *in vitro* culture are very useful in maintaining *ex situ* germplasm collections of plant

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species that have asexual propagation and of species that are impossible to keep as seeds or in field gene banks. Nowadays, biotechnology is also necessary to solve important problems of management, breeding and storage of plant genetic resources. Biotechnology provides new tools to select genotypes with desirable traits, to identify and incorporate important genes that induce disease resistance and tolerance to biotic and abiotic stress. It also offers diverse *in vitro* strategies to multiply and preserve plant genetic biodiversity outside its natural habitat. In a general context, biotechnological methods have been developed and used to conserve endangered, rare, crop, ornamental, medicinal, and forest species for short, medium and long-term (Cruz-Cruz et al. 2013). In addition, *in vitro* techniques offer a safe mean for the international exchange of germplasm, allow the establishment of extensive collections with minimal space requirements. They allow a valuable supply of materials for wild population recovery, they guarantee the storage of pathogen-free material and elite plants, and facilitate the performance of molecular investigations and ecological studies (Tandon and Kumaria 2005).

Until recently, most conservation efforts, apart from work on forest genetic resources, have focused on *ex situ* conservation, and particularly focused on seed genebanks. In the 1950s–1960s, the major advances in plant breeding due to the green revolution resulted in the wide-scale adoption of high-yielding varieties and genetically uniform cultivars of staple crops, particularly wheat and rice. Consequently, global concern about the loss of genetic diversity in these crops increased, because farmers abandoned their locally adapted landraces and traditional varieties, to replace them with improved, genetically uniform modern ones. In response to this concern, the international organizations related to agricultural research started to assemble germplasm collections of the major crop species, and coordinated a global effort to systematically collect and conserve the world's threatened plant genetic diversity (Engelmann and Engels 2002). As a result of this great effort, there are over 1750 genebanks worldwide today, about 130 of which hold more than 10,000 accessions each, and conserve around 7.4 million accessions (FAO 2009). There are also substantial *ex situ* collections in botanical gardens, of which there are over 2500 around the world, containing around 4 million accessions from over 80,000 species. In that way, botanical gardens and agricultural genebanks also represent important complementary strategies for *ex situ* conservation of plant biodiversity (Engels and Engelmann 1998). The establishment of field genebanks allows conserving species for which seed conservation is not appropriated or is impossible, but conservation in the field presents important drawbacks, either of biotic or abiotic character, and these limitations, seriously affect its efficacy and constitute a permanent threat to the safety of the germplasm conserved under these conditions. Therefore, the development of other storage technologies was a new common need for the international community (Engelmann and Engels 2002).

During the last 40 years, *in vitro* culture techniques have been extensively established and applied to more than 1000 different plant species (George 1996). Tissue culture techniques are of great interest for collecting, multiplication and storage of plant germplasm (Engelmann 1991). Tissue culture systems allow propagating plant material with high multiplication rates in an aseptic environment. In addi-

tion, virus-free plants can be obtained through meristem culture in combination with thermotherapy, thus ensuring the production of disease-free stocks and simplifying quarantine procedures for the international exchange of germplasm. The miniaturization of explants allows reducing space requirements and consequently labour costs for the maintenance of germplasm collections (Cruz-Cruz et al. 2013). However, the high multiplication rates achieved by *in vitro* culture procedures lead to the regular production of large amounts of plant material. This generates problems of managing large *in vitro* collections with a permanent risk of losing material through contamination or human error at each subculture, beside the risks of losing the genetic integrity of the plant material due to somaclonal variation induced by the prolonged time in culture (Scowcroft 1984). To avoid these difficulties, cryopreservation started receiving higher attention, and currently, it represents the safest alternative for long-term preservation of plant biodiversity without requiring continuous manipulations. In addition to germplasm conservation, cryopreservation techniques have proved useful to rejuvenate cultures, to cryoselect plant material with special properties, and to eliminate systemic plant pathogens such as virus, phytoplasmas and bacteria by cryotherapy (Engelmann 2004). However, the main stages involved in the cryopreservation procedures, such as cryoprotection, cooling/warming and recovery play a crucial role in attaining success, and each successive step must be optimized whatever the protocol selected and depending on the biological material used. In this chapter some technical aspects aiming at efficiently achieve cryopreservation are discussed, and some useful examples of its application are also presented.

8.2 Historical and Technical Aspects of Plant Cryopreservation

Plant cryopreservation techniques belong to the category of *ex situ* conservation strategies within the *in vitro* methods developed to guarantee the long-term storage of different biological materials. They are based on the use of tissue culture techniques, and on the conservation at ultra-low temperature, usually at −196 °C in liquid nitrogen, which produces the total arrest of all metabolic activity and cell divisions, ensuring that cells will not undergo genetic changes during storage (Gonzalez-Arnao et al. 2008).

At present, cryogenic research has deeply progressed as a broad discipline which includes the preservation of a wide spectrum of biodiversity and is extensively used in medical, horticultural, agricultural, aquaculture and forestry sectors (Benson et al. 2006). For centuries, cryobiology has functioned as an observational and empirical science. From at least 1787 the effects of cold temperatures on cells were investigated empirically (Luyet and Gehenio 1940), and the fortuitous discovery of the cryoprotective properties of glycerol in 1949 (Polge et al. 1949), resulted in a crucial moment for further development of cryopreservation research. However, the Russian scientist Nikolay Maximov was the first to describe the pro-

tective effects of glycerol in plants exposed to freezing temperatures (Maximov 1912), and he also identified the effect of other chemicals that eventually have become important cryoprotectants in contemporary research (Benson 2004). These pioneer investigations also strongly supported the studies performed later on plant cryoprotection (Finkle et al. 1985). Nevertheless, the first cited report on survival of plant tissues exposed to ultra-low temperatures was made by Sakai in 1956, who demonstrated that winter-hardy mulberry (*Morus* spp.) twigs could withstand exposure to liquid nitrogen. Another early report was the successful cryopreservation of flax cells published by Quatrano in 1968, but the most significant advances in the formulation of different cryogenic protocols occurred in the 1980s–1990s with the development of new innovative technological approaches such as a simple controlled-rate freezing method using a methanol bath (Withers and King 1980); the droplet-freezing technique (Kartha et al. 1982); vitrification (Sakai et al. 1990) and encapsulation-dehydration (Fabre and Dereuddre 1990). Currently, various improvements of these protocols and/or their combinations, have derived in the development of new vitrification-based procedures, and cryopreservation has been already developed for well over 200 plant species (Engelmann 2004), providing biotechnological alternatives for the storage and the potential use of plant biodiversity (Cruz-Cruz et al. 2013). Currently, the main challenge is to increase and improve its application at the cryobank level.

Today, it is well known that for any plant species, the first requirement to establish a cryopreservation protocols to have optimized its *in vitro* culture conditions. Operational tissue culture techniques ensure the regeneration, multiplication and recovery of plant material, providing a large number of samples with a relatively synchronized physiological state before and after cryopreservation (Engelmann 2011). Moreover, *in vitro* culture has made possible the production of a new category of germplasm, which includes cell suspensions, embryogenic callus and somatic embryos. These biotechnological products constitute a new source of material with important genetic information useful for breeding programs aiming at developing more productive and resistant plants to different stress conditions (Rao 2004), but they can only be safely conserved for long periods by using cryogenic storage.

The choice of starting material and its physiological characteristics play an important role to improve the tolerance to cryopreservation. Biological material can be sampled from *in vivo* or *in vitro* plants, but the use of *in vitro* mother-plants is generally preferable, since the explants are already miniaturized and free of superficial contamination (Engelmann 1991). However, recently, citrus and rose apices sampled from greenhouse material could be successfully cryopreserved. In case of citrus, apices were micrografted on *in vitro* rootstocks after rewarming (Volk et al. 2012), while growth recovery took place directly in case of rose apices (Le Bras et al. 2014).

The physiological state of *in vitro* mother-plants needs to be also optimized. For temperate species, a cold acclimation period, which triggers cold adaptation mechanisms, is often beneficial. For tropical, cold-sensitive species, this cold acclimation period can be efficiently replaced by a culture period on medium with high sucrose concentration (Engelmann 2014).

In the case of cell suspensions, the exponential growth stage is the most suitable for cells to successfully withstand liquid nitrogen exposure (Withers 1985). In that phase, cells are young, small, and contain only few vacuoles, which implies that they have low amounts of intracellular water, and are more tolerant. However, it is also important to take in mind as a general rule, that the long-term application of tissue culture techniques before cryopreservation may also significantly reduce the ability of biological material to survive after cryopreservation (Harding et al. 1991).

Another important parameter is the size of explants when dealing with organized structures as shoot-tips. Large explants usually maybe less tolerant to cryopreservation because they are difficult to get sufficiently or homogeneously dehydrated before their exposure to low temperatures, but at the same time, the dissection of too small meristems (about 0.2 mm) has to face the technical difficulties of excising, in addition to the main problem that such small explants do not always survive and regenerate new plants, especially when dealing with tropical species. Therefore, an important prerequisite is to define the smaller size of explant suitable to ensure viability of *in vitro* cultures.

Contemporary cryopreservation research has made a good use of the fact that sugars are natural plant cryoprotectants. Although the nature of the sugar employed during a preculture treatment can have a strong impact on recovery of cryopreserved explants, up to now, sucrose has played a major role in the acquisition of desiccation and cold tolerance in plants that are cryopreserved (Engelmann 1997). To date, there are many examples of its application at different stages for most cryopreservation protocols. In general, sucrose has been either successfully used alone or in combination with other cryoprotectants. Sucrose is also an important component for the formulation of loading, unloading and plant vitrification solutions (Kim et al. 2009a, b). Moreover, its addition to semisolid culture medium is often used for preconditioning of explants or of donor plants (Gonzalez-Arnao and Engelmann 2006). A 1-day-preculture of shoot-tips after dissection on semisolid medium supplemented with 0.3 M sucrose is the most commonly applied step of any cryopreservation procedure for organized tissues. In general, the action mode of sugars appears to be osmotic, but there are some evidences that they also have a crucial role in the stabilization of glasses and that their biochemical properties afford cell protection (Jitsuyama et al. 2002).

On the other hand, the unusual molecular characteristics of liquid water determine how it behaves throughout the cooling-warming cycle. Therefore, to provide an effective cryoprotection during a cryogenic process is also essential controlling the water phase transitions (Mazur 2004). In this sense, water removal and its interplay with the cooling and warming rates play a central role in preventing freezing injury.

Different methods exist to reduce the temperature depending on the cooling rate: ultra-rapid, rapid or slow cooling. In the latter case, a programmable freezing apparatus is usually used in order to obtain precise and reproducible cooling conditions. This characterizes the conventional methods for cryopreservation. Conventional protocols involve the pretreatment of samples with cryoprotective solutions composed by a single or a mixture of colligative chemical substances, followed by the

slow cooling (0.5–2.0 °C min^{-1}) up to a prefreezing temperature (usually around −40 °C) before rapid immersion of samples in liquid nitrogen (Gonzalez-Arnao et al. 2008). The conventional protocols induce a freeze-dehydration process by using a slow freezing regime, which aims at promoting the formation of an amorphous solid if the amount of remaining intracellular water at the moment of immersing samples in liquid nitrogen is so low that vitrifies (Benson et al. 2006). Compared to other techniques, controlled cooling is relatively costly to operate routinely (Ashmore 1997), but it has the advantage that many cryovials can be simultaneously bulk-handled during a cooling run, making the operation more time efficient compared to most vitrification methods that require operators to manually handle cryovials on a one-by-one basis.

Rapid and ultra-rapid cooling are characteristic of vitrification-based protocols, which involve some degree of dehydration and desiccation before cooling by exposure of samples to highly concentrated cryoprotective solutions and/or to physical drying conditions. As a result, most or all freezable water is removed during dehydration, and the internal solutes vitrify, when samples are rapidly immersed in liquid nitrogen (Engelmann 2000). Ultra-rapid cooling is achieved by using aluminium foil strips or aluminium cryoplates instead of cryovials to immerse samples directly into liquid nitrogen. This procedure significantly increases the probability of obtaining a vitrified state during cooling, and of avoiding devitrification during warming (Panis et al. 2005; Yamamoto et al. 2011).

Different vitrification-based procedures have been developed and have allowed cryopreservation of complex organs like shoot-tips and somatic embryos that could not be effectively cryopreserved following conventional protocols:

Vitrification: the vitrification technique involves treatment of samples with cryoprotective substances (loading), dehydration with a highly concentrated plant vitrification solution (PVS), rapid cooling and rewarming, removal of cryoprotectants and recovery. This procedure has been developed for apices, cell suspensions and somatic embryos of numerous different species (Sakai and Engelmann 2007; Sakai et al. 2008).

The most widely used Plant Vitrification Solution was developed by Sakai et al. (1990) and named PVS2, which consists of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, 15 % (w/v) DMSO and 13.7 % (w/v) sucrose in liquid medium.

Encapsulation/Dehydration: This is a technique in which shoot meristems or somatic embryos are encapsulated in calcium alginate beads (Fabre and Dereuddre 1990). The protective encapsulation process enables dehydration and desiccation to proceed which would otherwise be highly damaging or lethal to non-encapsulated samples (Gonzalez-Arnao and Engelmann 2006). The basic protocol comprises encapsulation, preculture of alginate coated samples in liquid medium with high (0.5–0.75 M) sucrose concentration, evaporative air or silica gel desiccation to a water content around 20 % (fresh weight basis), and rapid cooling in liquid nitrogen. Rewarming of the alginate encapsulated explants usually is performed at room temperature and for recovery the beads are placed onto standard culture medium without having to extract the shoots or embryos from their alginate coating (Engelmann et al. 2008; Sherlock et al. 2005).

Encapsulation-vitrification: This technique is a combination of encapsulation-dehydration and vitrification procedures, in which samples are encapsulated in alginate beads, and then treated and cooled as with the vitrification technique described above (Sakai and Engelmann 2007).

Droplet-vitrification is a protocol derived from the combination of the vitrification procedure with the droplet-freezing technique developed by Kartha et al. (1982) for cassava shoot tips (Gonzalez-Arnao et al. 2008). Samples are treated with loading and vitrification solutions, and then placed on an aluminium foil strip in minute droplets of vitrification solution or just in one small drop, and the aluminium foil strip is directly immersed with the samples in liquid nitrogen (Sakai and Engelmann 2007).

The most recent cryogenic procedure developed is termed Cryo-plate (Yamamoto et al. 2011), and combines the encapsulation-dehydration and droplet-vitrification techniques. In this method, shoot tips are attached with a thin calcium alginate layer to an aluminium cryo-plate, loaded, treated with PVS, and then cooled by direct immersion of cryo-plates in liquid nitrogen (Yamamoto et al. 2011, 2012).

The latest two cryopreservation techniques (Droplet-vitrification and Cryo-plate) have the common characteristic of providing higher cooling and warming rates compared to other vitrification-based procedures, since samples placed on aluminium foils or cryo-plates (with a very high thermal conductivity), are in direct contact with liquid nitrogen during cooling and with the unloading solution during warming (Engelmann 2014).

The pregrowth technique consists of culturing samples in the presence of cryoprotectants, followed by rapid immersion in liquid nitrogen. Dehydration consists of dehydrating explants usually by desiccation in the air current of a laminar airflow cabinet or with silica gel and then, direct immersion in liquid nitrogen. Pregrowth-Dehydration is the combination of the both previously mentioned methods. These techniques are mainly used for cryopreserving meristematic cultures, small size seeds, polyembryonic cultures, zygotic embryos or embryonic axes extracted from seeds, respectively (Gonzalez-Arnao et al. 2008).

The warming rate is also essential to avoid ice recrystallization, since during the temperature increase, ice nucleation can occur if samples are slowly rewarmed above the glass transition temperature (Tg) and homogeneous ice nucleation point ($\geq -40\,°C$). Therefore, rewarming should be performed rapidly, either by transferring the cryovials to a 40 °C water bath for several minutes or by plunging the aluminium foils with samples in the unloading solution at room temperature. Rapid rewarming allows preventing the destabilization of the non-crystalline vitrified glassy state, which limits devitrification and promotes a better post-storage recovery (Mazur 2004). However, this practice may cause stress fractures in the glass if rewarming is performed too rapidly. To reduce this possibility, a two phase approach may be useful: a first short phase (e.g. 1–2 s at ambient temperature) to allow glass relaxation without stress fracturing, followed by a rapid warming (at +45 °C) to ensure the speedy transition from glass to liquid without passing through an ice phase (Benson 2008).

8.3 Cryopreservation for Long-Term Storage of Plant Germplasm

A range of protocols have been widely applied to cryopreserve plant germplasm (Kaviani 2011).

Cryopreservation of seeds may be a very valuable strategy for the long-term conservation of tropical and subtropical forest species biodiversity, as it avoids problems related to embryo isolation and *in vitro* handling. However, the prerequisite requirement is that seeds be orthodox or intermediate. In addition, cryopreservation can be integrated as a complementary storage alternative for orthodox seeds. It was estimated that the shelf life of lettuce (*Lactuca*) seeds may be prolonged up to 20 times greater under cryogenic storage (Walters et al. 2004), than that predicted in a conventional seed bank at −20 °C (Roberts and Ellis 1989; Dickie et al. 1990). Some representative examples of seed cryopreservation are summarized in Table 8.1. Other applications of cryopreservation techniques to different *in vitro* forms: cell suspensions and callus cultures (Table 8.2), embryonic axes (Table 8.3), somatic embryos (Table 8.4) and shoot-tips (Table 8.5) of various plant species are also presented.

At present, there are a growing number of gene banks and botanic gardens where cryogenic techniques are already employed for different types of materials, representing good examples of large-scale application of cryopreservation (Cruz-Cruz et al. 2013).

8.4 Cryopreservation for Genetic Improvement

The development of biotechnology leads to the production of new germplasm including clones obtained from elite genotypes, cell lines with distinctive attributes and genetically transformed material (Engelmann 1992). This new germplasm is often of high added value and in some cases difficult to produce and maintain, therefore, its safe conservation is of paramount importance. In this context, application of cryopreservation techniques has been expanded to support some research breeding programs including those associated to genetic manipulation technologies. A good example is the one aimed at applying genetic manipulation for improving world cereal crops such as rice, wheat, barley and maize (Benson 2004).

Cryogenic storage has also been important to maintain stable mutant collections as the lines produced from the cell suspension cultures of *Arabidopsis thaliana* (Ribeiro et al. 1996). Another interesting strategy is to use cryopreservation to induce cold tolerance in sensitive species. Kendall et al. (1990) could select freezing tolerant calluses of *Triticum aestivum* by repeated exposures to liquid nitrogen, and the cryoselected calluses regenerated plants with enhanced cold hardiness. Cryoselection appears to involve, at least in part, selection for genetic rather than epigenetic variants (Engelmann 1991).

Table 8.1 Examples of plant species cryopreserved using seeds

Acacia sp.	Marzalina and Nashatul (2000); Salomão (2002); Touchell and Dixon (1994)
Abies procera	Walters et al. (2004)
Agathis spp.	Dickie and Smith (1995)
Albizia sp.	Salomão (2002)
Allium cepa	Walters et al. (2004)
Allocasuarina fraseriana	Touchell and Dixon (1994)
Amburana cearensis	Salomão (2002)
Anacamptis morio	Pritchard et al. (1999)
Anadenanthera colubrina	Salomão (2002)
Anemopaegma arvense	Salomão (2002)
Anigozanthos spp.	Merritt et al. (2005)
Apeiba tibourbou	Salomão (2002)
Apium graveolens	Gonzalez-Benito et al. (1995); Walters et al. (2004)
Apuleia leiocarpa	Salomão (2002)
Arachis hypogaea	Pritchard (1995)
Arabidopsis thaliana	Vernon (1999)
Aspidosperma spp.	Salomão (2002)
Astragalus spp.	Walters et al. (2004)
Astronium fraxinifolium	Salomão (2002)
Azadirachta indica	Berjak and Dumet (1996); Chaudhury and Chandel (1991)
Bambusa arundinacea	Marzalina and Nashatul (2000)
Banksia sp.	Touchell and Dixon (1994)
Beta vulgaris	Walters et al. (2004)
Bauhinia spp.	Salomão (2002)
Bletilla formosana	Hu et al. (2013)
Bowdichia virgilioides	Salomão (2002)
Brassica sp.	Perez-Garcia et al. (1996); Walters et al. (2004)
Bratonia	Popov et al. (2004)
Buchenavia tomentosa	Salomão (2002)
Burchardia umbellata	Touchell and Dixon (1994)
Byrsonima basiloba	Salomão (2002)
Camellia sinensis	Hu et al. (1994)
Capsicum annuum	Gonzalez-Benito and Perez-Garcia (2001); Walters et al. (2004)
Cariniana sp.	Salomão (2002)
Cassia spectabilis	Marzalina and Nashatul (2000)
Casuarina sumatrana	Marzalina and Nashatul (2000)
Cedrela fissilis	Nunes et al. (2003); Salomão (2002)
Chamaecrista desvauxii	Salomão (2002)
Chorisia pubiflora	Salomão (2002)
Citrullus lanatus	Walters et al. (2004)
Citrus spp.	Cho et al. (2002b); Lambardi et al. (2004); Makeen et al. (2005); Hor et al. (2005)
Cistus osbeckiifolius	Gonzalez-Benito et al. (1998b)
Coffea spp.	Dussert et al. (1997); Dussert et al. (1998)
Coronopus navasii	Gonzalez-Benito et al. (1998b)
Corylus avellana	Normah et al. (1994)
Crambe abyssinica	Walters et al. (2004)

Table 8.1 (continued)

Crotalaria cf.spectabilis	Salomão (2002)
Cucumis spp.	Walters et al. (2004)
Cyclolobium cf. blanchetianum	Salomão (2002)
Dactylorhiza spp.	Wood et al. (2003)
Dalbergia miscolobium	Salomão (2002)
Daucus carota	Pritchard (1995); Walters et al. (2004)
Dendrobium spp.	Wang et al. (1998); Vendrame et al. (2007)
Dendrocalamus spp.	Marzalina and Nashatul (2000)
Dialium divaricatum	Salomão (2002)
Dimorphandra mollis	Salomão (2002)
Dipterocarpus spp.	Marzalina and Nashatul (2000)
Dioscorea spp.	Salomão (2002)
Dodonaea hackettiana	Touchell and Dixon (1994)
Doritis pulcherrima	Thammasiri (2000)
Dyera costulata	Marzalina and Nashatul (2000)
Elettaria spp.	Chaudhury and Chandel (1995b)
Enterolobium spp.	Salomão (2002)
Eragrostis curvula	Walters et al. (2004)
Eriotheca gracilipis	Salomão (2002)
Elymus condensatus	Walters et al. (2004)
Eucalyptus loxophleba	Touchell and Dixon (1994)
Eulophia gonychila	Pritchard et al. (1999)
Festuca rubra	Walters et al. (2004)
Glycine max	Pritchard (1995)
Gossypium hirsutum	Gonzalez-Benito et al. (1998a)
Goodenia beardiana	Touchell and Dixon (1994)
Guettarda pohliana	Salomão (2002)
Guizotia abyssinica	Pritchard (1995)
Halimium spp.	Perez-Garcia and Gonzalez-Benito (2008)
Hardenbergia comptoniana	Salomão (2002)
Helianthemum spp.	Perez-Garcia and Gonzalez-Benito (2008)
Helianthus annuus	Pritchard 1995; Walters et al. (2004)
Hordeum vulgare	Pritchard (1995); Walters et al. (2004)
Indigofera australis	Touchell and Dixon (1994)
Jacaranda spp.	Salomão (2002)
Jacksonia floribunda	Touchell and Dixon (1994)
Kielmeyera coriacea	Salomão (2002)
Labichea teretifolia	Touchell and Dixon (1994)
Lactuca sativa	Walters et al. (2004)
Lafoensia pacari	Salomão (2002)
Lagestroemia floribunda	Marzalina and Nashatul (2000)
Lathyrus spp.	Cardoso-Almeida et al. (2000)
Lens culinaris	Cardoso-Almeida et al. (2000)
Lespedeza stipulacea	Walters et al. (2004)
Leucaena leucocephylla	Marzalina and Nashatul (2000)
Lilium ledebourii	Kaviani et al. (2009)
Lotus corniculatus	Walters et al. (2004)
Loxocarya 'gigas'	Touchell and Dixon (1994)
Luehea spp.	Salomão (2002)

Table 8.1 (continued)

Lupinus albus	Cardoso-Almeida et al. (2000)
Lycopersicum escluentum	Walters et al. (2004); Zevallos et al. (2013a, b)
Machaerium spp.	Salomão (2002)
Magonia pubescens	Salomão (2002)
Manihot sculentum	Marin et al. (1990)
Melaleuca cuticularis	Touchell and Dixon (1994)
Melanoxylum brauna	Salomão (2002)
Medicago sativa	Walters et al. (2004)
Mimosa spp.	Salomão (2002)
Musa balbisiana	Bhat et al. (1994)
Nicotiana tabacum	Walters et al. (2004)
Onobrychis viciifolia	Walters et al. (2004)
Onopordum acanthium	Gonzalez-Benito and Perez-Garcia (2001)
Ormosia fastigiata	Salomão (2002)
Oryza sativa	Walters et al. (2004)
Oxylobium atropurpureum	Touchell and Dixon (1994)
Papaver somniferum	Walters et al. (2004)
Paphiopedilum rothschildianum	Pritchard et al. (1999)
Passiflora spp.	Ospina et al. (2000)
Peltogyne confertiflora	Salomão (2002)
Pennisetum glaucum	Walters et al. (2004)
Petrophile dioaricata	Touchell and Dixon (1994)
Petroselium crispum	Walters et al. (2004)
Petunia spp.	Walters et al. (2004)
Phaius tankervilleae	Hirano et al. (2009)
Phaseolus vulgaris	Cardoso-Almeida et al. (2000); Walters et al. (2004); Cejas et al. (2012, 2013)
Pinus spp.	Pita et al. (1998)
Piper spp.	Chaudhury and Chandel (1994); Decruse and Seeni (2003)
Pistacia spp	Ozden-Tokatli et al. (2007)
Pisum sativum	Cardoso-Almeida et al. (2000)
Poa pratensis	Walters et al. (2004)
Plantago cordata	Pence and Clark (2005)
Platypodium elegans	Salomão (2002)
Ponerorchis graminifolia	Hirano et al. (2005)
Populus deltoides	Pence (1996)
Prunus spp.	Chaudhury (2000); Chmielarz (2009)
Pseudobombax cf. tomentosum	Salomão (2002)
Pterocarpus indicus	Marzalina and Nashatul (2000)
Pterodon emarginatus	Salomão (2002)
Pultenaea capitata	Salomão (2002)
Raphanus sativus	Walters et al. (2004)
Sclerolobium paniculatum	Salomão (2002)
Schinopsis brasiliensis	Salomão (2002)
Senna spp.	Salomão (2002)
Sesamum indicum	Pritchard (1995)
Siparuna guianensis	Salomão (2002)
Solanum melongena	Gonzalez-Benito and Perez-Garcia (2001)
Sorghum bicolor	Walters et al. (2004)

Table 8.1 (continued)

Spinacia oleracea	Walters et al. (2004)
Spondias mombin	Salomão (2002)
Sterculia striata	Salomão (2002)
Stryphnodendron sp.	Salomão (2002)
Stylobasium australe	Touchell and Dixon (1994)
Swainsona formosa	Touchell and Dixon (1994)
Swietenia macrophylla	Marzalina and Nashatul (2000)
Styrax camporum	Salomão (2002)
Tabebuia spp.	Salomão (2002)
Tectona grandis	Marzalina and Nashatul (2000)
Templetonia retusa	Touchell and Dixon (1994)
Thyrsostachys siamensis	Marzalina and Nashatul (2000)
Tocoyena formosa	Salomão (2002)
Trifolium spp.	Walters et al. (2004)
Triplaris gardneriana	Salomão (2002)
Triticum aestivum	Pritchard (1995); Walters et al. (2004)
Tuberaria major	Gonçalves et al. (2009)
Ulmus americana	Walters et al. (2004)
Vanda coerulea	Thammasiri and Soamkul (2007)
Vella pseudocytisus	Gonzalez-Benito et al. (1998b)
Vicia spp.	Walters et al. (2004); Cardoso-Almeida et al. (2000)
Vigna spp.	Normah and Vengadasalam (1992)
Warburgia salutaris	Kioko et al. (2000)
Wasabia spp.	Potts and Lumpkin (1997)
Zeyheria montana	Salomão (2002)
Zea mays	Walters et al. (2004)
Zinnia violacea	Walters et al. (2004)

Table 8.2 Examples of plant species cryopreserved using cell suspensions and callus cultures

Abies cephalonica	Aronen et al. (1999)
Asparagus officianalis	Nishizawa et al. (1993); Uragami et al. (1989); Jitsuyama et al. (2002)
Betula pendula	Ryynänen et al. (2002)
Brassica campestris	Langis et al. (1989)
Bromus inermis	Ishikawa et al. (1996)
Carica papaya	Tsai et al. (2009)
Castanea sativa	Corredoira et al. (2007)
Catharanthus roseus	Van Iren et al. (1995); Bachiri et al. (1995)
Citrus deliciosa	Perez et al. (1999); Aguilar et al. (1993); Engelmann et al. (1994)
Citrus sinensis	Sakai et al. (1990); Engelmann et al. (1994); Hao et al. (2003)
Citrus spp.	Perez et al. (1997)
Cyclamen persicum	Winkelmann et al. (2004)
Elaeis guineensis	Chabrillange et al. (2000); Dumet et al. (2000)
Festuca spp.	Wang et al. (1994)
Fragaria spp.	Wu et al. (1997)
Grape	Engelmann et al. (1994); Dussert et al. (1992)
Hevea brasiliensis	Engelmann and Etienne (2000)
Hordeum vulgare	Fretz and Lörz (1995)

Table 8.2 (continued)

Ipomoea batatas	Blakesley et al. (1996a); Bhatti et al. (1997); Shimonishi et al. (2000a); Golmirzaie et al. (2000)
Lolium spp.	Wang et al. (1994)
Mangifera indica	Wu et al. (2003)
Manihot esculenta	Danso and Fort-Lloyd (2004); Stewart et al. (2001)
Musa	Panis et al. (1990); Panis et al. (2007); Georget et al. (2009)
Nicotiana tabacum	Van Iren et al. (1995); Reinhoud et al. (1995); Schmale et al. (2006); Van Eck and Keen (2009)
Olea europaea	Lambardi et al. (2002)
Oryza sativa	Meijer et al. (1991); Jain et al. (1996); Huang et al. (1995); Lynch et al. (1995); Benson et al. (1995); Moukadiri et al. (2002); Wang et al. (2001); Watanabe and Steponkus (1995); Zhang et al. (2001); Zeng et al. (2009); Cornejo et al. (1995); Cho et al. (2007)
Papaver somniferum	Elleuch et al. (1998)
Picea abies	Nørgaard et al. (1993); Find et al. (1998); Högberg et al. (1998)
Picea glauca	Cyr et al. (1994); Kartha et al. (1988)
Picea mariana	Touchell et al. (2002)
Picea sitchensis	Kristensen et al. (1994); Find et al. (1998)
Pinus caribaea	Lainé et al. (1992)
Pinus patula	Ford et al. (2000b)
Pinus pinaster	Marum et al. (2004)
Pinus radiata	Hargreaves et al. (2002)
Pinus roxburghii	Mathur et al. (2003)
Pinus sylvestris	Haggman et al. (1998)
Prunus avium	Grenier-de March et al. (2005)
Pyrus pyrifolia	Gazeau et al. (1998)
Quercus robur	Chmielarz et al. (2005)
Saccharum spp.	Gnanapragasam and Vasil (1990)
Saccharum officinarum	Martinez-Montero et al. (1998)
Trifolium repens	Yamada et al. (1993)
Triticum aestivum	Chen et al. (1985); Fretz and Lörz (1995)
Vitis spp.	Dussert et al. (1992)
V. vinifera	Wang et al. (2004); Gonzalez-Benito et al. (2009); Vasanth and Vivier (2011)

Table 8.3 Examples of plant species cryopreserved using embryonic axes

Acer platanoides	Pukacki et al. (2009)
A. pseudoplatanus	Pukacki et al. (2009)
Aesculus hippocastanum	Pence and Dresser (1988)
A. glabra	Pence (1992)
Amaryllid	Sershen et al. (2007)
Anadenantha colubrina	Nery et al. (2009)
Arachis hypogaea	Gagliardi et al. (2002)
Araucaria hunstenii	Pritchard and Prendergast (1986)
Artocarpus heterophyllus	Chandel et al. (1995); Krishnapillay (1989); Thammasiri (1999)
Azadirachta indica	Berjak and Dumet (1996); Chandel et al. (1996)
Baccaurea motleyana	Normah and Marzalina (1995); Normah et al. (2000)
B. polyneura	Normah and Marzalina (1995); Normah et al. (2000)

Table 8.3 (continued)

Bactris gasipaes	Steinmacher et al. (2007)
Bletilla striata	Ishikawa et al. (1997)
Brassica napus	Withers (1982)
Byrsonima intermedia	Nogueira et al. (2009b)
Calamus manan	Krishnapillay et al. (1992)
Camellia japonica	Janeiro et al. (1996)
C. sinensis	Kim et al. (1998, 2002, 2005); Chandel et al. (1995); Wesley-Smith et al. (1992); Chaudhury et al. (1991); Kaviani (2009)
Capsella bursa-pastoris	Monnier and Leddet (1980)
Carva	Pence and Dresser (1988)
Carya illinoinensis	Abou-Taleb et al. (1992)
Castanea sativa	San-Jose et al. (2005); Corredoira et al. (2004); Pence and Dresser (1988); Pence (1992)
Citrus aurantifolia	Cho et al. (2002b)
C. halimii	Normah and Siti Dewi Serimala (1997)
C. hystrix	Normah and Laili Nordaini (1994)
C. latipes	Malik and Chaudhury (2006)
C. madurensis	Cho et al. (2001, 2002a, 2003)
C. macroptera	Malik and Chaudhury (2006)
C. medica	Cho et al. (2003)
C. reticulate	Normah and Laili Nordaini (1994)
C. sinensis	Sudarmonowati (2000); Santos and Stushnoff (2002, 2003)
C. suhuiensis	Makeen et al. (2005)
Cocos nucifera	Assy-Bah and Engelmann (1992a); Chin et al. (1989); Assy-Bah and Engelmann (1992b); Sajini et al. (2006)
Coffea spp.	Abdelnour et al. (1992); Dussert et al. (1997, 1999)
C. arabica	Martinez et al. (1996)
C. liberica	Normah and Vengadasalam (1992)
Corylus avellana	Gonzales-Benito and Perez (1994); Reed et al. (1994)
Durio zibethinus	Hor et al. (1990)
Ekebergia capensis	Peran et al. (2006)
Elaeis guineensis	Grout et al. (1983); Engelmann et al. (1995); Villa et al. (2007)
Elateriospermum tapos	Zaimah et al. (2007)
Euphoria longan	Fu et al. (1990)
Fagus	Pukacki et al. (2009); Pence and Dresser (1988)
Fortunella polyandra	Al-Zoubi and Normah (2009)
Fraxinus excelsior	Brearley et al. (1997)
Glycine max	Sudarmonowati (2000)
Hevea brasiliensis	Sam and Hor (1999); Yap et al. (1999); Normah et al. (1986)
Hopea odorata	Chai et al. (1994)
Hordeum vulgare	Withers (1982)
Howea forsteriana	Chin et al. (1988)
Ilex brasiliensis, *I. brevicuspis*, *I. dumosa*, *I. intergerrima*, *I. paraguariensis*, *I. pseudoboxus*, *I. taubertiana*, *I. theezans*	Mroginski et al. (2008)
Juglans regia	Pence and Dresser (1988)

Table 8.3 (continued)

Landolphia kirkii	Berjak et al. (1989)
Lansium domesticum	Normah et al. (2000)
Litchi sinensis	Sudarmonowati (2000); Chaudhury (2000)
Livistona chinensis	Wen and Song (2007a)
Manihot esculenta	Marin et al. (1990)
Melia azedarach	Kaviani (2009)
Musa acuminata *M. balbisiana*	Abdelnour-Esquivel et al. (1992)
Nephelium lappaceum	Hor et al. (1990)
Olea europaea	Gonzalez-Rio et al. (1994)
Paeonia lactiflora	Kim et al. (2004a)
Phaseolus vulgaris	Zavala and Sussex (1986)
Pinus radiata	Hargreaves et al. (2005)
Pisum sativum	Mycock (1999)
Poncirus trifoliata	Wesley-Smith et al. (2004a, b)
Prunus amygdalus	Chaudhury and Chandel (1995)
Prunus persica	de Boucaud et al. (1996)
Ptychospermum macarthurii	Normah and Marzalina (1995)
Quercus faginea	Gonzales-Benito and Perez-Ruiz (1992)
Q. falcata	Pence (1992)
Q. ilex	Gonzalez-Benito et al. (2002)
Q. leucotrichophora	Chaudhury (2000)
Q. macrocarpa	Pence (1992)
Q. nigra	Pence (1992)
Q. palustris	Pence (1992)
Q. robur	Berjak et al. (2000)
Q. rubra	Pence (1992)
Q. suber	Gonzalez-Benito et al. (2002)
Ricinus communis	Nogueira et al. (2009a)
Sechium edule	Abdelnour-Esquivel and Engelmann (2002)
Shorea leprosula	Chai et al. (1994)
Shorea odorata	Chai et al. (1994)
S. ovalis	Normah and Marzalina (1995)
S. parvifolia	Normah and Marzalina (1995)
Sterculia cordata	Nadarajan et al. (2006); Nadarajan et al. (2007)
Swietenia macrophylla	Marzalina (1995); Marzalina and Normah (2002)
Theobroma cacao	Pence (1991)
Triticum aestivum	Zavala and Sussex (1986); Kendall et al. (1993)
Veitchia merillii	Normah et al. (1986)
Vigna	Normah and Vengadasalam (1992)
Zea mays	Delvallée et al. (1989); Sudarmonowati (2000); Wen and Song (2007b)
Zizania palustris	Touchell and Walters (2000)
Z. texana	Walters et al. (2002)

Table 8.4 Examples of plant species cryopreserved using somatic embryos

Abies nordmanniana	Misson et al. (2006)
Aesculus hippocastanum	Jekkel et al. (1998); Lambardi et al. (2005)
Allium sativum	Sudarmonowati (2000)
Asparagus officinalis	Uragami et al. (1989, 1990b)
Brassica napus	Li et al. (1999)
Camellia japonica	Janeiro et al. (1996)
C. sinensis	Deepu et al. (2005)
Carya illinoinensis	Kumar and Sharma (2005)
Castanea sativa	Corredoira et al. (2004)
Citrus grandis	Oh (1997)
C. junos	Oh (1997)
C. platymamma	Oh (1997)
C. sinensis	Marin et al. (1993); Gonzalez-Arnao et al. (2003); Marin and Duran-Vila (1988)
Clitoria ternatea	Nair and Reghunath (2007)
Cnidium officinale	Cho et al. (1998)
Coffea arabica	Florin et al. (1995); Tessereau et al. (1994); Bertrand-Desbrunais et al. (1998); Mycock et al. (1995)
C.canephora	Hatanaka et al. (1994); Bertrand-Desbrunais (1991)
Coriandrum sativum	Popova et al. (2009)
Cucumis melo	Shimonishi et al. (1991, 2000a)
Daucus carota	Dereuddre et al. (1991); Lecouteux et al. (1991); Thierry et al. (1999)
Elaeis guineensis	Engelmann et al. (1985); Dumet et al. (1993, 1994)
Fraxinus angustifolia	Tonon et al. (2001)
Ipomoea batatas	Yoshinaga and Yamakawa (1994); Shimonishi et al. (2000b)
Iris nigricans	Shibli (2000)
Juglans regia	de Boucaud et al. (1994); Kumar and Sharma (2005); Lee (1989)
Macropidia fulginosa	Turner et al. (2000)
Manihot esculenta	Mycock et al. (1995); Stewart et al. (2001); Danso and Ford-Lloyd (2002)
Melia azedarach	Scocchi et al. (2007); Deb (2002)
Olea europea	Shibli and Al-Juboory (2000)
Paeonia lactiflora	Kim et al. (2006)
Phoenix dactylifera	Mycock et al. (1995)
Picea mariana	Bomal and Tremblay (2000)
P. glauca	Bomal and Tremblay (2000); Percy et al. (2001)
P. glaucaxengelmannii	Percy et al. (2001)
Pinus patula	Ford et al. (2000a)
Picea sitchensis	Gale et al. (2008)
Pisum sativum	Mycock et al. (1995)
Pometia pinnata	Sudarmonowati (2000)
Quercus robur	Martinez et al. (2003)
Q. suber	Fernandes et al. (2008); Valladares et al. (2004)
Saccharum spp.	Martinez-Montero et al. (2008)
Theobroma cacao	Fang et al. (2004, 2009a, b)
Vitis vinifera	Miaja et al. (2004)

Table 8.5 Examples of plant species cryopreserved using shoot-tips

Actinidia spp.	Wu et al. (2001); Sudarmonowati and Rosmithayani (1997); Suzuki et al. (1996); Bachiri et al. (2001)
Allium porrum	Niino et al. (2003)
Allium sativum	Makowska et al. (1999); Niwata (1995); Kim et al. (2004a); Volk et al. (2004)
Allium wakegi	Kohmura et al. (1994)
Amygdalus communis L.	Al-Ababneh et al. (2003)
Anthirrinium microphyllum	Gonzalez-Benito et al. (1998a)
Ananas comosus	Gonzalez-Arnao et al. (1998b); Thinh et al. (2000); Gamez-Pastrana et al. (2004); Martinez-Montero et al. (2012)
Arachis spp.	Gagliardi et al. (2003)
Armoracia rusticana	Punchindawan et al. (1997)
Asparagus officinalis	Uragami et al. (1990a)
Beta vulgaris	Vandenbussche et al. (2000)
Camellia sinensis	Kuranuki and Sakai (1995); Aoshima (1997)
Centaurium rigualii Esteve	Gonzalez-Benito and Perez (1997)
Ceratopetalum gummiferum	Shatnawi and Johnson (2004)
Cichorium intybus	Vandenbussche et al. (1993); Demeulemeester et al. (1992, 1993)
Citrus spp.	González-Arnao et al. (1998a); Wang and Deng (2004); Al-Ababneh et al. (2002); Cho et al. (2002a)
Chrysanthemum	Schnabel-Preikstas et al. (1992)
Chrysanthemum morifolium	Sakai et al. (2000)
Cocos nucifera	Hornung et al. (2001a)
Coffea racemosa	Mari et al. (1995)
Coffea sessiliflora	Mari et al. (1995)
Colocasia spp.	Takagi et al. (1997); Thinh (1997)
Cosmos atrosanguineus	Wilkinson et al. (1998)
Cymbidium spp.	Thinh and Takagi (2000)
Cymbopogon	Thinh et al. (2000)
Cynodon spp.	Reed et al. (2006)
Dendranthema grandiflorum	Fukai et al. (1994)
Dianthus caryophyllus	Dereuddre et al. (1988)
Digitalis obscura	Sales et al. (2001)
Dioscorea spp.	Mandal et al. (1996); Malaurie et al. (1998); Leunufna and Keller (2005)
Diospyros kaki	Matsumoto et al. (2001)
Ekebergia capensis	Peran et al. (2006)
Eucalyptus spp.	Poissonnier et al. (1992)
Fragaria spp.	Navatel and Capron (1997); Hirai et al. (1998); Hao et al. (2002); Reed and Hummer (1995); Kartha et al. (1980)
Gentiana spp.	Tanaka et al. (2004)
Grevillaria scapigera	Touchell and Dixon (1996)
Grevillaria cirsifolia	Touchell and Dixon (1996)
Holostemma annulare	Decruse and Seeni (2002)
Humulus lupulus	Martínez and Revilla (1999)
Ipomoea batatas	Towill and Jarret (1992); Pennycooke and Towill (2001); Hirai and Sakai (2003)
Lilium japonicum	Matsumoto et al. (1995b)

Table 8.5 (continued)

Limonium	Matsumoto et al. (1998b)
Lolium sp.	Chang et al. (2000)
Malus spp.	Wu et al. (1999); Niino et al. (1992b); Zhao et al. (1999a, b); Paul et al. (2000)
Manihot spp.	Charoensub et al. 1999, 2004
Mentha spp.	Towill and Bonnart (2003); Hirai and Sakai (1999a)
Morus spp.	Niino et al. (1992a)
Musa spp.	Thinh et al. (1999); Panis et al. (1996); Agrawal et al. (2004)
Olea europaea	Martínez et al. (1999)
Panax ginseng	Yoshimatsu et al. (1996)
Pelargonium spp.	Grapin et al. (2003)
Phoenix dactylifera	Bagniol and Engelmann (1991)
Picrorhiza kurroa	Sharma and Sharma (2003)
Populus alba	Lambardi et al. (2000)
Primula pubescens	Hornung et al. (2001b)
Prunus domestica	Brison et al. (1995); de Carlo et al. (2000)
Prunus dulcis	Shatnawi et al. (1999)
Prunus spp.	Niino et al. (1997)
Pyrus spp.	Scottez et al. (1992); Niino et al. (1992b); Chang and Reed (2000); Tahtamouni and Shibli (1999)
Ribes spp.	Reed (1990); Reed et al. (2005)
Rubus spp.	Reed and Yu (1995); Chang and Reed (1999); Rosa et al. (2010)
Saccharum spp.	Gonzalez-Arnao et al. (1993, 1996, 1999); Paulet et al. (1993)
Saintpaulia ionantha Wendl.	Moges et al. (2004)
Salix	Blakesley et al. (1996b)
Solanum spp.	Fabre and Dereuddre (1990); Lu and Steponkus (1994); Schäfer-Menuhr et al. (1994); Benson et al. (1989); Benson et al. (1996); Golmirzaie and Panta (2000); Hirai and Sakai (1999b); Yoon et al. (2006); Panta et al. (2009); Kaczmarczyk et al. (2011)
Solemostemon rotundifolius	Niino et al. (2000)
Sygysium francissi	Shatnawi et al. (2004)
Trifolium repens	Yamada et al. (1991)
Vitis vinifera	Plessis et al. (1993); Matsumoto and Sakai (2003)
Wasabia japonica	Matsumoto et al. (1998a)
Xanthosoma spp.	Thinh (1997)
Zoysia sp.	Chang et al. (2000)

Cryopreservation of embryogenic cultures is also essential for maintaining the totipotency of embryogenic material, and allows ensuring a consistent supply of material with a high regenerative ability to support the genetic transformation programs. Rice embryogenic cultures submitted to cryopreservation exhibited much higher regenerative potential than non-cryopreserved ones, and this positive effect has been also observed in several other plant species such as *Vitis vinifera* (Wang et al. 2002), *Citrus deliciosa* (Aguilar et al. 1993) and *Gentiana cruciata* (Mikuła et al. 2011). In this context, additional studies demonstrated that cryopreserved rice embryogenic callus had significantly higher transient gene expression level than non-cryopreserved controls (Moukadiri et al. 1999). Wang et al. (2005) showed that even when the transformation efficiency of grapevine cell suspensions was

similar between cryopreserved and non-cryopreserved controls, the regenerative potential of transformed cells was significantly higher after cryopreservation. These improvements appear to be related to the cell selection that may occur during a cryopreservation process (Aguilar et al. 1993; Wang et al. 2002).

At present, there have been a number of studies on cryopreservation of transgenic materials containing different target genes and including field crops like *Oryza sativa* and *Triticum aestivum*, fruit crops such as *Carica papaya*, *Citrus sinensis* and *Pyrus pyrifolia*, ornamental crops like *Betula pendula*, and medicinal crops like *Papaver somniferum* (Wang et al. 2012). As results, it has been so far indicated that cryopreservation does not affect expression of foreign genes in transgenic materials and that the productive ability of cryopreserved cells containing recombinant proteins also remains similar to that of non-cryopreserved cultures (Schmale et al. 2006; Cho et al. 2007). Therefore, transgenic materials can be safely stored in liquid nitrogen before being analyzed and evaluated (Spök and Karnar 2008).

On the other hand, the combination of genetic engineering techniques and cryopreservation can be useful to validate genes involved in tolerance to dehydration and freezing. Gonzalez-Arnao et al. (2011) reported recently the utilization of the transgenic technology to produce elevated levels of trehalose in plants *in vivo*, which led to improved tolerance to cryopreservation of chrysanthemum (*Dendranthema grandiflorum* Kitam.) *in vitro* apices compared to non-transgenic controls. Additionally, these studies allowed evaluating the effect of endogenous and exogenous presence of trehalose on regeneration of chrysanthemum shoot-tips subjected to cryopreservation (Osorio et al. 2014).

The cryogenic storage of embryogenic cultures with high regenerative potential is another important tool that facilitates the development of breeding programs based on somatic hybridization. The availability and maintenance of embryogenic callus is a major limitation for large-scale application of somatic hybridization for citrus breeding. However, using cryopreserved sweet orange callus as source for protoplast isolation, fusion and plant regeneration, no differences were found in protoplast yield, quality, growth and regeneration capacity in comparison with control non-cryopreserved callus. Plants regenerated from protoplasts of the two sources had the same phenotypic characters and no differences were detected by microsatellite analysis (Olivares-Fuster et al. 2000). Therefore, cryopreservation contributed to avoid the risks and high labour needs associated with the standard maintenance of a callus collection.

8.5 Cryopreservation for Elimination of Plant Pathogens (Cryotherapy)

In nature, plants are permanently exposed to attacks of a wide variety of pathogenic microorganisms (Cruz-Cruz and Peña-Rodríguez 2011), which cause considerable damage, as decrease in crop productivity, and economic losses of agricultural and horticultural crops (Bhojwani and Dantu 2013).

Plant diseases produced by systemic pathogens including viruses, viroids, phytoplasmas and bacteria are particularly problematic in vegetatively propagated species, because they are transmitted from generation to generation in the planted material (Agrios 2005; Xie et al. 2009); therefore, the acquisition and maintenance of pathogen-free plants is essential for controlling systemic diseases (Mink et al. 1998) and increasing agricultural productivity (Waterworth and Hadidi 1998).

A recent biotechnological approach termed cryotherapy is emerging as an alternative for elimination of systemic pathogens, and involves the use of cryopreservation techniques in shoot-tips to cure infected plants (Wang et al. 2009; Wang and Valkonen 2009; Feng et al. 2013). This method is based on exposing infected materials for a short time to liquid nitrogen, which results in destroying the more differentiated cells in which viruses and phytoplasmas are usually located. Regeneration of pathogen-free meristematic cells, able to withstand cryopreservation, allows obtaining a high frequency of pathogen-free regenerants (Engelmann and Dussert 2013), and consequently, an increased production of new healthy plants (Helliot et al. 2002; Wang et al. 2008). In addition, the efficiency of sanitation is mainly independent of shoot-tip size when using cryotherapy. Therefore, cryotherapy may result in obtaining high frequencies of pathogen-free plants and in avoiding the difficulties associated with the excision of small shoot-tips required when using meristem culture for the same purpose (Wang and Valkonen 2009). In this sense, cryotherapy could potentially replace this traditional method for eradication of plant pathogens (Wang and Valkonen 2009; Feng et al. 2013).

Cryotherapy of shoot tips comprises six major steps to produce pathogen-free plants: (i) introduction of infected plant materials into *in vitro* culture; (ii) excision of shoot tips; (iii) cryopreservation;(iv) post-culture for plant regeneration; (v) indexing for pathogens in regenerated plants after cryotherapy; and (vi) establishment of pathogen-free nuclear stock plants (Feng et al. 2013).

To date, cryotherapy of shoot tips has been successfully applied in several economical important crops such as *Prunus* spp. (Brison et al. 1997), banana (*Musa* spp.; Helliot et al. 2002), grapevine (*Vitis vinifera*; Wang et al. 2003), potato (*Solanum tuberosum*; Wang et al. 2006), *Citrus* spp. (Ding et al. 2008), raspberry (*Rubus idaeus*; Wang et al. 2008), and sweet potato (*Ipomoea batatas*; Feng et al. 2011).

Pathogen eradication by cryotherapy of shoot tips can be readily tested with species and genotypes for which cryopreservation protocols are already available, and this represents a very promising method to be incorporated in cryobank management in order to ensure the good phytosanitary status of stored samples. Another important advantage is that cryotherapy may facilitate national and international germplasm exchange once its effectiveness in the production of pathogen-free plants is confirmed (Feng et al. 2011). Cryotherapy of shoot tips is becoming an innovative biotechnological tool, which simultaneously will contribute to guarantee the long-term storage of plant germplasm and the regeneration of pathogen-free plants (Wang et al. 2006).

8.6 Conclusion

In this chapter we have attempted to illustrate how cryostorage has advanced and become an important biotechnological tool with the realistic target of supporting the management and the long-term conservation of plant biodiversity. Since the first report on plant cryopreservation, the most important progress has been related to the successful application of cryogenic techniques to different germplasm forms of species which previously could not tolerate cooling in liquid nitrogen. In addition, cryopreservation currently provides complementary solutions for the elimination of systemic pathogens, and potentially ensures plant germplasm security useful to breeding programs.

As reviewed in this chapter, various criteria should be considered to select the most appropriate cryopreservation procedure depending on the biological material. In fact, the formulation of any cryopreservation protocol always requires the input of theoretical and practical expertise from diverse disciplines, in order to define the most suitable and adaptable cryogenic (cryoprotectants and cooling rate) and non-cryogenic (pre- and post-storage culture) factors.

Although there are general guidelines to follow, there is no 'universal protocol' that can be used for all groups of plants, and this is due to the specific physiological and biochemical characteristics of each species, which demand optimizing protocols according to each individual behavior. However, the standard procedures already developed are a very helpful starting point, because they can often be adapted to other groups of species with only minor modifications. In this context, it is important to continue advancing our understanding of different protective mechanisms, such as the conflicting effect of protection and toxicity resulting from the use of highly concentrated cryoprotective solutions and their role in enhancing a glassy state to avoid intracellular crystallization of water and lethal injuries. The better understanding of the stress conditions involved in cryopreservation protocols will allow improving the tolerance of explants to dehydration and cryopreservation, thereby improving the efficiency of cryostorage for present and future use.

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Chapter 9
Biotechnology in Biodiversity Conservation: Overview of its Application for Conservation of Endangered African Tree Species

Thierry D. Houehanou, Achille E. Assogbadjo and Brice Sinsin

Abstract Over the world, one of perspective challenges in biodiversity conservation is how to meet effective conservation of threatened species. In this frame, endangered African tree species is becoming a priority that should attract development of conservation strategies.

Since biotechnology is developing rapidly as conservation strategies of biodiversity targets these last decades, it has been questioned to know (i) the current situation concerning biotechnology and endangered African tree species, (ii) the problems that prevent using of biotechnology in conservation of endangered African tree species and (iii) perspectives to help biotechnology to conserve endangered African tree species. Thus an overview on these questions showed that endangered African tree species have not taken advantages of biotechnologies strategies yet. Few biotechnologies researches based on endangered African tree species have been undertaken until now. This state of knowledge is explained by some difficulties that have been highlighted. Those difficulties concerned mostly characteristics of seeds of endangered African tree species, cost of biotechnologies strategies and bad integration of biotechnology discipline with other ones. They are preventing wide use of biotechnology strategies to conserve endangered African tree species. Considering them, some recommendations have been addressed as perspectives of conservation of endangered African tree species by biotechnology.

Keywords Biotechnology · Biodiversity · Endangered African tree species · Conservation

9.1 Background

Over the world perspective challenges of conservation biology is to protect biological diversity and the processes that sustain it in a context of human disturbance effect (Moritz 2002). Indeed, biodiversity crisis expressed by increasing of number of

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threatened species or those at risk in one part and extinction of many species (animals and plants) in other part is becoming more perceptible. Extinction rate was estimated 100–200 times higher than historical natural level (Dudley and Parish 2006). According to Engelmann (2010), the number of plants recorded as critically endangered on IUCN Red List has increased by 60 %during the period 1996–2004. However, forest species are among the one taxon that is mostly at risk. In Africa, over 1000 tree species have been reported to be threatened with various levels of threat (WCWM 1998). Regarding the human population growth trend, biodiversity declining will be more and more exacerbated in Africa in future. Indeed, in 1900, the population of Africa was about one-quarter that of Europe one; by 2000, the two regions had about the same demographic weight, and by 2050, Africa population will be three times larger than Europe (Hirschman 2005). Thus, conservation of biodiversity in general and particularly threatened African tree species, is an important task concerning the human scientist population worldwide and mainly the ones in Africa. In this frame, plant biodiversity is receiving great importance. In fact, plant biodiversity is a natural source of medicinal and food products. It provides raw materials for several industries and is able to supply genetic information required for developing sustainable use, management and conservation of most crops (Rao 2004; Cruz-Cruz et al. 2013).

Moreover, biotechnology is become a rapid developing field that has received considerable attention since discover of DNA. Thus, their activities have been intensively focused on plant resources in these last decades. It is applying on major plant crop species as well as on tree species. Its use is allowing breeding programs for improving crop species productivity and their resistance against biological and environmental stress. Although biotechnology activities on plants resources have recently increased rapidly (Dawson and Jaenicke 2006), tree species received little attention on this matter. Biotechnology has been developed on few tree species and those concerned were mostly from high income countries. However, African tree species have been seldom emphasized by biotechnologies activities. This situation is more exacerbated in case of endangered African tree species.

Additionally threatened tree species and particularly those endangered are receiving priorities in biodiversity conservation. Thus, conservation of endangered African tree species by biotechnology should require a critical analysis for efficient using of this tool in frame of biodiversity conservation. What is the actual situation concerning biotechnology and endangered African tree species? What are problems that prevent using of biotechnology in conservation of endangered African tree species? How can we do in perspective to help biotechnology to conserve endangered African tree species?

9.2 State of Knowledge on Endangered African Tree Species (EATS)

Since conservation of endangered tree species represents important task in conservation biology of biodiversity, several researches have focused worldwide on these tree species this last decade to conserve them. However, in Africa endangered

tree species are missing sufficient data for managing and designing their sustainable use or conservation. Indeed, although some African tree species were reported as threatened with various levels of threats on international Red List of UICN, it remains also many others ones without sufficient data and therefore their status is misunderstood. In the tropics, in general and in Africa mainly, the flora was least described (Maxted et al. 1997). Thus, many African tree species that are endangered were not always mentioned in a report due to the lack of information on their distribution, biology, ecology etc. Considering endangered African tree species that have been listed by international or local reports, knowledge related to conservation ecology has been mostly highlighted on these tree species. For instance, in spite of the fact that several African studies (Sinsin et al. 2004; GlèlèKakaï et al. 2009; Adjonou et al. 2010; Houehanou et al. 2011, 2013), focused on endangered African tree species, those studies addressed mostly ecological researches questions. Moreover, few applications were delighted from these ecological researches to secure conservation or sustainable uses of these tree species. This is a general problem of researches in low-income countries. Indeed, researches in developing countries are mostly funding by high-income or developed countries and therefore, applications are not always easy to be done.

Over the world, biotechnology activities as conservation strategies are advancing. Regarding taxa coverage by biotechnologies activities, more than 140 genera received biotechnologies activities in the entire world but most of them took place in high-income regions (Dawson and Jaenicke 2006). FAO (2004) recorded biotechnologies activities in 76 countries with 71 and 3 % undertaken respectively in high-income countries and in Africa. Most of biotechnologies activities of Africa were concentrated in South Africa (FAO 2004). Few biotechnological studies were undertaken in others parts of Africa on endangered African tree species. Those existent studies, concerned characterization of genetic structure of some endangered tree species such as *Adansonia digitata* (Kyndt et al. 2009), *Milicia excelsa* (Bizoux et al. 2009) and *Vitis vinifera ssp. Sylvestris* (Zoghlami et al. 2013). This state of knowledge is showing that African taxa are very few covered at present by biotechnologies activities. Consequently, from state of knowledge it can be concluded that endangered African tree species have not taken advantage of biotechnologies strategies yet, although their conservation is a priority in biodiversity conservation frame.

9.3 What is Biotechnology?

Biotechnology could be split up in two simply words such as biology and technology. It is then “any technological application that uses, living organisms or derivatives, to make or modify products or processes for specific use”. Thus, it concerns a range of scientific tools that, provides powerful methods for the sustainable development of agriculture, fisheries and forestry (Dawson and Jaenicke 2006).

Biotechnology is not sum up to genetic engineering as considered by many people but it includes genetic engineering and is much wider than this one (Dawson and Jaenicke 2006). Hence, according to Dawson and Jaenicke (2006), biotechnology can come at present into four areas such as (i) tissue culture and micro propagation, (ii) characterizing genetic diversity, (iii) genetic maps, marker assisted selection and genomics and (iv) genetic modification.

9.4 Summary of Biotechnology Conservation Strategies

Four main conservation strategies are developing biotechnologically to promote management, sustainable use and conservation of plant biodiversity. There are generally grouped into (i) tissue cultures and micro propagation, (ii) characterizing genetic diversity, (iii) genetic maps, marker assisted selection and genomics and (iv) genetic modification.

- Tissue culture and micropropagation
  This area is also called *in vitro* techniques and includes the use of cryopreservation and other storage methods for *in vitro* conservation of plant material. However, *in vitro* culture covers a wide range of techniques involving the growth under sterile conditions of plant germplasm (especially shoot tips, meristems, somatic embryos or embryogenic callus) (Paunescu 2009). In general, *in vitro* culture could be divided into two categories such as: (i) slow growth procedures, where germplasm accessions are kept as sterile plant tissues or plantlets on nutrient gels; and (ii) cryopreservation where plant material is stored in liquid nitrogen (Rao 2004). Cryopreservation is the technique that ensures the safe and long-term conservation of the germplasm of species.
- Characterizing genetic diversity
  Characterizing genetic diversity includes biotechnologies areas applications that partition variation within and between populations of a species and aims to (i) assess reproductive biology (gene flow, breeding systems) of species and (ii) detect the relationships between taxa (phylogenetics),hybridization and human impacts on plant populations (Dawson and Jaenicke 2006). Molecular approaches based on genetic variation by determination polymorphism in DNA have become more available in these two last decades (Jamnadass et al. 2009; Dawson et al. 2009). Indeed, DNA-based techniques introduced over the past two decades have potential to identify polymorphisms represented by differences in DNA sequences. These methods have advantages, to analyze variation at the DNA level itself, excluding all environmental influences (Rao 2004).
- Genetic maps, marker assisted selection and genomics
  This area of biotechnology activity starts since 1990s, and aims to develop molecular markers and genetic linkage maps for identifying statistical associations between markers and genes that control a proportion of the genetic and phenotypic variation of a given trait (Dawson and Jaenicke 2006).

- Genetic modification
  Genetic modification is the use of recombinant DNA and asexual gene transfer methods that alter the structure or expression of specific genes and traits (Dawson and Jaenicke 2006). A genetic modified organism or transgenic, is one that has been obtained by the insertion of one or more genes from another organism. Active research in this area has been ongoing since the 1980s.

9.5 State of Knowledge on Application of Biotechnology Strategies on Endangered African Tree Species

As biotechnology strategies are receiving interest in biodiversity conservation these last decades, it become important to highlight an overview on how its application are growing with endangered African tree species. As different conservation strategies of biotechnology exist, they have not been applied with the same importance on endangered African tree species. Biotechnology area of *in vitro* culture and cryopreservation has been used easily to collect and conserve genetic resources that are difficult to conserve as seeds (Rao 2004). This has involved development of several *in vitro* techniques for storage of species that propagate by vegetative or that produce recalcitrant seed. While cryopreservation has been established for conserving mostly vegetative propagation species, it is much less advanced for recalcitrant seed species (Rao 2004). Indeed, according to this precedent author, recalcitrant seeds are high sensitive to desiccation and their developmental stage and water content at maturity are complex and heterogenic. In comparison with crop species, only limited research has been performed on rare and endangered species with this biotechnology strategy. Moreover, although propagation technique could be used in conservation of endangered species, this technique was not widely applied in Africa (Wala and Jasrai 2003; Mng'omba et al. 2008). Numerous endangered plants on which researches have been focused for applying biotechnology strategy like *in vitro* technique have been from temperate and tropical origin (without Africa) until now (Engelmann 2010).

Characterizing genetic diversity strategy has been applied widely on tree species over the world. In Africa, it has been used for some specific cases. For instance domestication program and conservation approaches of bush mango species from Central and West Africa have been guided with genetic diversity approach (Lowe et al. 2005). However, regarding genetic diversity study of endangered tree species in Africa, this is enough limited.

The two remaining biotechnologies areas ((i) Genetic maps, marker assisted selection and genomics and (ii) Genetic modification) are least applied in Africa on plant genetic resources until now. Consequently endangered African tree species have not been well focused by these biotechnologies researches areas.

9.6 Updating of Conservation Approaches on Endangered African Tree Species

There are two approaches (*in situ* and *ex situ*) for conservation, which are globally used to conserve plant biodiversity in the entire world. *In situ* conservation involves maintaining plant biodiversity in their natural areas while *ex situ* conservation on the other hand, involves conservation outside the natural area (Rao 2004). According to this last author *ex situ* conservation approaches is generally suitable to conserve in danger species population. Therefore, *ex situ* conservation strategies should be used mostly on endangered tree species. However, as the global strategy of plant conservation states that at least 60% of threatened plant species should be within protected areas (Vellak et al. 2009) there is increasing concern about the extent to which *in situ* conservation strategies contribute to conserve mostly endangered plant species. Thus, *in situ* and *ex situ* conservation approaches should be combined to design conservation strategies and secure conservation of endangered tree species particularly the African ones.

By past, most biotechnologies activities for conservation approaches focused on crop species and agroforestry ones. This is revealing that wild tree species were neglected in conservation strategies. Although, *ex situ* conservation approaches is seen sometime as the only option for conserving some highly endangered and rare species (Ramsay et al. 2000), the traditional conservation approach of *in situ* is used generally to conserve wild tree species. Then, in the case of wild tree species, conservation approaches of *ex* and *insitu* could be combined for their conservation. In this context, botanic gardens that play important role in *ex situ* conservation of plant biodiversity (Engelmann 2010), are able tool that combine sometimes *ex* and *in situ* conservation approaches. Indeed, in the botanic garden, some plants that are present can be in their natural areas while others ones can be outside of their natural area. Highlighting importance of botanic gardens in plant biodiversity conservation, it has been estimated that botanic gardens conserve more than one third of the world's flowering plants which among more than 15,000 threatened species have been identified (UNEP 1995; Engelmann 2010; http://www.bgci.org/ourwork/1977/). This is involving that Botanic gardens over the world are guarantying conservation of most endangered African tree species.

Apart of Botanic gardens, value of agroforestry ecosystems for conserving plant biodiversity through tree species diversity, has become more widely recognized by several researches (Kindt 2002; Kirschenmann 2007; Dawson et al. 2009). This approach named *circa situ* conservation permitted conservation of lots tree species among which some endangered African tree species, in agricultural landscape such as *Adansonia digitata, Milicia excelsa* etc. Thus, conservation of diversity of endangered African tree species by agroforestry ecosystems is promoting world widely.

9.7 Biotechnology Strategies in Relationship with Conservation Approaches of Biodiversity

Each of biotechnology strategies contributed to each conservation approach of plant biodiversity. Biotechnology strategies like seed storage, field gene banks, DNA and pollen storage contribute exclusively to *ex situ* biodiversity conservation. They are used to conserve biodiversity plant outside their natural area. However, characterizing genetic diversity is used to assist in developing both *in situ* and *ex situ* conservation of biodiversity. More generally results of this biotechnology area are used to develop genetic conservation strategies of biodiversity plants in their natural area. Moreover, comparing *ex* and *in situ* conservation approach of plant biodiversity, it has been argued that *in situ* approach has some advantages (Maxted et al. 1997). Firstly, it allows the process of evolution to continue in relationship with other biological systems of concerned species habitat. Indeed, none species is static but is continually interacting with physical environment and is competing with other species in the ecosystem. Secondly, it is much easier to conserve viable population of species in their natural habitat rather than in an *ex situ* situation. Therefore, using characterizing genetic diversity should be more accounted for *in situ* conservation approach of endangered African tree species. Then, although biotechnologies strategies that contribute to *ex situ* conservation have focused lowly on endangered African tree species until now, it would be better to develop in future mostly research that will be able to contribute to their *in situ* conservation.

9.8 Difficulties Linked to Biotechnologies Strategies Application on EATS

As various biotechnology strategies are being used for biodiversity conservation, application of each of them defined above, has some advantages and disadvantages in the context of endangered African tree species. Disadvantages allow application to be difficult on endangered African tree species and are summarized in below points.

- Characteristics of seeds of endangered African tree species: As a large number of important tropical and sub-tropical tree species produce recalcitrant seeds; seed storage strategies are not possible (Roberts 1973). According to Engelmann (2010) numerous forest tree species, especially from tropical origin, produce recalcitrant seeds (i.e. seeds that cannot be dried to sufficiently low moisture level to allow their storage at low temperature; see Roberts 1973). Then, conservation of endangered African tree species by *in vitro* technique would be very difficult. This situation is reinforced by the fact that tropical and sub-tropical tree species are ecologically viable in environment of high temperature. Consequently it would be more difficult to conserve their seed in very low temperature comparatively to seeds of temperate regions that are naturally adaptive to low

temperature. Therefore, conservation with *in vitro* technique would be more suitable for trees of temperate regions.

- Cost of biotechnology strategies: All biotechnology strategies are costly and time consuming and are generally applied to one species at a time (Dawson et al. 2009). Additionally, many biotechnology techniques require considerable investment in infrastructure, consumables, staff salaries and training (Dawson and Jaenicke 2006). This is preventing application of biotechnologies strategies on most endangered African tree species and explains why until now most biotechnology researches are conducted in high-income countries.
- Biotechnology strategies require to be integrated approach: Although characterizing genetic diversity can reveal great detail about genetic variation in trees species, application of results has been very limited (FAO 2004; Dawson et al. 2009). This situation has been explained by the fact that molecular approaches did not combined generally others aspects such as social, ecological or economical. In the context of endangered African tree species those aspects will need to be integrated to make easy application of results.

9.9 Future Recommendations for Better Conserving Endangered African Tree Species with Biotechnologies

Considering difficulties linked to biotechnologies application on endangered African tree species, and requirement of its conservation, it would be better to take into consideration some issues.

- Updating of list of endangered African tree species for allowing conservation researches on more exhaustive list. Thus, each African country should work for establishing local red list of tree species. Such list could be accounted for a regional or country scale list of endangered African tree species.
- African countries should develop their own funding biotechnology research on priorities endangered tree species. Such a self-funding research would enhance facility in results application. Then, after establishing list of endangered tree species, a prioritizing species for biotechnology strategies will become important. Regarding context of limited financial resources in Africa, prioritizing should be required on endangered African tree species for developing biotechnology strategies efficiently.
- As biotechnologies strategies are generally costly and time consuming and applied to one species at a time, alternative would be researching on technique that can be more easily used on many species.
- Regarding application of biotechnologies results that was limited in Africa and particularly on endangered African tree species, biotechnologies specialists should work in future in collaboration with other disciplines such as social, economic, ecology etc. Interdisciplinary science will reinforce application of biotechnologies results on endangered African tree species.

- Developing partnership between African and high-income countries should be an opportunity to enhance biotechnology research on endangered African tree species.
- Enhancing genetic diversity characterization researches of endangered African tree species will be opportunity researches in future.
- Prioritizing *in situ* conservation approach on endangered African tree species by enhancing research on prioritizing area for conservation of endangered African tree species should be required. Such research would be a part of a new concept (*gap analysis of protected area*) process for biodiversity conservation.

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Chapter 10
Biotechnology for Endangered Plant Conservation

Anca Manole-Paunescu

Abstract The rhythm of biodiversity loss is estimated to be between 1000 and 10,000 times higher than the natural extinction rate. This is often referred to as "the sixth extinction crisis" after the five known mass extinction events in geological history. Plant species are known to be particularly vulnerable to present biotic crisis. These figures indicate that research for improving existing, and developing new effective conservation strategies are necessary. Advances in biotechnology provide new methods for plant diversity conservation and evaluation. In the past decade a significant number of plant species, either wild or crop, threatened, medicinal or ornamental, were conserved by biotechnological means. Therefore, the challenges to support conservation by biotechnology are many and varied. Direct application of biotechnological tools like *in vitro* culture and cryopreservation proved to be valuable means for large-scale propagation, storage, and reintroduction of endangered plant species. The *in vitro* techniques for plant conservation include photoautotrophic micropropagation, somatic embryogenesis, cell culture and embryo rescue techniques, as well as *in vitro* cold storage and cryopreservation methods. This chapter presents their application in the development of *ex situ* collections and their contribution toward an integrated system to conserve endangered plant species.

Keywords *In vitro* conservation · Endangered plants · Recalcitrant seeds

10.1 Introduction

Concerns about biodiversity loss have made necessary complex studies and approaches. As a result, in March 2005, the *Millennium Ecosystem Assessment* (MA) was released. This is a comprehensive report drawn up by 1300 researchers from 95 nations over 4 years, and funded by the Global Environment Facility, the United

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Nations Foundation, the World Bank and others. One of the key conclusions of this report is that "Everyone in the world depends on nature and ecosystem services to provide the conditions for a decent, healthy, and secure life." In the same report is also highlighted that substantially and largely irreversible loss in the diversity of life on Earth, occurred in the last century. The *Millennium Ecosystem Assessment* analyzed for the first time the conditions and the trends in the world ecosystems and services, they provide and underline two distinct focal points of biodiversity and ecosystem functioning (BEF) and biodiversity and ecosystem services (BES) research. These researches reveals how species and functional diversity of organism control basic ecological processes and also how major habitat modifications influenced provisioning and regulating services of ecosystems (Cardinale et al. 2012).

According to the latest version of the Red List of Threatened Plants issued by International Union for Conservation of Nature (IUCN), from the 18,292 listed species, 10,065 are threatened (IUCN 2013). These figures indicate that approximately 4% of described plant species are threatened.

Causes of plant endangerment are numerous and include:

- habitat alteration, fragmentation or destruction
- introduction of exotic species, intentionally or accidentally, with competitive advantages over native species
- over-collecting, overexploiting and unsuitable use
- air, soil, and water pollution
- urbanisation
- unsuitable agriculture, farming and forestry practices
- global warming, severe droughts, salinization
- natural causes (overspecialisation, loss of genetic diversity, catastrophic events) (Pitman and Jorgensen 2002).

The most vulnerable plant species originate mainly from resource-poor areas of the world, from global biodiversity hotspots and island countries. In the past, 15.7% of the earth's land surface was covered by the 34 global biodiversity hotspots which host more than 50% of the world's endemic plant species. Presently, global biodiversity hotspots area covers only 2.3% of the earth's land surface. Each hotspot faces extreme threats and has already lost at least 70% of its original natural vegetation (Reed et al. 2011). A broad international initiative for an integrate plant conservation was taken under The *Convention on Biological Diversity* by adopting the recommendations of the *Gran Canaria Declaration* for a *Global Strategy for Plant Conservation,* which presently is signed by 220 countries. Under the Target 8, proposed for period 2011–2020, is stipulated that *At least 75% of threatened plant species in ex situ collections, preferably in the country of origin, and at least 20% available for recovery and restoration programmes* (GSPC 2011). As a consequence several conservation strategies were developed mainly in the terms of *in situ* and *ex situ* conservation.

In situ conservation refers to on-site conservation of species and populations on their natural habitats. Protection of natural areas is the main way to preserve natural

biological resources. Protected areas remain one of the cornerstones for promoting biodiversity, ecosystem services and human well-being. By 2012, the 177,547 nationally designated protected areas around the world, listed in the *World Database on Protected Areas* cover 12.7% of the world's terrestrial area and 1.6% of the global ocean area. They store 15% of the global terrestrial carbon stock, assist in reducing deforestation, habitat and species loss, and support the livelihoods of over one billion people (Bertzky et al. 2012).

In situ biodiversity preservation through the establishment of protected areas offers distinct advantages over *ex situ* methods in terms of coverage, viability of the resource, and the economic sustainability of the methods. Establishing a conservation area does not mean that its biodiversity is under complete protection and without risk. Some risks, both natural and anthropogenic, remain. Habitat degradation or elimination to make way for human settlement and associated development activities is the most important risk factor contributing to the biodiversity loss. These hazards can only be met with a full array of conservation programs, including those that use *ex-situ* methods.

Ex situ (off-site) conservation involves preservation and maintenance of samples of living organisms outside their natural habitat, in the form of whole plants, seed, pollen, vegetative propagules, tissue or cell cultures. Ex *situ* techniques are generally used to complement *in situ* methods but in some cases are the only possible techniques to conserve certain species (Ramsay et al. 2000). Among *ex situ* conservation methods, the most common are cultivation in botanic gardens, seed storage, and *in vitro* cultivation.

The world's 2500 botanic gardens cultivate more than 100,000 species meaning almost one third of the known vascular plant species of the world. Although cultivation in botanic gardens is an efficient way to conserve endangered species *ex situ*, it is limited in time and space and it has to overcome acclimatisation and accommodation problems.

Among the various *ex situ* conservation methods, seed storage seems to be one of the most convenient for long-term conservation. This involves desiccation and storage at low temperatures. Many plant species produce so-called orthodox seeds which support desiccation and can be stored for long periods. For example, an experiment by the Arava Research Institute (Israel), an ancient seed of *Phoenix dactylifera* (naturally desiccated in Israel's warm and dry environment) has been germinated and a date palm seedling produced. The seeds were recovered from a jar during the excavations of Masada in the 1970s and were then kept in store. Radiocarbon dating has confirmed that the seeds are around 2000 years old, making this the oldest seed to have been successfully germinated (Sallon et al. 2008). Currently, all over the world are established seed collections onto so-called seed banks. For example, almost 24% of the endangered Spanish flora is stored in the Plant Germplasm Bank of the Universidad Politecnica de Madrid as seed accessions (Gonzales-Benito and Martin 2011) as well as more than 200 of endangered Australian plant species which are accessioned in the Threatened Flora Seed Centre and Western Australian Seed Technology Centre (Kaczmarczyk et al. 2011). The

most extensive projects for seed collections are *Millennium Seed Bank Partnership* and The *Svalbard Global Seed Vault*. *The Millennium Seed Bank Partnership* is considered the largest *ex situ* wild plant conservation project in the world. With a network of partners across 80 countries, within this project over 11 % of the world's wild plant species seeds were already banked. Also, aims to save 25 % of those plant species with bankable seeds until 2020 (about 75,000 species). *The Svalbard Global Seed Vault*'s mission is to preserve genetic diversity of the world's food crops and to provide a safety net against accidental loss of diversity in traditional gene banks. Presently, more than 770,000 different seed samples are deposited in the vault for long-term storage.

However, there are a large number of threatened species, which produce immature, sterile or recalcitrant seeds that quickly lose viability and do not survive desiccation. Predominantly tropical and sub-tropical species have recalcitrant or intermediate seeds, which do not support desiccation and low temperature treatments, hence conventional seed storage strategies are not suitable (Engelmann and Engels 2002). There are estimated to be 5000 or more endangered species for which conventional conservation methods are not adequate (Pence 2013). For these species, biotechnological tools offer a valuable alternative for conservation. The concept of "biotechnology" encompasses a wide range of procedures, but when refers to plants (green biotechnology) is associated mainly with recombinant DNA technology and with the designing of transgenic plants. Plant biotechnologies include advanced tools like designing synthetic promoters, "tunable" transcription factors, genome-editing tools and site-specific recombinases, as well as assembly and synthesis of large DNA molecules, plant transformation with linked multigenes and design plant artificial chromosomes in order to produce new products in plants and to generate plants with new functions. When refers to conservation, biotechnologies include mainly *in vitro* techniques like cell and tissue culture, micropropagation as well as cold storage and cryopreservation. Several *in vitro* techniques have been developed, mostly for vegetatively propagated and recalcitrant seed producing species, with recent establishment of extensive germplasm collections (Engelmann and Engels 2002; Sarasan et al. 2006; Paunescu 2009b). This chapter aims to summarise the biotechnological tools implemented worldwide for plant conservation with specific examples of some successful accomplishments.

10.2 *In Vitro* Conservation of Plant Germplasm

The term *in vitro* culture covers a wide range of techniques involving the growth under sterile conditions of plant tissues (especially shoot tips, axillary meristems, somatic embryos or embryogenic callus) on artificial semi-solid culture media. Although each species require specific protocols, there are some common steps in establishing an *in vitro* collection: culture initiation, maintenance and multiplication, followed by long-term storage.

10.2.1 Culture Initiation

The potential explant (the starting tissue originated from the donor plant) consist mostly of shoot meristem (apical or axillary), leaf tissues (lamina segments with ribs, petiole), flower pieces, immature embryos, hypocotyl fragments or cotyledons. Generally, younger, more rapidly growing tissue or tissue in early developmental stage are the most effective. Therefore, the initial quality of the explants will determine the success of the conservation procedure. The criteria for a good quality explants are: normal, true to type donor plant, vigorous and disease free (Fay 1992). Plant fragments are initiated into axenic culture from various sterilization procedures depending of the tissue used. As a common rule, fragile tissues (meristems, immature embryos, cotyledons, hypocotyls) requires less exposure to sterilizing agents than seeds or lignified organs. A successful sterilization is achieved when the explant is fully decontaminated and remains viable. An alternative for obtaining uncontaminated explants is to obtain explants from seedlings, which are aseptically grown from surface-sterilized seeds. Another approach, known as *in vitro* collecting, is to introduce the explant *in vitro* under aseptic conditions directly in the field (Withers 1995, 2002). This method is a valuable alternative for rare and endangered species since this material is limited in supply and seed collection is restricted (Cruz-Cruz et al. 2013). Also, *in vitro* collecting allows germplasm collection of species without seeds, or storage organs, or when buds would quickly loose viability or is highly contaminated (Engelmann and Engels 2002). Disinfestation protocols vary considerably from single step to more complex procedures involving a variety of sterilizing agents including chemicals (liquids or gases) or physical agents. Most procedures consists in pre-treatments with 70–95 % ethanol, followed by immersion in broad-spectrum biocide solution like hypochlorite solutions (sodium or calcium), sodium dichloroisocyanurate, mercuric chloride, silver nitrate, bromine water, hydrogen peroxide, silver nitrate, etc. (Sarasan et al. 2006; Paunescu 2009b). More elaborated protocols could include gas sterilization, ultrasonic cleaner and ethanol "dip and flame". Such protocols were reported to be successful in establishment of embryo or ovule *in vitro* cultures of some endangered Hawaiian taxa (Sugii 2011).

After sterilization step, the explant is placed on a sterile culture media containing mineral nutrients, vitamins, carbohydrates, and plant growth regulators (Murashige and Skoog 1962). Plant growth regulators are the critical media components in determining the developmental pathway of the plant cells. A balance between auxin and cytokinin growth regulator is most often required for initiation of the morphogenetic events. The interactions found are often complex and, more than one combination of substances is likely to produce the desired results. Seven major types of growth regulators are generally recognised:

- auxins, usually involved in cell enlargement and differentiation
- cytokinins associated with cell division
- gibberellins stimulate cell elongation, seed germination
- abscisic acid involved in dormancy by inhibiting cell growth
- ethylene often associated with senescence

- brassinosteroids involved in gravitropism, stimulates cell elongation and division
- jasmonates associated with growth inhibition, senescence promotion, wound responses

Auxins and cytokinins are the most widely used growth regulators in plant tissue culture, usually being utilised together. The ratio between auxin and cytokinin is essential for the type of culture established. Generally, the indirect pathway (via callus) is avoided because of the increased risk of somaclonal variation occurrence. Although, somaclonal variation could provide some adaptive advantages, this is not a desired outcome for conservation in tissue culture.

10.2.2 Culture Maintenance and Multiplication

One major step in establishing *in vitro* germplasm collections is induction and multiplication of shoots. This step could be often critical for some species, particularly woody plants. The media composition, especially the growth regulators, mineral salts and supplement factors are of paramount importance to successfully obtain viable tissue culture. In the last 20 years, there were many reports in developing micropropagation protocols for endangered plant species, all over the world (Table 10.1). According to Sarasan (Sarasan et al. 2006), at Conservation Biotechnology Unit (previously known as Micropropagation Unit) from Royal Botanic Gardens, Kew, are micropropagated and maintained more than 3000 endangered plant taxa, from all over the world. Under a national initiative several research Spanish groups have developed micropropagation protocols for more than 60 endemic and endangered species, most of them belonging to *Plumbaginaceae* and *Asteraceae* families (Gonzales-Benito and Martin 2011). Since 1998 the Hawaiian Rare Plant Program (Lyon Arboretum, Honolulu, Hawaii) has been successful in establishing *in vitro* cultures of 135 endangered plant taxa (Sugii 2011). Some successful multiplication strategies were developed for a number of endangered plants in Romanian flora (Paunescu 2009b). A recent overview of *in vitro* conservation technologies for Eastern Australian endangered plant taxa was published by Ashmore et al. (2011) including *Alloxylon flammeum*, *Citrus* spp., *Davidsonia* spp., *Diploglotis campbellii*, *Macadamia tetraphylla*, *Pimelea spicata*, *Stackhousia tryonii*, *Syzygium* spp., *Tectaria devexa*, *Wollemia nobilis* and ten selected orchid taxa. A significant number of other rare native species from Australian flora are also preserved *in vitro* collections in Kings Park and Botanic Garden, Australia (Kaczmarczyk et al. 2011). During the last 25 years of research within Tropical Botanic Garden and Research Institute, Palode (India) were developed *in vitro* protocol for rapid regeneration and establishment of more than 40 medicinal rare and threatened plants from Western Ghats (Krishnan et al. 2011). As well, some examples of successful micropropagated endangered plants from South Africa, was recently reviewed, including *Haworthia limofolia* and *Siphonochilus aethiopicus* (Berjak et al. 2011). Most of the reviewed culture protocols were developed onto Murashige and Skoog basal

Table 10.1 Selected endangered species for which micropropagation protocols has been reported within last two decades

Species	Explant type	Culture media	Author
Dianthus bohemicus	Nodal segments	MS BAP—1 mgl−1	Kovác (1995)
Globularia ascanii	Apical buds	MS BAP—1 mgl−1 NAA—0.1 mgl−1	Cabrera-Pérez (1995)
Trillium persistens	Dormand buds	1/2MS BAP—1 mgl−1 NAA—0.1 mgl−1	Pence and Soukup (1995)
Vella lucentina	Apical buds	MS BAP—0.5−2 mgl−1	Lledó et al. (1995)
Leontopodium alpinum	Young inflorescences	MS BAP—2 mgl−1 IAA—2 mgl−1	Zapartan (1996)
Gentiana cerina *G. corymbifera*	Axillary buds	MS BAP—0.05−0.5 mgl−1	Morgan et al. (1997)
Saussurea lappa	Shoot apex	MS TDZ—0.1 mgl−1	Johnson et al. (1997)
Hypericum foliosum	Axillary buds	CM BAP—1 mgl−1 NAA—0.5 mgl−1	Moura (1998)
Holostemma annulare	Shoot apex, nodal segments	MS BAP—1 mgl−1 NAA—0.1 mgl−1	Sudha et al. (1998)
Dianthus superbus	nodal segments	MS BAP—1 mgl−1	Mikulik (1999)
Astragalus péterfii	Nodal segments, foliar and flower cuttings	MS BAP—0.5 mgl−1 NAA—0.3 mgl−1	Suteu et al. (1999)
Heracleum candicans	Axillary buds	MS BAP—0.5 mgl−1 NAA—0.1 mgl−1	Wakhlu et al. (1999)
Salvia blancoana	Shoot apex Nodal segments	MS 2iP—1 mgl−1	Cuenca and Amo-Marco (2000)
Salvia valentina	Shoot apex Nodal segments	MS Kin—1−2 mgl−1	Cuenca and Amo-Marco (2000)
Primula scotica	Seedlings cuttings	MS BAP—1 mgl−1 IAA—0.1 mgl−1	Benson et al. (2000)
Piper barberi	Nodal segments	MS BAP—1 mgl−1 Kin—0.5 mgl−1	Anand and Rao (2000)
Ochreinauclea missionis	Nodal segments	MS BAP—2 mgl−1	Dalal and Rai (2001)
Ensete ventricosum	Nodal segments	MS BAP—2−4 mgl−1	Negash et al. (2001)
Allium wallichii	Shoot cuttings	MS Ze—4 mgl−1	Wawrosch et al. (2001)
Aerides maculosum	Leaf cuttigs	MS BAP—2 mgl−1	Murthy and Pyati (2001)

Table 10.1 (continued)

Species	Explant type	Culture media	Author
Arnica montana	Nodal segments	MS 2iP—1 mgl−1 NAA—1 mgl−1	Butiuc-Keull and Deliu (2001)
Turbinicarpus laui	Seedling cuttings	MS BAP—2−3 mgl−1 NAA—0.5 mgl−1	Rosas et al. (2001)
Quercus leucotrichophora şi Q. glauca	Immature embryo, cotyledonar cuttings	MS BAP—5 mgl−1	Purohit et al. (2002)
Andryala levitomentosa	Petiole cuttings	MS Kin—1 mgl−1 2,4D—1 mgl−1	Paunescu and Vantu (2002)
Limonium cordatum	Young inflorescences	MS BAP—0.1 mgl−1	Casazza et al. (2002)
Psoralea corylifolia	Fragmente radiculare	MS BAP—3 mgl−1 NAA—2 mgl−1	Chand and Sahrawat (2002)
Dianthus spiculifolius	Nodal segments	MS BAP—1 mgl−1 NAA—0.1 mgl−1	Cristea et al. (2002)
Nothapodytes foetida	Hypotyl cuttings	MS TDZ—0.5 mgl−1	Ravishankar Rai (2002)
Calophyllum apetalum	Nodal segments	MS BAP—2 mgl−1	Nair and Seeni (2003)
Cypripedium formosanum	Protocorm cuttings	MS TDZ—1 mgl−1 2,4D—1 mgl−1	Lee and Lee (2003)
Ipsea malabarica	Rhizome buds	MS Kin—1.5 mgl−1	Martin (2003)
Kniphofia leucocephala	nodal segments	MS BAP—2 mgl−1	McCartan and van Staden (2003)
Dianthus callizonus	Nodal segments	MS BAP—1 mgl−1 NAA—0.1 mgl−1	Paunescu and Holobiuc (2003)
Dianthus simonkaianus	Flower buds and nodal segments	MS BAP—1 mgl−1 NAA—0.1 mgl−1	Miclaus et al. (2003)
Dionaea muscipula	90 Days old shoot cuttings	1/3MS Kin—0.5 mgl−1	Jang et al. (2003)
Centaurea reichenbachii	Nodal segments	MS BAP—1 mgl−1 NAA—0.1 mgl−1	Cristea et al. (2004)
Eryngium foetidum	Nodal segments and flower buds	MS Kin—0.5–1 mgl−1 NAA—1–2 mgl−1	Martin (2004)
Hydrastis canadensis	shoot cuttings	MS BAP—2 mgl−1	Liu et al. (2004)
Decalepis hamiltonii	Leaf cuttings	MS BAP—4 mgl−1 Ze—3 mgl−1	Giridhar et al. (2005)

Table 10.1 (continued)

Species	Explant type	Culture media	Author
Sternbergia fischeriana	Dormand buds and immature embryo	MS BAP—4 mgl−1 NAA—0.2 mgl−1	Mirici et al. (2005)
Rotula aquatica	Shoot cuttings	MS 2,4D—0.1 mgl−1	Chithra et al. (2005)
Eucalyptus phylacis	Shoot cuttings	1/2MS Ze—0.1 mgl−1	Bunn et al. (2005)
Decalepis hamiltonii	Axillary buds	MS 2iP—1 mgl−1	Giridhar et al. (2005)
Decalepis arayalpathra	Nodal segments	MS BAP—3 mgl−1 NAA—0.5 mgl−1 2iP—0.5 mgl−1	Sudha et al. (2005)
Dianthus tenuifolius	Nodal segments	MS BAP—1 mgl−1 NAA— 0.1 mgl−1	Paunescu and Holobiuc (2005)
Cerastium transsilvanicum	Nodal segments	MS BAP—1 mgl−1 NAA—0.1 mgl−1	Păunescu (2005)
Notocactus magnificus	Shoot cuttings	MS BAP—1 mgl−1 2,4 D—0.1 mgl−1	Medeiros et al. (2006)
Rauvolfia micrantha	Young root cuttings	MS BAP—0.2 mgl−1 NAA—0.1 mgl−1	Sudha and Seeni (2006)
Bupleurum kaoi	Nodal segments	MS BAP—0.25 mgl−1	Chen et al. (2006)
Lippia filifolia	nodal segments	MS BAP—1 mgl−1 NAA—0.001 mgl−1	Pereira et al. (2006)
Potentilia potanii	Hypocotyl and cotyledon cuttings	MS BAP—5 mgl−1 NAA—1 mgl−1	He et al. (2006)
Swertia chirayita	Nodal segments	MS BAP—1 mgl−1 2iP—0.3 mgl−1	Joshi and Dhawan (2007)
Pinus armandii	Embrioni imaturi	MS BAP—0.2 mgl−1 2,4 D—0.6 mgl−1	Maruyama et al. (2007)
Curculigo orchioides	Shoot apex	MS BAP—1.5 mgl−1 IBA—0.25 mgl−1	Francis et al. (2007)
Saussurea involucrata	Foliar cuttings	MS BAP—2 mgl−1 NAA—0.5 mgl−1	Guo et al. (2007)
Vitex agnus-castus	Mature apical meristem and nodal explants	MS BAP—2 mgl−1 Kin—0.1 mgl−1	Balaraju et al. (2008)
Encyclia mariae	Leaves from in vitro germinated seedlings	MS BAP—5 mgl−1 NAA—2 mgl−1	Santos Diaz and Carranza Alvarez (2009)

Table 10.1 (continued)

Species	Explant type	Culture media	Author
Alyssum borzaeanum	Seedling cuttings	MS Kin—1 mgl−1 NOA—0.1 mgl−1	Paunescu (2009a)
Marsilea quadrifolia	Rhizome buds	½ MS hormone free	Banciu et al. (2009)
Cistus clusii	Shoot tips and nodes	MS BAP—0.5 mgl−1	Ruta and Morone-Fortunato (2010)
Daucus carota L. subsp. halophilus	Shoot tips from in vitro germinated seedlings	MS BAP—1 mgl−1	Tavares et al. (2010)
Campanula polymorpha	Nodal segments	MS BAP—1 mgl−1 NAA—0.1 mgl−1	Paunescu (2010)
Gomortega keule	Zygotic embryos	MS BAP—1 mgl−1 NAA—0.1 mgl−1	Muñoz-Concha and Davey (2011)
Teucrium polium	Axillary buds	MS BAP—2 mgl−1 Kin—1.6 mgl−1	Al-Qudah et al. (2011)
Xyris tennesseensis	Seedling cuttings	1/3MS Kin—1 mgl−1 NAA—0.1–0.5 mgl−1	Johnson et al. (2012)
Arum palaestinum	Corm bud sprouts	MS BAP—1 mgl−1 NAA—1 mgl−1	Shibli et al. (2012)
Curcuma vamana	Rhizome nodal segments	MS BAP—1 mgl−1	Bejoy et al. (2012)
Lilium pumilum	Leaf cuttings	MS BAP—2 mgl−1 NAA—0.5 mgl−1	Jin et al. (2013)
Viola pilosa	Buds	MS BAP—0.5 mgl−1 TDZ—0.5 mgl−1 GA3—0.5 mgl−1	Soni and Kaur (2013)
Zeyheria montana	Isolated mature zygotic embryos Nodal segments	MS GA3—2 mgl−1 1/4MS BAP—0.1 mgl−1 GA3—0.5 mgl−1	Cardoso and Teixeira da Silva (2013)
Celastrus paniculatus	Nodal segments	MS BAP—0.5 mgl−1 NAA—0.1 mgl−1	Senapati et al. (2013)
Ceropegia evansii	Nodal segments	MS BAP—4 mgl−1 IAA—0.3 mgl−1	Chavan et al. (2013)
Sphaerophysa kotschyana	Nodal segments	MS BAP—0.5−2 mgl−1 TDZ—0.05−0.4 mgl−1 Ze—0.2−1 mgl−1	Erisen and Oncel (2013)

BAP 6-benzylaminopurine, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, GA_3 gibberellic acid, *Kin* kinetin, *MS* Murashige-Skook basal media, *NAA* 1-naphthaleneacetic acid, *NOA* 1-naphthoxyacetic acid, *TDZ* thidiazuron, *Ze* zeatin, *2iP* isopentenyl adenine, *2,4D* 2,4-dichlorophenoxyacetic acid

medium (Murashige and Skoog 1962) supplemented with different combinations of cytokinins and auxins. One of the most efficient cytokinin in shoot regeneration was 6-benzylaminopurine (BAP) followed by Kinetin (Kn), in concentration ranging from 0.5 to 5 mgL^{-1}. In most of the cases cytokinin was added in combination with low amounts of auxin, like indole-3-acetic-acid (IAA) or 1-naphthalenacetic acid (NAA).

10.2.3 Storage of Collections

In vitro storage techniques include the medium-term storage (for a determined period—a few months up to a few years) using slow growth strategy or artificial seed production, and long-term storage (tentatively for an indeterminate period of time) using cryopreservation. In slow growth, cultures are kept under the level of optimal growth conditions. Generally, there are three recognised methods for reducing *in vitro* growth rates, including physical (reduced temperature and light conditions), chemical (using growth retardants), and a combination of the two (Engelmann and Engels 2002). Temperature will vary upon the origin of stored species; temperate species may be stored at 4 °C, whereas the tropical plants are requiring temperatures in the range of 15–20 °C. Light conditions may be darkness or a 12–16 h photoperiod, the light intensity varying upon the light requirement of the species stored. The humidity should be between 40–50 %. Some effective approaches for slow growth include reduction of the oxygen level available achieved by covering explants with a layer of liquid medium, paraffin or mineral oil, or by placing them in controlled atmosphere (Engelmann and Engels 2002). By optimization of all parameters, the subculture time is greatly enhanced, the quality of the stored material is maintained and the recovery of the shoot proliferative potential is assured. The subculture periods could be extended from 12 month up to 4 years for many plant species (Ashmore 1997). Although slow growth techniques are available for a wide range of plant species, there are used in practice only for a limited number of endangered species. This is mainly because the need for regular subculture raises the risk of contamination and occurrence of somaclonal variation. For example, this technique is applied only for a very few Malaysian species like *Nephelium lappaceum* (shoot cultures at 18 °C with subcultures at 12 weeks), and *Lansium domesticum* seeds onto full-strength Murashige and Skoog medium (Murashige and Skoog 1962) at 25 °C with two subcultures in 15 months (Normah et al. 2011). Slow growth storage strategy is also used to preserve some endangered phytotaxa in the Institute of Biology (Bucharest) *in vitro* collections. The endangered species conserved by medium-term tissue culture are *Artemisia tschernieviana, Astragalus pseudopurpureus, Cerastium transsilvanicum, Dianthus callizonus, D. spiculifolius, D.tenuifolius, Erigeron nanus, Hieracium pojoritense,* and *Marsilea quadrifolia* (Paunescu 2009b). Minimum growth techniques have been also used for some Spanish endemics (*Centaurium rigualii, Coronopus navasii, Lavatera oblongifolia, Limonium calaminare, L. catalaunicum, L. dichotomum, L. dufourii, L. estevei*, and *L. gibertii*) with relative success, reduction of temperature being the most effective

way of decreasing growth (Gonzales-Benito and Martin 2011). Slow growth conditions were employed for medium term conservation of 16 threatened species and subspecies of the genus *Turbinicarpus* (Cactaceae), all native to the Chihuahuan Desert in Mexico, using osmotic agents (sorbitol and mannitol) and temperatures of 4 °C (Pérez-Molphe-Balch et al. 2012).

Artificial seeds (synthetic seeds, manufactured seeds) were first introduced in the 1970s as a novel analogue to the plant seeds, useful for propagation and medium term storage (Redenbaugh et al. 1988). Artificial seeds are produced by encapsulating a plant propagule in a matrix, which will allow it to grow into a plant. A typical synthetic seed has following parts:

- plant propagule
- matrix (synthetic gametophyte)
- artificial coat (membrane)

Plant propagules may consist of shoot buds, shoot tip, somatic embryos, or any other competent aggregate cells, able to regenerate the whole plant. The most used plant regenerative units in artificial seed production are somatic embryos in post-heart or early cotyledonary stage. They are enclosed in gel agents like: alginate, agarose, polyoxyethylene, urethane polymers, guar gum, sodium pectate, carrageenan, polyacrylamide, etc. Among these, alginate encapsulation was found to be more suitable and practicable. Alginate hydrogel is frequently selected as a matrix for synthetic seed because of its moderate viscosity and low spinnability of solution, low toxicity for propagules and quick gellation, low cost and bio-compatibility characteristics. The chosen propagules are mixed with sodium alginate and the suspension is dropped into the calcium salts solution. The principle involved is when sodium alginate dropped into the calcium salt solutions it from round firm beads due to the ion exchange between Na^+ in sodium alginate and Ca^{2+} in calcium slat solutions and sodium alginate form calcium alginate. The rigidity of capsular membranes depends on concentration of the two solutions. Generally, a 3 % (w/v) sodium alginate solution combined with a 75 mM calcium salt, generates a firm and efficient membrane. The matrix of encapsulation should be enriched with nutrients and growth regulator, which will serves as an artificial endosperm. This will increase the efficiency of germination and of seed viability. Superfluoro chemical oils are used as oxygen carrier and are often mixed with gel. It increases oxygen supply and helps in keeping the seed viable for longer time. Other materials like fungicides, pesticides, herbicides, insecticides, antibiotics and mycorrhizal fungi or bacteria can also be incorporated. Generally, there are recognized various types artificial seeds which include: (i) uncoated non-quiescent somatic embryos, (ii) non-quiescent somatic embryos in a hydrated encapsulation and, (iii) dehydrated quiescent somatic embryos (Ravi and Anand 2012).

When cultured, the seed coat softness allowing the propagule to resume growth, enlarging and emerging from the encapsulation. The advantages of propagules encapsulation include multiplication of recalcitrant or non-seed producing species, easier manipulation of fragile tissues, and direct protection during dehydration and thawing during the cryopreservation trial (Saiprasad 2001). Synthetic seed technol-

ogy was applied for conservation purposes to various plant species. Particularly, the method was successfully applied for a number of endangered orchid species. It involves protocorms-like bodies (orchid somatic embryos, Lee et al. 2013) encapsulation and shows high efficiency, for example 70% viability after more than 6 months storage at 4 °C (Devi et al. 1998).

Despite of their availability the *in vitro* medium term storage techniques are used routinely in a few botanical gardens, research institutes, universities, or other worldwide conservation centres. This is because such techniques require high tech equipment, trained staff and large storage spaces. Also, culturing with periodic refreshment of medium is laborious and subsequent culturing increases the risk of microbial contamination and somaclonal variation. Another disadvantage is that under medium term storage the material could be preserved only for a limited period (up to a few years).

To avoid the genetic alterations that may occur in long cultures storage, experimental protocols have been developed to preserve germplasm at very low temperatures known as *cryopreservation* (Martin et al. 1998). The temperature used are those of liquid nitrogen (−196 °C) or its vapour phase (−150 °C). At these temperatures, all metabolic activity is suppressed minimizing the risk of genetic alterations and eliminating the requirement for refreshing the culture medium. Furthermore, cryopreserved material is stored in a small volume for a theoretically indefinite time. Also, it requires minimal space and maintenance, such as replenishment the storage container with liquid nitrogen (Kaczmarczyk et al. 2011). The technology is effective for a broad range of phytotaxa including unicellular to flowering higher plants (Reed 2008). Cryopreservation provides a safe and cost-effective method for the long-term storage of genetic resources. Apart from the use in plant long-term conservation, exposure to extreme low temperature proved to be very effective in elimination of systemic plant pathogens (viruses, phytoplasmas and bacteria), procedure known as cryotherapy (Wang and Valkonen 2009). Cryopreservation as a conservation tool has been underlined by a number of authors (Engelmann 2004; Stacey et al. 1999) and presently is recognised as the most effective technique for long-term storage of plant germplasm. Under low temperatures tissues proved to remain viable for very long periods of time. Recently, was published a report concerning whole plant regeneration using *in vitro* tissue culture of a species originate from Arctic tundra (*Silene stenophylla* Ledeb., Caryophyllaceae) from maternal, fruit tissue, of Late Pleistocene age. The fruits were isolated from permafrost deposits of about 30,000-year-old. The regenerated plants successful developed flowers and fertile seeds (Yashina et al. 2012). Presently, a number of studies are dedicated to the effect of low temperatures upon living tissues and its applicability in plant long-term conservation. Pioneer researches about successful cryopreservation of plant cell suspension (Quatrano 1968) and regeneration of somatic embryos from cryopreserved cells (Nag and Street 1973) have led to numerous studies on cryopreservation of plant system (Frinkle et al. 1985; Kartha 1985; Prithcard and Predergast 1986). Cryopreservation technique is based on the removal of all freezable water from tissues by physical or osmotic dehydration, followed by ultra-rapid freezing, resulting in vitrification of intracellular solutes. Vitrification refers to transition of

water directly from the liquid phase into an amorphous phase, whilst avoiding the formation of crystalline ice (Fahy et al. 1984). Vitrification-based procedures offers some advantages being more appropriate for complex structures (shoot tips, buds, embryos) which contain a variety of cell types, each with unique requirements under condition of freeze-induced dehydration (Engelmann 2011). The main advantages are simplicity and the applicability to a wide range of genotypes. Currently, are identified seven different vitrification-based procedures: (1) encapsulation-dehydration, (2) vitrification, (3) encapsulation-vitrification, (4) dehydration, (5) pregrowth, (6) pregrowth-dehydration, and (7) droplet-vitrification (Engelmann 2004, 2011). The *encapsulation-dehydration* procedure is based on the technology developed for the production of artificial seeds. The method is suitable for shoot apices, cell suspensions and somatic embryos (Engelmann 2011). This technique was employed also for nodal explants of some Spanish endemic species (Gonzales-Benito and Martin 2011). *Vitrification* consists of placing explants in the presence of highly concentrated cryoprotective solution followed by rapid freezing. *Encapsulation–vitrification* is a combination of the first two, where explants are encapsulated in alginate beads and treated with vitrification solutions before freezing. Vitrification methods were successfully used for cryopreservation of more than 30 species of Western Australian endangered plants (Kaczmarczyk et al. 2011) as well as for shoot tips of *Centaurea ultreiae* a critically endangered species from Spain (Gonzales-Benito and Martin 2011).

Dehydration is the simplest procedure and consists of dehydrating explants and freezing them rapidly by direct immersion in liquid nitrogen and it may be used for freezing zygotic embryos or embryonic axes extracted from seeds (Engelmann 2004; Dumet et al. 1997). It was also successfully used for freezing the seeds of 68 endangered Western Australian species (Touchell and Dixon 1993), several endangered species originate from Eastern Australia including indigenous *Citrus* (Ashmore et al. 2011) as well as for the seed of some rare temperate orchid species (Nikishina et al. 2007). *Pregrowth* involves *in vitro* culture of explants in the presence of cryoprotectants followed by rapid freezing by immersion in liquid nitrogen. *Pregrowth-dehydration* follows the same steps like pregrowth method with the difference that explants are dehydrated under laminar airflow or with silica gel prior the immersion in liquid nitrogen. *Droplet-vitrification* is the newest developed and consist in pre-treatment of the explants with vitrification reagents then placed in minute droplets of vitrification and finally frozen in liquid nitrogen (Engelmann 2011). This technique has been used for shoot apices of *Thymus moroderi*, an endemic of Southeast Spain (Marco-Medina et al. 2010), shoot tips of wild potatoes (Yoon et al. 2007) and for two *Diospyros* species (Niu et al. 2009).

A critical step in developing a reliable cryopreservation protocol is the regeneration of plants after a determined period (as long as possible) of exposure to extreme low temperatures. Plant regeneration is dependent on genotype, age and physiological state of the culture, cryoprotectant treatment, rate of freezing, method of thawing, etc. (Tsukara and Hirosawa 1992). The pregrowth phase of plant cells is considered a critical one because various changes may occur at the cellular level, including a decrease in cell and vacuole size, changes in the flexibility and thickness of cell walls and alteration of metabolic activities (Withers 1978). Somatic embryogen-

esis is particularly affected by the temperature of the pretreatment. Lower temperatures prevent embryos from maturing and, thus extend embryogenic tissue recovery (Kong and Aderkas 2011). Currently, are available some viability tests which are successfully applied to cryopreserved material. The most used are flourescein diacetate (FDA) and triphenyltetrazolium chloride (TTC) test. The FDA is converted to flourescein as a result of esterase activity. Cells with uninjured plasma membrane fluoresce green in ultraviolet light as the larger molecules of fluorescin are unable to pass through the membrane. The TTC reduction is based on the mitochondrial respiratory efficiency of cells that converts the tetrazolium salt to insoluble form azon, which is extracted and measured spectrophotometrically.

Until 2010 were published about 40 reports dealing with 52 wild endangered plant species from which complete cryopreservation protocols were established (Berjak et al. 2011).

10.3 Genetic Stability of Preserved Germplasm

In vitro conservation, although medium term or cryostorage, comprises multiple manipulations, starting with culture initiation followed by proliferation, maintenance, subcultures, pregrowth, osmotic treatments, cryoprotection, desiccation, freezing/vitrification, thawing, recovery, regrowth and finally, regeneration. An increase number of manipulation raises the risk of material alteration in terms of genetic and epigenetic changes (somaclonal variation) or even viability loss. Apart from genetic changes, epigenetic modifications may play an important role in plant growth and development. Epigenetic modification refers to a mechanism that controls gene expression without altering DNA sequence and leads to genetic modifications by DNA methylation, histone and chromatin changes. Studies show that changes in DNA methylation are quite stable and are frequently transmitted during meiosis and mitosis (Smulders and De Klerk 2011). Under these circumstances there is an increasing requirement to determine whether genotypes preserved by *in vitro* cultures are "true to type". To achieve the objective of maintaining the genetic integrity of the sample, the stored germplasm should be monitored visually every 1–3 month, depending on the species. Initial characterization of the accession is essential for comparison. In case that any abnormalities are found, the tissue culture plants must be grown for an entire cycle in greenhouse examining any morphological changes. Since the visual examination is not reliable, germplasm that has been kept in collection for more than 1 year should be assessed by biochemical and/or molecular methods. Confirmation of the genetic stability is prerequisite to the sustainable management of the preserved bioresources and their future use. The term of "cryobionomics" describes the re-modelled concept of genetic stability and the re-introduction of cryopreserved plants into the environment. This evolving concept deals with two practical aspects of cryostorage: (i) cryoinjury and genetic stability and (ii) unaltered integrity of the reintroduced material in natural environment at phenotypic, histological, cytological, biochemical and molecular level (Harding 2004, 2010).

Protein markers, usually named "biochemical markers" (seed storage proteins and isozymes) are generated through electrophoresis, taking advantage of the migrating proprieties of proteins, and subsequently revealed by specific histochemical stains. Genetic stability has been successfully studied using seven isozymes systems in the endangered Spanish plant *Centaurium rigualii* (Iriondo and Perez 1996). A total of about 90 isozyme systems have been used for plants assessment, with isozyme loci being mapped in many cases. However, the major limitation of isozyme analysis is the reduced number of markers analysed. Another disadvantage of isozyme analysis consists in the phenotypical dependence of the markers. As such, the biochemical markers are limited due to their modifications by environment exposure and during the development.

Molecular (DNA) markers are derived from the initial DNA and considered to provide the best measure of genetic variation. DNA polymorphism can be detected in nuclear and organelle DNA and is not modified by environmental exposure. Further, the analysis can be carried out at any time during plant development and it may cover the entire genome. Many molecular markers have been developed for plant genetic diversity assessment (Karp et al. 1997). Commonly, DNA polymorphism assessment techniques based on polymerase chain reaction (PCR) and restriction fragment length polymorphism are applied to endangered species (Chase and Fay 1997). In particular, microsatellites have been effectively deployed to establish the genetic stability of long-term maintained germplasm. Microsatellite markers are favoured, since their preferential association with low copy regions of plant genomes (Morgante et al. 2002).

As a complementary method to molecular markers, flow cytometry is used to detect the possible changes in ploidy levels and DNA content (Fukai et al. 2002; Mallon et al. 2010).

10.4 Conclusions

The past two decades have witnessed significant advancement of biotechnology and currently new methods are available to conserve the threatened plant germplasm. The new developed techniques offer new options and facilitate conservation in the form of seeds, pollen, embryos, and *in vitro* tissue cultures. *In vitro* techniques and storage methods are enabling the establishment of extensive collection using minimum space. All-over the world, germplasm collections are present in the form of genebanks, biobanks or cryobanks. Although technology for the *in vitro* preservation is available it is under-utilised as a conservation strategy for endangered plants. Technologies like micropropagation and cryostorage are used mainly for crop, medicinal, ornamental or other commercial species, for supplying nurseries, for secondary metabolites production, for educational displays, etc. Establishing biobanking facilities for endangered plant at national and international level should be considered as a priority to complement *in situ* conservation strategy. This will have an immediate benefit by reduction the collection pressure on the wild populations. The developed active collections will allow for continuous supply of

valuable material for wild population recovery, molecular investigations, ecological studies, or economic uses. As biodiversity loss pressure is increasing and the more endangered species are identified, an integrated strategy should be undertaken by a holistic approach using all the technologies available for both *in situ* and *ex situ* conservation.

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Chapter 11
Biotechnological Approaches to Medicinal Plants of Aravalli Hills: Conservation and Scientific Validation of Biological Activities

Shaily Goyal, Jaya Arora and Kishan G. Ramawat

Abstract Aravalli hills are hot spot of subtropical plant biodiversity. The tribal people of the region partially or fully depend upon herbal drugs for primary healthcare. Overexploitation of these plants has made several of them as endangered species. The present paper aimed to document the biotechnological approaches being used to conserve ethnomedicinal plants of Aravalli Hills, the bioactive molecules present in them and their traditional uses and the modern scientific validation/assay of biological activities. Plants of Aravalli hills are showing various promising biological activities and bioactive molecules. Though various biotechnological methods are attempted for enhanced production of these bioactive molecules and for their micropropagation, yet the approaches are insufficient at mass scale level as some of the endangered species may have unusual growth requirements and thus may require modified procedures for in vitro culture. The review will be supportive in deciding the priorities at various decision-making levels and further technology development for sustainable use and conservation of these plants.

Keywords Biotechnology · Biodiversity · Conservation · *Bacopa* · *Centella* · *Commiphora* · *Celastrus* · *Curculigo* · *Chlorophytum*, · *Withania* · *Pueraria*

11.1 Introduction

The demand for medicinal plants is continuously increasing not only in developing countries but also in developed countries as drug, food supplement (nutraceuticals) and cosmetics (Ramawat et al. 2004). Tyler defines ‘herbal medicines as crude drug

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of vegetable origin utilized for the treatment of diseased state often of a chronic nature or to attain or maintain a condition of improved health' (Tyler 1994). If we look at socio-economical scenario of the Asian and African countries, modern medicine is neither affordable nor in reach of many villagers and tribes inhabiting remote areas and deep forests (Katewa and Jain 2006). Two of the largest users of medicinal plants are China and India. Traditional Chinese medicine utilizes over 5000 plant species while the major classical systems of medicine in Indian sub continent like Ayurveda, Siddha, Unani, altogether use about 1200 plant species to treat human ailments, but the tribals of India are using over more than 7500 plant species (Pushpangadan 1994). However, India's share in the world market is US $ 1 billion as compared to China's share of 6 billion. Indigenous medicinal herbs provide about 75% of need for medicines of the third world countries (Rajshekharan 2002).

India is one of the world's 12 hot spots having the largest plant biodiversity; it has almost 45,000 plant species, out of which 15,000–20,000 are used for medicinal value (Ramawat and Goyal 2008). Aravalli ranges have about 8% of the flora of India, consisting of 1378 species belonging to 126 families (Tiagi and Aery 2007). Reserve sanctuaries of the region (Sitamata, Sajjangarh, Phulwari-ki-naal) harbor rich biodiversity. Aravalli ranges dissect the state of Rajasthan into two parts: (1) North-western desert, and (2) South-eastern hilly semi-arid forest. These ranges lie between 25°N to 73°30′E running approximately 800 km from Delhi to Gujarat states having highest peak—Guru Shikhar in Mt Abu at 1722 m. These geographical conditions provide variable habitat for a wide range of flora including bryophytes, pteridophytes, a lone gymnosperm—*Ephedra foliata*, and subtropical angiospermic flora (Arora et al. 2010a).

Historically, herbal drugs were used as tinctures, poultices, powders and teas followed by formulations, and lastly as pure compounds. Medicinal plants or their extracts are used by humans since time immemorial for different ailments and provide valuable drugs such as analgesic (morphine), antitussive (codeine), antihypertensive (reserpine), cardiotonic (digoxin), antineoplastic (vinblastine and taxol) and antimalarial (quinine and artemisinin). Some of the plants which continues to be used from Mesopotamian civilization till today are *Cedrus* species, *Cupressus sempervirens*, *Glycyrrhiza glabra*, *Commiphora wightii*, and *Papaver somniferum* (Ramawat and Goyal 2008; Ramawat et al. 2009; Gurib-Fakim 2006). During 2000–2005, about two dozen new drugs derived from natural sources have been approved by Food and Drug Administration, USA and put in market, which include drugs for cancer, neurological, cardiovascular, metabolic and immunological diseases, and genetic disorders. Seven plant derived drugs currently used clinically for various types of cancers are taxol from *Taxus* species, vinblastine and vincristine from *Catharanthus roseus,* topotecan and irinotecan from *Camptotheca acuminata,* and etoposide and teniposide from *Podophyllum peltatum*. It is estimated that market potential for herbal drugs in the world is forecast to reach $ 107 billion by the year 2017, spurred by growing aging population and increasing consumer awareness about general health and well being, according to a new report from Global Industry Analysts.

Overexploitation of medicinal plants by herbalists and traders associated with urbanization leads to their removal from many localities; some of them enlisted as an endangered plant such as *Commiphora wightii*, *Curculigo orchioides* and *Chlorophytum borivilianum*. This prompted biotechnologists to develop alternative technology for the production of their bioactive molecules and micropropagtion of these plants. In this article we attempted to assess the success achieved and lessons learned in this endeavor towards sustainable use of traditional medicinal plants. We have also presented the data about to what extend traditional medicinal plant's knowledge hold true on modern scientific parameters.

11.2 Biotechnology and Conservation

Biotechnology in plant science is generally regarded as the use of *in-vitro* techniques in conjunction with molecular tools. In present day the science of biotechnology has availed us with the tools and techniques for mass cloning and improvement in medicinal plants. *In-vitro* culture and micropropagation hold promise because of the following advantages. (1) All the year-round disease free plantlets production. (2) Rapid mass cloning of genotypes. (3) Efficient mean of germplasm exchange, storage and maintenance. (4) Provide ready protocol for the application of genetic engineering tools for plant improvement. (5) Plant material generated are "synchronized", miniaturized and relatively homogenous in terms of size, cellular composition and physiological state. (6) Micropropagation of those species which are difficult to multiply by conventional propagation methods.

These benefits of modern biotechnological tools play important role in conservation of threatened species of Aravallis. Although it is believed that species conservation is achieved most effectively through the management of wild populations and natural habitats (in situ conservation) but ex-situ techniques can be used in assistance to in situ methods and for some plants, may be the only option.

11.2.1 Micropropagation

Micropropagation refers to in vitro mass production of plant propagules from any plant part or cell. Such propagules are used to raise whole plants. The attraction of micropropagation, as an alternative to other propagation methods, lies in its ability to multiply elite clonal material very rapidly. Micropropagation exploits the "totipotency" nature of plant cells and tissues. Currently many tissue culture protocols have been established for conserving widely consumed medicinal plants of Aravalli Hills. Although for many of these plants standard micropropagation methods are available while some of the endangered species may have unusual growth requirements and thus may require modified procedures for in vitro culture. Here we summarize the efforts done by different scientific groups for conserving the important medicinal plants of Aravallis.

Aegle marmelos (Rutaceae), commonly known as "Bel", is a medium sized tree (6–9 m), growing wild in several parts of India, including Aravalli Hills. Its root-bark, leaves and fruits are of high medicinal value (Dev 2006). The plant is cited as one of the red-listed medicinal species of South India (Ravikumar and Ved 2000) due to its overexploitation in Ayurvedic medicines. It is propagated either through seeds or vegetatively by root cutting or sucker formation, which is a rare occurrence. Micropopagation and fidelity of regenerants for superior fruit quality and conservation of this species has been reported by several workers. Multiple shoots (91.23 %) were obtained from cotyledons and shoot tips on Murashige and Skoog's (MS 1962) medium supplemented with 2.0 mg/6-Benzylaminopurine (BAP) and 0.2 mg/l α-Naphthalene Acetic Acid (NAA) (Das et al. 2008). The rooted shoots can give 100 % establishment after hardening as reported by Puhan and Rath (2012). Genetic uniformity of micropropagated plants with that of the mother tree was confirmed by molecular markers (Mishra et al. 2008). Even the seed priming has also been reported as an efficient method for increasing of seed vigour and improvement of germination and seedling growth, thus helping in plant conservation (Venudevan and Srimathi 2013).

Similarly another plant with very high morphogenic potential and ever increasing demand is *Bacopa monnieri* (Scrophulariaceae), commonly known as "Brahmi". Several protocols have been established for the rapid multiplication of shoots and also for the micropropagation of the plant (Rajani 2008). Efficient adventitious shoots regeneration have been reported from different internodes and leaf explants cultured on MS medium containing different combination of BA and NAA followed by rooting and acclimatization with a 100 % survival rate. Even the rooted plantlets were successfully acclimatized in water of various pH levels between 4.0–10.00 (Karatas et al. 2013). All regenerates were monomorphic as assessed by molecular markers (Ramesh et al. 2011). A slow growth protocol was also developed for medium-term conservation using mineral oil (MO) overlay. Single node explants were implanted on MS medium supplemented with 0.2 mg l^{-1} BA and were covered with MO. Subculture duration could be enhanced from 6 to 24 months, on the above medium. Normal plants without any variation from the parent plant were regenerated from conserved cultures and were successfully established in soil (Sharma et al. 2012). Similarly, cryopreservation technique was also employed in *Bacopa* (Sharma et al. 2011b). Besides growth in flasks *Bacopa* shoots were grown in Growtek bioreactor, thereby scaling up the process of micropropagation without loss of secondary metabolites content (Jain et al. 2012).

Another plant *Cayratia trifolia* (Vitaceae, syn. *Vitis trifolia* Linn., *Cissus carnosa* Lam.), a liana, is reported from India to southern China and through the Malaya to the Moluccas and the Caroline Islands. There is not much research done for the conservation of this plant. Yet we can find some initiative efforts in the form of increasing the metabolite content in the in vitro grown plant roots and callus cultures. Cell suspension cultures of *C. trifolia* were maintained in liquid MS medium supplemented with NAA (0.25 mg/l) and kinetin (0.2 mg/l) and 250 mg/l casein hydrolysate. Root cultures were maintained in MS medium supplemented with 0.5 mg/l NAA, 0.1 mg/l kinetin. Occurrence of stilbenes in callus cultures of

C. trifolia (Roat et al. 2008), their production as influenced by plant growth regulators (Roat and Ramawat 2009a) and biotic and abiotic elicitors in cell cultures (Roat and Ramawat 2009b; Arora et al. 2010b) and in root cultures (Arora et al. 2009) was reported. Use of an angiospermic elicitor was a novel finding in enhancing the stilbene content of the cell cultures (Arora et al. 2010b).

Unlike *C. trifolia, Centella asiatica* (Apiaceae), popularly known as 'Mandukaparni' have different micropropagation protocols reported. The plant is a prostrate, stoloniferous herb grown in marshy areas. In India, it is found at the altitude of 600 m. The wild stock of this species has been markedly depleted due to its large scale and unrestricted exploitation, and as a result, it is listed as threatened species by International Union for Conservation of Nature and National resources (IUCN) (Pandey et al. 1993) and an endangered species (Singh 1989). Hence, there is an urgent need to conserve this valuable germplasm. Micropropagation work of *C. asiatica* includes multiple shoot regeneration from nodal and shoots tip explants on MS medium containing either BAP alone or in combination with Indole-3-acetic acid (IAA), NAA and Kn. Maximum multiple shoots were found on MS supplemented with 1.0 mg/l BAP and 0.4 mg/l NAA. Profuse healthy rooting was obtained on MS medium containing 0.2 mg/l Indole butyric acid (IBA). The well rooted plantlets were successfully transplanted to the garden soil and their survival rate under natural conditions was 90–95 % (Jaheduzzaman et al. 2012). Multiple shoot induction and in vitro flowering in *C. asiatica* was also reported (Gaddaguti et al. 2013).

Celastrus paniculatus (Celastraceae), commonly known as "Malkangni" is a woody climber, traditionally used to stimulate intellect and to sharpen the memory. *C. paniculatus* is valued for its seeds and seed oil is used commercially. Indiscriminate collection of this plant has threatened the species and ex-situ conservation is the urge of the present scenario. To develop micropropagation methods, adventitious root formation in semi hardwood cuttings with a 57 % response was obtained on the MS medium containing 3.0 g/l IAA, rooted cuttings exhibited 100 % survival in the experimental field (Raju and Prasad 2010). In a highly efficient protocol, maximum percentage of shoot multiplication (83.4 %) with 8.2 shoots/explants was achieved on MS basal medium supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA. After rooting and acclimatization, 91 % of these plantlets survived at the natural conditions. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) marker study confirmed genetic stability of *in vitro* raised plantlets by showing 100 % monomorphism (Senapati et al. 2013). Similarly, shoot multiplication was also optimized up to 47 shoots while confirming the genetic stability of the plants (Phulwaria et al. 2013). Thus, these efforts help in process of conservation of this plant.

Clitoria trenatea (Fabaceae), commonly known as "Shankpushpi", is a perennial climbing vine found in India, China, Philippines and Madagascar. The primary method of propagation is by seed, but this method result in considerable variability in phenology and reproductive traits among accessions. A reproducible protocol for rapid mass propagation using cotyledonary nodal explants was developed. The highest frequency (100 %) and the maximum number of shoots (10.1) were induced on MS medium supplemented with 1.5 mg/l BAP. The highest rooting (100 %) and

maximum number of roots (5.3) per shoot was obtained when shoots were dipped in IBA solution (500 mg/l) for 5 min and further subcultured on half-strength MS medium. Acclimatized plants grew normally in the field without showing any morphological variation (Singh and Tiwari 2010). In vitro clonal propagation was achieved by employing decapitated embryonic axes (DEAs) explants (Singh and Tiwari 2012). Further work with different explants and thidiazuron on micropropropagation of *C. ternatea* resulted in establishing a protocol, which gave about 88 % survival rate of in vitro developed plants in the fields (Mukhtar et al. 2012).

Chlorophytum borivilianum (Liliaceae) is a popular herb in traditional Indian medicine commonly known as "Safed Musli" and constitutes a group of herbs used as 'Rasayan' or adaptogen. Due to poor seed set and viability, the plant is propagated by root tubers containing a part of stem. This is a slow, tedious, labor oriented method producing low yield of the root tubers (Arora et al. 2004). Therefore, many in-vitro methods have been developed to conserve this beneficial plant including somatic embryogenesis (Arora et al. 1999) and organogenesis (Dave et al. 2003, 2004). Through this protocol 15,000 plantlets were produced with 90 % survival in field conditions and the method was found cost effective (Dave et al. 2003, 2004). Further, method of encapsulation and analysis of fidelity of the regenerants was done using RAPD (Mathur et al. 2008; Arora et al 2006; Lattoo et al. 2006). Factors like propagule size, subculture strategy, gelling agents, liquid pulse treatment of BAP and vessel type were optimized for in vitro shoot multiplication in *C. borivilianum*. These plants showed comparable or better growth and 90 % survival than the greenhouse hardened plants (Joshi and Purohit 2012). RAPD and ISSR analysis of regenerated plants showed genetic similarity to mother plant (Kumar et al. 2010a). *In vitro* tuberization was also reported in *C. borivilianum* using solid and liquid culture systems (Farshad et al. 2013). These methods enable the cultivation of the plants at very large acreage.

Commiphora wightii (Burseraceae), commonly known as "Guggul", is a slow growing tree of arid regions of western India. It is listed in IUCN's Red Data List of threatened plants and now it is becoming endangered. The plant exhibits poor regeneration and its population is fast depleting in its natural habitat, primarily due to over-exploitation, unsustainable and destructive methods of gum-extraction (Jain and Nadgauda 2013). Research on *C. wightii* has been supported by the Ministry of Science and Technology (India) for the past 30 years. Many Biotechnological attempts have been made in order to conserve this important medicinal plant (Kulhari et al. 2012). Organogenesis has been induced through axillary shoot proliferation from nodal segments, seedling explants, shoot tips, internodes and leaves, by different group of workers (Soni 2010; Kant et al. 2010; Singh et al. 2010). Less number of plantlet formation was major constraint in all these studies with low rate of establishment in the field (Kulhari et al. 2012). But in recent years few studies showed some progressive propagation and field survival percentage of in vitro raised plants from nodal segments of mature plants (Parmar and Kant 2012). With such not so competent micropropagation protocol, alternative studies have been done which indirectly assist in plant conservation by providing the important secondary metabolites (guggulsterones) for which the plant is being destroyed.

Guggulsterone production has been reported in callus cultures as influenced by medium nutrients (Mathur et al. 2007) and plant growth regulators (Tanwar et al. 2007). Cell suspension cultures were grown in medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) 0.5 mg/l and kinetin (0.25 mg/l) for maintenance. For the high guggulsterones production the cultures were grown in production medium containing modified MS medium supplemented with 0.1 mg/l kinetin and 0.1 mg/l (2,4-D) in shake flasks and bioreactor (Mathur and Ramawat 2008, 2007). Field grown plant population from Rajasthan and Gujarat state (India) showed high genetic variability by RAPD marker and also in guggulsterone content (Suthar et al. 2008). This genetic variability was also recorded in somatic cells (Dass and Ramawat 2009a). The production of guggulsterones can also be regulated by calcium ions (Dass and Ramawat 2009b), plant gums (Dass and Ramawat 2009c) and by exogenous supply of growth retardants in fed batch cultures (Suthar and Ramawat 2010).

Some plants have advantage over others in long term in vitro conservation as in the case of *Curculigo orchioides* also called as "Kali Musli". It is an herbaceous monocot plant. In nature, its multiplication is slow as root tubers produce a few plants. Biotechnological methods used for micropropagation involve regeneration in static cultures (Suri et al. 1998; Thomas 2007), anther culture (Augustine et al. 2008), bulbils induction in MS medium with 1 mg/l BAP and 0.1 mg/l morphactin (Suri et al. 2000; Nema et al. 2008). In another study, establishment of plantlets in soil was about 80% due to rapid tuberous roots development (Suri et al. 2000). The effect of alar and CCC incorporated in Murashige and Skoog (MS) medium was studied on bulbil induction from leaf explants and subsequent germination of bulbils. Growth retardants markedly reduce the bulbil formation, yield and fresh weight of bulbils. Incorporation of retardants resulted in 60% germination inhibition, thereby prolonging the storage conditions (Malviya et al. 2011) and hence helping conservation.

As observed in case of *Commiphora, Pueraria tuberosa* also lacks efficient protocol for its micropropagation. *Pueraria tuberosa* (Fabaceae), commonly called as Vidarikand, produce underground tubers up to 20 kg. Biotechnological approaches assisting its conservation include micropropagation of this wild plant towards domestication and production of isoflavonoids through callus and cell cultures. Multiple shoot formation was reported using surface-sterilized nodal shoots. These explants were incubated on MS medium supplemented with 2 mg/l BAP, 50 mg/l ascorbic acid, and 25 mg/l of each of citric acid and adenine sulphate. These shoots can be rooted ex-vitro and were acclimatized in the greenhouse (Rathore and Shekhawat 2009). The cell cultures were maintained on the medium containing modified MS medium with 0.1 mg/l 2,4,5-triphenoxy acetic acid (2,4,5-T) and 0.1 mg/l kinetin (Goyal and Ramawat 2008a, b). Production of bioactive isoflavonoids in cell cultures using *Pueraria* and allied species has been scaled up-to the bioreactor level (Luczkiewicz 2008). Production of isoflavonoids was markedly influenced by abiotic and biotic elicitors (Goyal and Ramawat 2008a; Goyal et al. 2011). A maximum yield of isoflavonoids ~82-folds (80 mg/l) was obtained in cultures grown at 0.1 mg/l morphactin and 5.0 mg/l of N^6-(2-Isopentenyl) adenine (2iP) supplemented

medium (Goyal and Ramawat 2008b). These cultures were scaled up-to 2 L stirred tank bioreactor (Sharma et al. 2009). Shoots developed from explants callus were grown in growtek bioreactor with different aeration volume and maximum puerarin content was recorded with 20% v/v aeration, which was ~2.3 fold higher than puerarin content recorded in control cultures (cultures grown in growtek without aeration) (Sharma et al. 2011a).

Withania somnifera (Solanaceae), commonly known as Ashwagandha, (means smell like a horse) is an annual plant and its tuberous roots are used for medicinal purposes. According to a report multiple shoot cultures were developed from nodal explants of field-grown plants on MS medium supplemented with BAP and IAA with the addition of polyamine, spermidine. A total of 46.4 shoots were obtained from nodal explants. After rooting these rooted plants were successfully hardened and acclimatized with a survival rate of 100% (Sivanandhan et al. 2011). In another study, cotyledonary nodes derived from axenic seedlings were used for micropropagation. MS medium supplemented with BA was found to be optimum for production of multiple shoots (100% shoot proliferation frequency and 16.93 shoots per explant). Regenerated shoots were best rooted (95.2%, 38.7 roots per shoot) in half-strength MS medium supplemented with IAA. The plantlets were successfully acclimated and established in soil. RAPD and ISSR analysis revealed a homogeneous amplification profile for all micropropagated plants (Nayak et al. 2013). Besides direct regeneration from explants, callus mediated regeneration was also studied in *W. somnifera*. Among different types of calli, best shoot regeneration was observed on green, compact calli produced on MS medium with a combination of BA and IBA. MS medium supplemented with BAP (2.0 mg/l) showed highest frequency (98%) of shoot bud regeneration in the cultures. The micro-shoots were efficiently rooted and rooted plants were transferred to soil-vermi-compost (1:3; w/w) medium in greenhouse for acclimatization (Chakraborty et al. 2013a). Hairy root cultures of the plant were also reported for the production of useful metabolites (Praveen and Murthy 2013; Kumar et al. 2005). These efforts clearly demonstrate the feasibility of micropropagation technique in conserving these medicinal plants. Novel approaches like use of morphactin as one of the plant growth regulator and biotic elicitor like plant gum and *Cuscuta* are highlights of these works. Nove combinations may result in enhanced production of secondary metabolites.

11.2.2 Somatic Embryogenesis

Somatic embryogenesis is a process whereby a cell or group of cells from somatic tissue forms an embryo. The development of somatic embryos nearly replicates the process of zygotic embryo formation. Somatic embryogenesis mostly occurs indirectly via an intervening callus phase or directly from initial explant and can grow into seedlings on suitable medium. The primary somatic embryos are also capable of producing more embryos through secondary somatic embryogenesis. Somatic embryogenesis is an important method for mass production of plants and for the

development of artificial seeds. These artificial seeds can be used for germplasm conservation and large scale clonal propagation, breeding of plants producing non-orthodox seeds or non-seed producing plants and facilitate the storage and transportation of samples making handling and direct planting easier.

Although standard in vitro propagation methods are, in general, accessible, endangered species may have unusual growth requirements and, thus, may need modified procedures for in vitro culture. In addition, the limited amount of plant material available from rare and endangered species poses major challenges in the application of in vitro techniques. Plant regeneration via somatic embryogenesis has been demonstrated in many medicinal plant species. Effective in vitro regeneration of *Bacopa* has been reported via young leaf derived somatic embryo cultures. Leaf explants cultured in 2,4-D and BAP medium, initiated high frequency somatic embryogenesis. Regenerated plantlets were successfully transferred to soil with 100% survival rate (Chakravarthy et al. 2013). Similarly, in *Clitoria* an efficient plant regeneration protocol has been developed from embryogenic callus derived from cotyledonary explants. Optimum embryogenic callus (75%) was induced on MS medium supplemented with 2, 4-D. On subculturing the callus on MS medium supplemented with BA, NAA, and ABA the highest embryogenic response, frequency of 83% and mean number of 37 embryos per gram callus, was observed. Synthetic seeds were produced by encapsulating embryos in calcium alginate gel and were germinated with 92% frequency. The synthetic seeds were stored at 4 °C and lab conditions (25 ± 2 °C) up to 5 months. The synthetic seeds kept at 4 °C showed 86% viability even after 5 months of storage. Both somatic embryos and synthetic seeds germinated and were transferred to soil successfully (Kumar and Thomas 2012). In *Chlorophytum*, moderate to good callus induction was observed on MS medium containing kinetin and 2,4-D using in vitro grown seedlings. Regular subculturing of callus on kinetin and 2,4-D supplemented medium induced somatic embryogenesis. Modified solid embryogenic medium and liquid embryogenic medium supported better somatic embryo production and maturation. Highest germination (57.5%) was observed at inoculum density of 0.4 g/40 ml of liquid medium. RAPD analysis of *C. borivilianum* plants regenerated through somatic embryogenesis revealed that they were genetically similar to the mother plant (Rizvi et al. 2012). Another plant with a hard to grow in nature, *Commiphora*, has also produced somatic embryogenesis (Kumar et al. 2006b). Development of resin canal (Kumar et al. 2004) and guggulsterone synthesis during somatic embryogenesis has been observed in these cultures (Kumar et al. 2006a). Similarly, in *Curculigo* embryogenic callus mediated somatic embryogenesis has been reported with a frequency of 90% embryos being developed into complete plantlets and with 65–70% field survival rate (Nagesh et al. 2010). Genetic fidelity of somatic embryogenesis derived regenerant using RAPD was also assessed (Patel et al. 2011).

It is evident from the above account that complete protocols have been developed for *A. marmelos, B. monniera, C. asiatica, C. borivilianum, C. orchioides* and *W. somnifera*, and these protocols are used for the production of plants while more work is required for other plants. Production of bioactive molecules is being carried out in our laboratory and more inputs are being tested to make the technology viable.

Out of ~1400 species known to occur in Rajasthan, a brief account of 11 species is presented above and other 15 species, also used by tribals and exploited biotechnologically, are summarised in Table 11.1. *In vitro* response of these species involves use of MS medium except a few who used B5 or WPM medium and plant growth regulators. The response varied from callus induction to shoot formation and rooting of regenerated shoots. Hence most of the work still requires refinement of technology for the effective use.

11.3 Scientific Validation of Traditional Medicinal Plant Usage

Plants have been used for prevention and cure of many ailments since time immemorial. And according to their usage as medicines many of them have been categorized as medicinal plants. Most of our present day knowledge of medicinal plants comes from there folk uses and there uses by our ancestors. In the current scenario when the natural products are gaining importance due to their less or negligible side effects more and more research is being done on the scientific validation of these traditional plants. Many of the research have proved that these traditional usages of plants hold true when tested on scientific parameters. Now to find the herbal based formulations in the pharmacy stores is quite common. Below we have summarized the scientific validations of few important medicinal plants of Aravallis.

11.3.1 Aegle marmelos (Linn.) Corr.

Fruits of *A. marmelos* are highly valued for treatment for chronic dysentery, diarrhoea, and are considered as gastric stimulant. Charak Samhita (Vedic medicinal text) has classified the fruits as curative of piles, haemorrhoids, oedema and swelling. This is one of the ingredients of Dasmoolarisht (ten roots, a standard Ayurvedic medicine for loss of appetite and inflammations of uterus). Fresh leaf juice is effective in diabetes, asthama and fever, and body malodour.

It contains diverse chemicals such as essential oils, coumarins (marmain, aurapten, umbelliferone, marmenol), furoquinoline, alkaloids, triterpenoids, tannins and sterols etc. (Dev 2006; Samarasekera et al. 2004). A new molecule, 24-epibrassinolide has also been reported from *A. marmelos* (Sondhi et al. 2008).

The plant shows many biological activities. Its antidiarrhoeal activity has been reported in rat model (Mazumder et al. 2006) and antimicrobial activity against diarrhoea causing microbes (Brijesh et al. 2009). The plant's unripe fruit extract shows anti-inflammatory, antioxidant, and mast cell stabilizing effects thus demonstrating protective effect in inflammatory bowel disease (Behera et al. 2012). Other biological activities of the plant extract include cardioprotective (Krushna et al. 2012) analgesic, antipyretic, sedative, anticonvulsant (Dev 2006), contraceptive

Table 11.1 Selected plant species of Aravalli Hills with their *in vitro* response on different media. *MS*, Murashige and Skoog medium; *WPM*, Woody plant medium; *BAP*, 6-benzylaminopurinne; *NAA*, α-napthaleneacetic acid; *Kn*, kinetin; *IBA*, indole-3-butyric acid; *IAA*, 3 indole-3-acetic acid

Plant species	Plant part used	Medium	Regeneration	Reference
Abrus precatorius	Node Callus *In- vitro* shoots	MS + BAP (5.0 mg/l) + NAA (0.5 mg/l) MS + BAP (3.0 mg/l) + Kn (0.5 mg/l) + NAA (0.5 mg/l) ½ MS + IBA (1.0 mg/l)	Callus Shoot regeneration Rooting, the rooted plantlets were transferred to soil after proper acclimatization	Biswas et al. (2007) -do- -do-
Acacia nilotica	Node Excised shoots (2–3 cm)	MS + NAA (0.6 mg/L) + Kn (1.0 mg/L) ½ MS + IBA (0.5 mg/L)	Shoot proliferation Rooting, micropropagated plantlets were successfully transferred to natural conditions with 75% survival rate	Dhabhai et al. (2010) -do-
Achyranthes aspera	Leaf	MS + 2,4-D (1.0–2.0 mg/l) + NAA (0.5 mg/l)	Callus	Kayani et al. (2008)
Asparagus racemosus	Node *In-vitro* shoots	MS + 2-isopentyl adenine (3.69 μM) MS + NAA (1.61 μM) + Kn (0.46 μM) + adenine sulfate (98.91 μM) + 500 mg/l malt extract + phloroglucinol (198.25 μM)	Shoot proliferation 85% rooting, tissue-cultured plants were transferred to the field with a 100% survival rate	Bopana and Saxena (2008)
Balanites aegyptiaca	Axillary bud *In-vitro* shoots Axillary meristems	MS + BAP (2.5 mg/l) + NAA (0.1 mg/l) MS + IBA (20 mg/l) MS + BAP (0.45 μM)	Shoot regeneration Rooting, rooted shoots acclimated and were successfully transferred into soil, with 48% of the plantlets surviving Shoot regeneration	Ndoye et al. (2003) -do- Rathore et al. (2004)
Boerhavia diffusa	Shoot tip and Node *In-vitro* shoots Callus *In-vitro* shoots	MS + BAP (1.5 mg/l) + NAA (0.5 mg/l) ½ MS + IBA (1.0 mg/l) + IAA(1.0 mg/l) MS + NAA (1.0 mg/l) + BAP (1.0 mg/l) MS + NAA(0.25 mg/l) + I BA (0.25 mg/l)	Multiple shoot regeneration Rooting, about 80% rooted plantlets survived under field conditions Shoot regeneration Rooting, Regenerated shoots were morphologically similar to the shoots of field grown plants	Roy (2008) -do- Gupta et al. (2004) -do-

Table 11.1 (continued)

Plant species	Plant part used	Medium	Regeneration	Reference
Boswellia serrate	Cotyledonary node *In-vitro* shoots	MS + BAP (0.5 mg dm−3) + NAA (0.5 mg dm^{-3}) + antioxidants ¼ MS + IBA (0.5 mg dm^{-3}) + IAA (0.25 mg dm) + antioxidants	Multiple shoot proliferation 80 % rooting, rooted plantlets were transferred to pots after hardening and acclimatization	Purohit et al. (1995) -do-
Butea monosperma	Cotyledonary node	WPM + BAP (5 mg/l) + fructose (10 mg/l) as an additive	Shoot regeneration without shoot tip necrosis	Kulkarni and D'Souza (2000)
Ficus bengalensis	Node Excised shoots	MS + BAP(1.0 mg/L) + NAA (0.1 mg/L) + 20% (v/v) coconut milk ½ MS + IBA (0.5 mg/L)	Mulliple shoot regeneration Rooting, the complete plantlets thus obtained were successfully transferred to soil	Munshi et al. (2004) -do-
Helicteres isora	Callus obtained from nodal explants *In-vitro* shoots	BAP (2.22 μM) + Kin (2.32 μM) ½ MS + IBA (4.90) μM	Shoot organogenesis Rooting, rooted plantlets were hardened and acclimatized	Shriram et al. (2008) -do-
Phyllanthus emblica syn Emblica officinalis	Node Mature embryo	MS + BAP (4.44 μM/L) + IBA (2.46 μM/l) MS + 2,4-D (1–4 mg/l) + kinetin (0.05 mg/l) and MS + NAA (1–4 mg/l) + kinetin (0.05 mg/l)	Shoot regeneration Callus	Goyal and Bhadauria (2008) Tyagi and Govil (1999)
Phyllanthus fraternus	Shoot tips *In-vitro* shoots	B_5 medium + BAP (10^{-5} M) ½ B_5 medium + IBA (10^{-6} M)	Multiple shoot regeneration Rooting	Rajasubramaniam and Pardha Saradhi (1997)
Salvadora persica	Cotyledonary node Shoot	MS + BAP (4.0 mg/l) + IAA (0.5 mg/l) + adenine sulphate (40 mg/l) + glutamine (100 mg/l) + thiamine HCl (10 mg/l) ½ MS + IBA (3.0 mg/l)	Shoot regeneration Rooting, micropropagated plantlets were successfully transferred to natural conditions with 60 % survival rate	Mathur et al. (2002) -do-
Tinospora cordifolia	Node *In-vitro* shoots	WPM + BAP (8.87 μM) ½ MS + IAA (2.85 μM)	Multiple shoot regeneration Rooting, rooted plantlets were successfully transferred to sand and established with 80 % survival rate	Raghu et al. (2006) -do-

(Chauhan and Agarwal 2009), antidiabetic (Panaskar et al. 2013), hepatoprotective (Verma et al. 2013), antiproliferative (Lampronti et al. 2003) and chemopreventive (George et al. 2014).

It is also anticancerous and works through apoptosis of epithelial cancer cells and activation of tumour necrosis factor -TNF-α (Subramaniam et al. 2008). In other study, *A. marmelos* extract has shown the potential to reduce chemical-induced skin papillomas by enhancing the antioxidant defense system (Agrawal et al. 2012). The plant extract provides protection against γ-radiation (Jagetia et al. 2003) and coxsakie virus B1–B6 (Badam et al. 2002). *A. marmelos* is also among the plants which have demonstrated promising anti-HIV potential (Sabde et al. 2011).

11.3.2 Bacopa monnieri (L.) Pennell.

It is a small prostrate herb that grows wild in marshy places throughout India. The plant contains a complex mixture of dammarane type of triterpenoidal saponins with jujubogenin or pseudojujubogenin moiety as aglycone. The saponins differ in the sugar moieties. Important saponins include bacoside A_1, A_2, A_3, bacopasaponins A-G, bacopaside I-VIII, bacopaside N1,N2,X and jujubogenin. Other chemical constituents of the plant are hersaponin, betulic acid, alkaloids- brahmine and herpestine, flavonoids- luteolin-7-glucoside, glucuronyl-7-apigenin and glucuronyl-7-luteolin, luteolin-7-*O*-β-glucopyranoside, a triterpene bacosine (lup-20(29)-ene-3α-ol-27-oic acid) and several common phytosterols (Rajani 2008; Zhao et al. 2007; Bhandari et al. 2006). High Performance Liquid Chromatography (HPLC) was used to quantify several bacopa saponins (Murthy et al. 2006). A method of enrichment of bacopa saponins from the plant has been patented for its use as memory enhancer (Kahol et al. 2004).

Various investigations have attempted to substantiate and identify a scientific basis for the reputed effects of memory enhancing (Howes and Hughton 2009). A number of *in-vivo* studies have shown *B. monnieri* extracts to improve cognitive function. The mode of action to explain these effects has yet to be fully elucidated. Some studies suggest that the antioxidant effects of *B. monnieri* may protect the Central Nervous System (CNS) from oxidative damage. In a study, the rat experimental model of neonatal hypoglycaemia, *Bacopa* extract improved alterations in Dopamine D1, D2 receptor expression, cAMP signaling and cell death resulting from oxidative stress (Thomas et al. 2013). It has shown beneficial effect in hypoxia and epilepsy management by down regulating glutamate receptor gene expression in rat model (Paulose et al. 2008). In another study, alcoholic extract of *B. monnieri* treatment ameliorated olfactory bulbectomized (OBX) induced cognition dysfunction in mice via a mechanism involving enhancement of synaptic plasticity-related signaling and brain-derived neurotrophic factor (BDNF) transcription and protection of cholinergic systems from OBX-induced neuronal damage (Le et al. 2013). Although the majority of relevant studies, which have investigated the reputed cognitive-enhancing effects, have focused on extracts rather than isolated

constituents, it is the triterpenoid saponins that have been associated with the activity. Triterpenoid saponins, a mixture known as bacoside A which includes bacoside A_3, have been shown to protect rat brains from smoking-induced apoptosis and from structural and functional impairment of mitochondria (Anbarasi et al. 2005).

Ten years of research at Swinburne University at Melbourne, with the extract of *B. monnieri,* showed it to be a safe and efficacious cognitive enhancer. Studies using this extract indicate that it has several modes of action on the human brain. Promising indications for use in humans include improving cognition in the elderly and in patients with neurodegenerative disorders (Stough et al. 2013). Some studies also suggest *B. monnieri* to be an efficient antidepressant which is comparable to well accepted antidepressant drug Fluoxetine hydrochloride (Hazra et al. 2013) and also showed a potential as a possible anti-Parkinsonian agent (Jadiya et al. 2011). It is therefore possible that *B. monnieri* may exert multiple beneficial effects on the CNS and brain ageing (Aguiar and Borowski 2013; Singh et al. 2008; Howes and Hughton 2009).

11.3.3 Cayratia trifolia (L.) Domin

It is traditionally used for boils, for curing bone fracture, as an asrtringent and for the treatment of leukorrhea (Choudhary et al. 2008). Phytoalexins from the Vitaceae constitute a rather restricted group of polyphenolic secondary metabolites belonging to the stilbenes family (piceid, resveratrol, viniferin). Stilbenes production is mainly studied in cell cultures of *Vitis vinifera* (Waffo-Teguo et al. 2008).

In plants, stilbenes play significant role in constitutive and inducible defense mechanisms including antibacterial and antifungal activities (Jeandent et al. 2002; Kostecki et al. 2004). Stilbenes possess a broad spectrum of pharmacological and therapeutic effects such as anti-epileptic effect (Wu et al. 2009), antioxidative, anticancer, anti-atherosclerosis activities, as well as having cardioprotective, hepatoprotective, and neuroprotective effects (Nassiri-Asl and Hosseinzadeh 2009; Kumar et al. 2011; Waffo-Teugo et al. 2008; Baur and Sinclair 2006; Delmas et al. 2006). Some studies also showed that *C. trifolia* extract possesses antiulcerogenic as well as ulcer healing properties, which might be due to its antisecretory activity (Gupta et al. 2012). In another study water extract of *C. trifolia* leaf promised as a cost effective and potent larvicidal agent against *Culex quinquefasciatus* (Chakraborty et al. 2013b). The role of stilbenes in management of cognitive impairment through increasing the activity of choline acetyl transferase and antioxidative mechanism has been identified (Ruan et al. 2009). Antimicrobial activity of stilbenes against oral pathogens has also been explored (Yim et al. 2009). Low incidence of atherosclerosis in red wine drinking society is often co-related with significant level of stilbenes in red wine (Renaud et al. 2004; Waffo-Teguo et al. 2008).

11.3.4 Centella asiatica (L.) Urb.

It is reputed to restore youth, memory, longevity and several minor ailments. Several pentacyclic triterpenoids of ursane subtype (viz., asiatic acid, madasiatic acid, brahmic acid, isothankunic acid and their glycosides) are present in *C. asiatica.* The herb contains an essential oil rich in sesquiterpenes (b-caryophyllene, trans-b-farnesene etc.), flavonoids, steroid and an alkaloid (Dev 2006; Yu et al. 2006). Methods of quantification of some of these compounds have been established (Rafamantanana et al. 2009; Zhang et al. 2008).

The pharmacological basis to explain the reputed anti-amnesic effects of *C. asiatica* has been explored in a number of studies (Howes and Houghton 2009). Based on its traditional use as memory and intellect promoting plant, several works showed its prominent effect in improving cognitive functions and protection in Alzheimer's disease. It also has anti-epileptic, anabolic, antiviral and antitumor activity, and is mild sedative and has beneficial effects in psoriasis and ulcer (Dev 2006). Modern studies proved its antipyretic and anti-inflammatory effects (Wan et al. 2013). It has been shown that asiatic acid plays an important role in cancer apoptosis by inducing mitochondrial death apoptosis cascade (Tang et al. 2009). Similarly, the other results indicate that derivatives of asiatic acid induces inhibition of cell proliferation *via* down regulation of the Ras/Raf/MEK/ERK pathway and cell cycle arrest at G1/S and G2/M (Wang et al. 2013). Madecassoside, a triterpenoid isolated from the herb, exhibits prominent antioxidant activities in collagen induced arthritic conditions in mice model (Li et al. 2009). The plant's activity was explored in epilepsy, stroke and other degenerative conditions (Krishnamurthy et al. 2009). An anxiolytic effect of *C. asiatica* extract was demonstrated in non-stressed mice subjected to acute stress in all behavioral tests employed. These effects could be mainly accounted by madecassoside and asiaticoside, thereby suggesting a possible use of this extract for the treatment of both acute and chronic anxiety in the pathological state (Wanasuntronwong et al. 2012). *C. asiatica* was also found to attenuate the neurobehavioral, neurochemical and histological changes in transient focal middle cerebral artery occlusion rats (Tabassum et al. 2013). The plant extract render radioprotection to DNA and membranes both in *in-vitro* and *in-vivo* conditions (Joy and Nair 2009). Moreover, *C. asiatica* has also shown to protect against UVB-induced HaCaT keratinocyte damage through microRNA expression changes (An et al. 2012). In a study, asiaticoside enhanced the initial skin cell adhesion, induced an increase in the number of normal human dermal fibroblasts thereby promoting skin cell behaviors involved in wound healing (Lee et al. 2012). *C. asiatica* extract capsule can be used for wound healing promotion and also suppress the scar in diabetic wound patients. These capsules can shorten the course of diabetic wound and can be prescribed to the diabetic patients safely (Paocharoen 2010).

11.3.5 *Celastrus paniculatus Willd.*

The plant's seeds and seed oil are considered analgesic, sedative, anti-inflammatory, antirheumatic, diuretic, alterative, nervine tonic and aphrodisiac, and beneficial in gout, and paralysis (Dev 2006). Characteristic secondary metabolites of the plant are a range of esterified bicarbocyclic sesquiterpene polyols occurring in the seeds and seed oil. These polyols e.g., malkanguniol, are esterified with one or more of the following acids: acetic acid, benzoic acid and cinnamic acid (e.g., celapanine) (Dev 2006). A new sesquiterpene polyol ester has also been characterized from the seeds (Borbone et al. 2007).

In a study, seed oil of *C. paniculatus* has shown to improve memory (Kumar and Gupta 2002). Methanol extract of flowers from *C. paniculatus* has shown to be anti-inflammatory (Ahmad et al. 1994), which may also have some relevance in the management of neurodegenerative disorders. A poly-herbal formula (Abana®) containing *C. paniculatus* as a component amongst other herbs is used in Ayurvedic medicine, and dose-dependently improved memory in both young and aged rodents and reversed scopolamine- and diazepam-induced amnesia (Parle and Vasudevan 2007). The contribution of each of the component herbs of this formula to the observed effects, or if any synergistic effect occurred, is unknown. In other studies, *C. paniculatus* extract protected neuronal cells by virtue of their free radical scavenging properties, reducing lipid per oxidation, and also by their ability to induce the antioxidant enzyme catalase (da Rocha et al. 2011). In addition, aqueous extracts of its seed have dose-dependent cholinergic activity, thereby improving memory performance. This enhanced cognition may be due to increased acetylcholine level in rat brain (Bhanumathy et al. 2010). Besides, well known neurological benefits *C. paniculatus* also exhibits different pharmacological activities like analgesic (Debnath et al. 2012), hypolipidemic (Patil et al. 2010) and antiproliferative (Weng et al. 2013). Seed extract has also shown potent relaxant effect in isolated rat and human ileum (Borrelli et al. 2009) that could explain the traditional use of this herb in the treatment of intestinal spasms.

11.3.6 *Clitoria ternatea L.*

It is a major component of an Ayurvedic formulation 'Shankhpushpi', a memory booster (Sethiya et al. 2009). Several flavonoids including quercetin, robinin, and ternatin C1–C5, D3 and preternatin were isolated from *C. ternatea* flowers (Terahara et al. 1998). A novel beta-D-galactosides specific lectin was also purified from seeds (Naeem et al. 2007). A sensitive and precise method has been developed for the determination of taraxerol in the plant (Kumar et al. 2008b).

Extract of *C. ternatea* flowers is used as a component of cosmetics because of its antioxidant activity (Kamkaen and Wilkinson 2009). Its extract possesses a wide range of pharmacological activities including antimicrobial, antipyretic, antidiabetic, anti-inflammatory, insecticidal, diuretic, blood platelet aggregation inhibiting,

and for use as a vascular smooth muscle relaxant properties (Mukherjee et al. 2008). Its antipyretic property was established by yeast induced pyrexia in albino rats (Parimaladevi et al. 2004). The methanolic extract was found to possess nootropic, anxiolytic, antidepressant, anticonvulsions, antistress (Jain et al. 2003), hepatoprotective (Nithianantham et al. 2013) and larvicidal activity (Mathew et al. 2009) and the leaves fresh juice showed anthelmintic activity (Nahar et al. 2010). Another group studied the chemosensitizing activities of cyclotides from *C. ternatea* in paclitaxel-resistant lung cancer cells (Sen et al. 2013). Oral administration of the hydroalcoholic extract of the roots and seeds of the plant resulted in a significant ($p < 0.05$) reduction of serum total cholesterol, triglycerides, very low-density lipoprotein cholesterol, and low-density lipoprotein cholesterol levels. This antihyperlipidemic activity might be attributed to increased biliary excretion and decreased absorption of dietary cholesterol (Solanki and Jain 2010a). Its leaf and flower extracts exhibit antihyperglycaemic effect in rats with alloxan-induced diabetes mellitus (Daisy and Rajathi 2009). The root extract showed antiasthmatic property (Taur and Patil 2011) and profound immunosupressive activity in male albino rat model. The antioxidant and anti-inflammatory activities of plant may be playing major role in immunoinhibition (Solanki and Jain 2010b). Bioassay-guided fractionation of effective extracts may result in identification of useful molecules responsible for these activities. The roots of the plant have a reputation for promoting intellect. Memory enhancing property of root extract of plant was shown in neonatal rat pups (7 days old) by improved retention and spatial learning performance (Rai et al. 2001). This reputed effect may be related to effects on cholinergic activity in the CNS (Howes and Houghton 2009). Further studies are necessary to establish the mechanism of action to explain the observed effects of the root extract on the CNS, and also to identify the compounds responsible for activity.

11.3.7 Chlorophytum borivilianum Sant. and Fern.

Chlorophytum species are reported as diploid, triploid, tetraploid and polyploid with basic chromosome number 7 or 8 (Arora et al. 2004). The tuberous roots (mostly powdered) are widely used in Indian system of medicine for the following properties: as a non-hormonal restorative tonic, in fatigue, general debility, weakness and as a general purpose tonic, in impotency and sterility and to enhance male potency, as cardiac and brain tonic, as curative agent in various diseases like piles, diabetes, albuminorrhoea- leucorrhoea, menorrhoea, and as anti-pyretic, sialogogue, galactogue, diuretic, hemostatic (Arora et al. 2004). These properties have been inferred on the basis of its use in folk medicine, traditional medicine and experiments on man volunteers (Jain 1991; Arora et al. 2004). An Ayurvedic medicine known as 'Musli power extra' has become a house hold energetic medicine for all age groups.

Tuberous roots of *C. arundinaceum,* contain steroidal saponins (neohecogenin, neotigogenin, stigmasterol, tokorogenin), a bibenzyl xyloside (2,4,4'-trihydroxy-2-xylopyranosyl bibenzyl), a disubstituted tetrahydrofuran (4-hydroxy-8,11-

oxidoheneicosanol), nonacosone, tetracosanoic and triacontanoic acid, pentacosyl docosanoate, galactoglucan and sugars (arabinose, glucose and xylose) (Tandon and Shukla 1995; Kaushik 2005). Four new spirostan-type saponins named borivianosides E-H were recently isolated from ethanol extract of the roots of *C. borivilianum* (Acharya et al. 2009).

Most of the traditional uses of *C. borivilianum* have been proved on scientific parameters. Extract prepared from the dried root tubers of *C. borivilianum* inhibited ^{3}H-dopamine—uptake in striatal synaptosomes, and thereby could lead to enhanced dopaminergic tone in the CNS and act as psychostimulants. This has a beneficial effect on brain and resultantly human body by increasing alertness, mental ability and intelligentsia, sexual and maternal characters (our unpublished results). Improvement in sexual behaviour has been observed recently in rat model as reflected by increased penile erection index and improved sperm counts and related behavior (Thakur et al. 2009, 2011; Kenjale et al. 2008). Recently, a study demonstrated that co-administration of *C. borivilianum* root extract with cyproterone acetate enhanced male reproductive potentiality in cyproterone acetate-induced sub fertile wistar strain male albino rat and prevented the negative deviations after the treatment with cyproterone acetate by means of increasing oxidative defence and maintaining homeostasis in testicular apoptosis process (Ray et al. 2013). The ethanolic extract of the plant has shown a strong immunomodulatory (Thakur et al. 2007), antistress and antioxidant effects (Kenjale et al. 2007). Feeding the root powder caused increased super oxide dismutase and ascorbic acid level in mice (Visavadiya and Narasimhacharya 2007a). The methanolic extract of root tubers showed potent hepatoprotective effect against hepatotoxicity induced by CCL_4 in albino rats (Panda et al. 2011). The root extract also have anti-tumour, anti-mutagenic and chemomodulatory effects (Kumar et al. 2010b).

11.3.8 Commiphora wightii (Arnott.) Bhandari

It provides guggul, an oleo-gum-resin, whose medicinal and curative properties are mentioned in the classic Ayurvedic medical text, the Sushruta Samhita 3000 years ago (Dev 2006). The active component of gum guggul, are E- and Z-guggulsterones.

Due to presence of guggulsterones, it has antioxidant, hepatoprotective, neuroprotective and thyroid stimulatory effects (Shishodia et al. 2008), and have therapeutic potential against inflammation, hyperlipidemia and associated cardiac disorders such as hypertension and ischaemia, skin disorders, cancer and urinary disorders (Shah et al. 2012). Several clinical studies conducted in India and abroad have demonstrated that administration of guggul significantly lowers LDL-cholesterol and triglyceride levels in patients with hyperlipidemia (Dev 2006; Shishodia et al. 2008; Yu et al. 2009; Siddiqui and Mazumder 2012). Although the exact mechanism of lipid lowering is far from clear, guggulsterone has been shown to modulate the nuclear receptors, farnesoid X receptor (FXR), pregnane X receptor (PXR), CYP 2b10 gene expression, and the bile salt export pump for cholesterol

elimination (Urizar et al. 2002; Capello et al. 2008). Besides this, guggulsterone also exhibits potent cancer chemopreventive activities (Almazari and Surh 2013). Guggulsterone inhibits smokeless tobacco and nicotine-induced NF-κB and STAT3 pathways in head and neck cancer cells (Macha et al. 2011). It suppresses the pro-inflammatory transcription factor, NF-κB and NF-κB-regulated gene products involved in anti-apoptosis (IAP1, xIAP, Bfl-1/A1, Bcl-2, cFLIP, and survivin), proliferation (cyclin D1 and c-Myc), and metastasis (MMP-9, COX-2, and VEGF) of tumour cells (Shishodia et al. 2008; Lv et al. 2008; Lee et al. 2008). In colon cancer cells, guggulsterone significantly increased apoptosis by activating caspases-3 and -8 (An et al. 2009). Guggulsterone mediates gene expression through regulation of various transcription factors including NF-κB, STAT-3 and C/EBPa and various steroid receptors such as androgen receptor and glucocorticoid receptors (Ahn et al. 2008; Kim et al. 2008; Leeman-Neill et al. 2009). In addition to these activities, guggulsterone has also found to exert a melanogenic inhibitory effect through the downregulation of tyrosinase expression (Koo et al. 2012).

11.3.9 Curculigo orchioides Gaertn.

Tuberous roots are widely used as tonic for health, vigour and vitality because of the presence of flavanone glycosides and other steroidal saponins. Several bioactive compounds isolated from the plant include flavones, glycosides, steroids, saponins and triterpenoids (Tandon and Shukla 1995). New curculigosides from *in-vitro* tubers of *C. orchioides* (Valls et al. 2006), and orchioside D and curculigoside E from roots were isolated (Dall'Acqua et al. 2009).

The medicinal property of the herb is mainly attributed to curculigosides and *Curculigo* saponins (Xu et al. 1992), which is used along with *Withania somnifera*, *C. borivilianum* and *Asparagus racemosus* in several herbal formulations. Increased activity in terms of reduction of mount latency, increase in mount frequency, increased penile erection index and enhanced attraction towards female was recorded from ethanolic extract of the plant (Thakur et al. 2009; Chauhan et al. 2007). Similarly, the aqueous extract of the herb improved sexual performance in streptozotocin induced hyperglycemic and subsequent sexual dysfunctional male rats (Thakur et al. 2010). Curculigoside can improve cognitive function in aged animals, possibly by decreasing the activity of acetylcholinesterase in the cerebra and inhibiting the expression of BACE1 in the hippocampus (Wu et al. 2012). Curculigoside also improved osteogenesis and inhibited osteoclastogenesis of human amniotic fluid-derived stem cells (hAFSC), suggesting its potential use to regulate hAFSC osteogenic differentiation for treating bone disorders (Liu et al. 2014). The methanolic extract of *C. orchioides* has shown to enhance the antioxidant defense against reactive oxygen species produced under hyperglycemic conditions, hence protecting the liver, pancreatic and kidney tissue injuries (Anandakirouchenane et al. 2013). Ethanolic extract of the rhizome exhibited potent activities such as—significant hypoglycemic activity in normal and streptozotocin-induced diabetic rats (Jain et al.

2010), antihypertensive (Joshi et al. 2012), antiosteoporosis (Jiao et al. 2009; Cao et al. 2008), immunostimulant and anti-inflammatory activity (Venkatesh et al. 2009), and estrogenic activity (Vijayanarayana et al. 2007; Nie et al. 2013).

11.3.10 Pueraria tuberosa D.C.

Its tuber contains isoflavonoids (puerarin, genistin, daidzein, and genistein etc.) and is highly valued in Ayurveda since the time of *Samhitaas* (Dev 2006). Besides this, kudzu root (*Pueraria lobata*) is a well-known Chinese herbal medicine, which is being extensively investigated. Traditionally it is used as contraceptive, cardiotonic, rejuvenating and in rheumatism.

These roots are the important crude drugs in the pharmaceutical industries (Dev 2006; Keung 2002). *P. tuberosa* extract have significant anxiolytic and anti-stress properties (Pramanik et al. 2010). Recent findings reveal that puerarin exerts the hypoglycemic and hypolipidemic roles and is potential anti-diabetic which is associated with elevating insulin expression and maintaining metabolic homoeostasis (Wu et al. 2013). Anti-osteoprotic action of puerarin is independent of the estrogen receptor mediated pathway (Michihara et al. 2012). Besides these activities, puerarin is also beneficial against neurological disorders by preventing the dysfunction of the neuronal cholinergic system and ameliorates the increase of β-amyloid caused by estrogen deficiency (Zhang et al. 2013). Puerarin has anti-Parkinson's (Zhu et al. 2014) and cardioprotective activity (Chung et al. 2008). Puerarin successfully reverses hepatotoxicity in CCl_4-induced HF rats via the underlying mechanisms of regulating serum enzymes and attenuating TNF-α/NF-κB pathway for anti-inflammation response, as well as improving metabolic function in liver tissues (Li et al. 2013; Xu et al. 2013; Peng et al. 2013). The other compound of interest is the genistein, which is a promising anticancer agent that inhibits platelet aggregation and induces apoptosis (Lin et al. 2009). Genistein and daidzein both show phytoestrogenic activities (Dixon and Ferreira 2002) and exhibit effective antioxidant and antiosteoporotic properties (Dai et al. 2008). Besides these molecules, tuberosin also have efficient antioxidant properties (Pandey and Tripathi 2010).

11.3.11 Withania somnifera (L.) Dunal.

The plant is cultivated in Rajasthan and Madhya Pradesh states of India. It is highly valued in Ayurveda as an alternative, restorative and as an anabolic agent. It has diuretic, antidepressant and cardioprotective activities. Characteristic compounds present in the plant are steroids with ergostane skeleton, which have been named withanoloides. More than 45 withanololides such as withanolide-A, withaferin-A, sitoinodoside-IX, sitoinodoside-X, somniferine, somniferinine have been isolated from the leaves, fruits and roots of *W. somnifera* (Dev 2006; Mirjalili et al. 2009). This plant has several geno- and chemo-types and hence, the nature and percentage

of secondary metabolites occurring in plant materials of different origin varies greatly (Bandyopadhyay and Jha 2003).

W. somnifera could be developed as a potential preventive drug for ionizing irradiation induced hepatotoxicity disorders via enhancing the antioxidant activity and induction of HO-1 (Mansour and Hafez 2012). Its anti-inflammatory and antioxidant activity also provide protective effect against collagen-induced arthritis (CIA) in rats (Gupta and Singh 2014). It is known to increase spermatogenesis (Mishra 2004), activity in cancer chemoprevention (Stan et al. 2008), cardioprotection (Kulkarni and Dhir 2008) and osteoporosis (Nagareddy and Lakshmana 2006). *W. somnifera* also exhibits beneficial effects as diuretic, antidepressant, hypoglycaemic, and is used in the treatment of skin diseases (Zanwar et al. 2013), CNS disorders, asthma and hypertension (Dev 2006; Visavadiya and Narasimhacharya 2007b). A *W. somnifera* root extract exerts neuroprotective effect against β-amyloid and HIV-1Ba-L (clade B) induced neuro-pathogenesis (Kurapati et al. 2013) and reverses Alzheimer's disease pathology by enhancing low-density lipoprotein receptor-related protein in liver (Sehgal et al. 2012). Different phytochemicals from *W. somnifera* exhibited growth inhibition and cytotoxic activity against human lung cancer cell line (NCI-H460), with withaferin A being the most potent (Choudhary et al. 2010). Low concentrations of *W. somnifera* root extracts (WRE) standardized to Withaferin A (sWRE) inhibit cancer metastasis potentially through epithelial to mesenchymal transition inhibition. Moreover, these doses of sWRE have nearly no toxicity in normal mouse organs, suggesting the potential for clinical use of orally administered WRE capsules (Yang et al. 2013).

There is a surge in publications related to biological/pharmacological activities of these hitherto lesser known plants as evident by data presented in Table 11.2. These plants are from diverse taxa, contain a variety of bioactive molecules as alkaloids, polyphenolics and terpenoids. These data show wide ranging activities like antimicrobial and antidiarrhoeal, immunostimulatory or immunosuppressive, anti-inflammatory and antiarthiritic, antiplasmodial, antiproliferative and anticancerous, osteogenic, antidiabetic, and so on and newer ones are being reported. These traditional ethnomedicinal plants prove to be useful sources of newer biological activities leading to identification of bioactive molecules.

Conservation of plant biodiversity can be accomplished in many ways. Presently, advances in plant biotechnology, especially those associated to in vitro culture and molecular biology provides a good option. Tropical plants often cannot be stored as seeds and must be conserved as growing plants. In vitro conservation is especially important for vegetatively propagated and for non-orthodox seed plant species.

11.4 Future Prospects

Plants of Aravalli hills are showing various promising biological activities such as anticancerous, antitumour, anti-inflammatory, antimicrobial, hypolipidemic, hypocholesteremic, etc. An array of prototype bioactive molecules of different classes

Table 11.2 Selected plant species of the Aravalli Hills, their bioactive molecules, traditional usage and scientific validation

Plant species (family)	Local name (part/s used)	Bioactive molecules	Traditional uses	Biological activities	References
Abrus precatorius (Fabaceae)	Chirmi (Leaves, root)	Abrine, abricin, arbidin, precatorine, choline	Abortifacient, antifertility, aphrodisiac, in leucoderma	Mitochondrial apoptosis induced by the peptide fraction of abrin Lectins-immunostimulatory Seed oil-antimicrobial activity	Bhutia et al. (2009a) Ramnath et al. (2009) Bhutia et al. (2009b) Adelowotan et al. (2008); Zore et al. (2007)
Acacia nilotica (Mimosaceae)	Babul (Leaves, Stem bark, gum, flower)	Kaempferol (AN-5), D-pinitol, a sex hormone viz 3 ß-acetoxy-17-hydroxy-androst-5-ene, acanilol A,B, triterpene lupenone, gallic acid, ellagic acid, epicatechin, rutin	In asthma, cholera, diabetes, diarrhoea, liver complication, leprosy	Antioxidant Immunosuppressive Chemopreventive potential Anti-inflammatory Antiplasmodial Antidiarrhoeal Antimicrobial activity against multi-drug resistant bacterial and fungal strains Inhibitory effect on hepatitis C virus (HCV) protease Antihypertensive and antispasmodic activities	Kalaivani and Mathew (2009) Aderbauer et al. (2008) Singh et al. (2009a) Chaubal et al. (2006) Kirira et al. (2006) Agunu et al. (2005) Khan et al. (2009) Hussein et al. (2000) Gilani et al. (1999)
Achyranthes aspera (Amaranthaceae)	Andhijara, undhokanto (Root, leaves)	Ecdysterone, Betaine	Stimulant, in ulcer, piles, snake antidote, hypoglycaemic, diuretic	Antiparasitic activity of leaf ethyl acetate extract Treatment of leprosy, fistula-in-ano, bronchial asthma Post coital antifertility activity Immunity enhancement Anti-inflammatory Antiarthritic Cancer chemopreventive Prothyroidic, antiperoxidative	Zahir et al. (2009) Goyal et al. (2007) Vasudeva and Sharma (2006) Chakrabarti and Vasudeva (2006) Vetrichelvan and Jegadeesan (2003) Gokhale et al. (2002) Chakraborty et al. (2002) Tahiliani and Kar (2000)

Table 11.2 (continued)

Plant species (family)	Local name (part/s used)	Bioactive molecules	Traditional uses	Biological activities	References
Asparagus racemosus (Liliaceae)	Satawari (Roots)	Steroidal saponins like shatavaroside A (1),B(2), shatavarins VI-X, and saponin like filiasparoside C, Racemoside A,B,C, racemofuran (3) asparagamine A (1), racemosol (2)	Antiageing, intellect promoting, aphrodisiac, improves digestion, abaptogen, treatment of diarrhoea and dysentery	Potent antioxidant activity Aphrodisiac activity Immunomodulatory Elimination of excess cholesterol and elevation of hepatic antioxidant status in hypercholesteremic conditions Chemopreventive Antidepressant effect Racemoside A is a potent anti-leishmanial agent Root extracts have wide-ranging stimulatory effects on physiological insulinotropic pathways Antiulcerogenic agent Antidiarrhoeal potential Antibacterial efficacy	Visavadiya et al. (2009) Thakur et al. (2009) Gautam et al. (2009) Visavadiya and Narasimhacharya (2009) Agrawal et al. (2008) Singh et al. (2009b) Dutta et al. (2007) Hannan et al. (2007) Bhatnagar and Sisodia (2006) Venkatesan et al. (2005) Mandal et al. (2000)
Balanites aegyptiaca (Balanitaceae)	Hingota (Seed kernel, fruit, root, bark)	Balanitin-6 and-7: diosgenyl saponins	Snake antidote, in skin disorders, urine complications, insect bite, pneumonia	Antitumor activity Larvicidal Anti-inflammatory, antinociceptive, antioxidant Fasciolicidal	Gnoula et al. (2008) Chapagain et al. (2008) Speroni et al. (2005) Koko et al. (2000)
Boerhavia diffusa (Nyctaginaceae)	Punarnavaa (Whole plant)	Nonprenylated rotenoids viz boeravinones G(1), H(2), I(10), J(11), punarnavoside, liriodendrin	Diuretic,analgesic, laxative, anti-inflammatory, curative for bronchitis, jaundice, gonorrhoea	Antiproliferative and antiestrogenic properties Cell mediated immune response Radioprotective Spasmolytic effects Immunomodulatory activity Antifungal activity Antidiabetic activity with improvement in antioxidant status Cancer chemopreventive	Sreeja and Sreeja (2009) Manu and Kuttan (2008) Manu et al. (2007) Borrelli et al. (2006) Manu and Kuttan (2009) Agrawal et al. (2004) Satheesh and Pari (2004) Bharali et al. (2003)

Table 11.2 (continued)

Plant species (family)	Local name (part/s used)	Bioactive molecules	Traditional uses	Biological activities	References
Boswellia serrata (Burseraceae)	Salar (Resin)	Incensole acetate, boswellic acids	Frankincense preparations used to cure inflammatory diseases	Anti-depressive, immunomodulatory , neuroprotective Apoptotic effects Cathepsin G as functional target in anti-inflammatory activity Anticancerous Prevent hyperlipidemia and atherosclerosis Antiosteoarthritis	Moussaieff and Mechoulam (2009) Liu and Duan (2009) Tausch et al. (2009) Pang et al. (2009); Bhushan et al. (2009); Kunnumakkara et al. (2009) Tripathi (2009) Sengupta et al. (2008a)
Butea monosperma (Fabaceae)	Dhak (Leaves, flowers, bark, seed , gum)	Butrin, isobutrin, buteaspermin A,B buteaspermanol monospermoside, monospermin, stigmasterol dihydromonospermoside	Antihelmenthic appetizer, aphrodisiac, laxative	Antidiabetic and antioxidant potential Antimycobacterial activity Osteogenic activity Thyroid inhibitory, antiperoxidative Anti-inflammatory activity Chemopreventive hepatic carcinogenesis Dermal wound healing	Sharma and Garg (2009) Chokchaisiri et al. (2009) Maurya et al. (2009) Panda et al. (2009) Shahavi and Desai (2008) Sehrawat and Sultana (2006) Sumitra et al. (2005)
Cocculus hirsutus (Menispermaceae)	Jal-jammi (Leaves)	Isoquinoline alkaloid d-trilobine, dl-coclaurine, cohirsinine, jamtinine, cohirsutine	In eczema, dysentery and in urinary problems, eye diseases	Diuretic larvicidal activity against *Anopheles subpictus* and *Culex tritaeniorhynchus*. Antidiabetic and spermatogenic	Badole et al. (2009) Elango et al. (2009) Sangameswaran and Jayakar (2007)
Ficus bengalensis (Moraceae)	Bar (Aerial roots, bark, latex, fruits)	Flavonoids, viz. leucopelargonin, leucocyanin derivative, quercetin	In urinary problems, ulcers, sores, antipyretic, anti-inflammatory	Antidiabetic Antiallergic and antistress in asthma Antiatherogenic Antioxidant and hypolipidaemic	Singh et al. (2009c) Taur et al. (2007) Daniel et al. (2003) Shukla et al. (2004)

Table 11.2 (continued)

Plant species (family)	Local name (part/s used)	Bioactive molecules	Traditional uses	Biological activities	References
Helicteres isora (Sterculiaceae)	Marorphali (Bark, fruit, root)	Saponins and sapogenin, flavonoid glucuronides viz isoscutellarein 4'-methyl ether 8-O-beta-D-glucuronide etc.	Antidiabetic, antispasmodic activity, in anaemia, asthma, gastrointestinal complications	Hypoglycaemic effect Improving hyperlipidaemia and hyperglycaemia by increasing the gene expression of adipsin, Glut4 and PPARgamma Hypolipidaemic activity Antinociceptive activity Hepatoprotective activity	Kumar et al. (2009); Bhavsar et al. (2009) Kumar and Murugesan (2008) Venkatesh et al. (2007) Kumar et al. (2006a)
Phyllanthus emblica syn Emblica officinalis (Euphorbeaceae)	Amla (Fruit, leaves)	Emblicanin-A, B, gallic acid, ellagic acid, pyrogallol, apigenin 7-0-(6' butyryl-ß-glucopyranoside), quercetin, putranjivain A, phyllaemblicin-A,B,E,F, 4'-hydroxyphyllaemblicin B (1)	Memory and intellect enhancer, nervine tonic, antifatigue, diuretic, promoter of hair growth, and good eyesight	Induce specifically programmed cell death of mature osteoclasts without altering the process of osteoclastogenesis Potential therapeutic agent for viral myocarditis Potent antioxidant Chondroprotective activity in osteoarthritis Antimicrobial, virucidal action against HIV-1NL4.3 and HPV infections Hepatoprotective Cancer chemopreventive Memory improvement and reversal of memory deficits Preventive role in prefibrogenesis of liver Healing activity Radioprotective effect Hypercholesterolemia and atherosclerosis	Piva et al. (2009); Penolazzi et al. (2008) Wang et al. (2009) Poltanov et al. (2009) Sumantran et al. (2008) Talwar et al. (2008); Srikumar et al. (2007) Reddy et al. (2009); Pinmai et al. (2008); Arulkumaran et al. (2007); Deep et al. (2005) Vasudevan and Parle (2007) Mir et al. (2007); Kumar et al. (2008a); Jindal et al. (2009) Kim et al. (2005)

Table 11.2 (continued)

Plant species (family)	Local name (part/s used)	Bioactive molecules	Traditional uses	Biological activities	References
Phyllanthus fraternus (Euphorbeaceae)	Bhumi amla (Whole plant)	E,E-2,4-octadienamide, E,Z-2,4-decadienamide, niruriside, phyllanthin	In jaundice, cough, laboured breathing, malaria, inflammation	Hepatoprotective Antinociceptive Antiplasmodial	Sailaja and Setty (2006); Khatoon et al. (2006) Catapan et al. (2000) Sittie et al. (1998)
Salvadora persica (Salvadoraceae)	Miswak (Stem)	Four benzylamides of which N-benzyl-2-phenylacetamide is pharmacologically important	Chewing sticks, in toothache, mouth ulcer, antimalarial	Antifungal properties against oral *Candida* strains Carries prevention Antiplasmodial Anticonvulsant and sedative effects Antiulcer Hypolipidaemic	Noumi et al. (2009) Sofrata et al. (2008); Darmani et al. (2006) Ali et al. (2002) Monforte et al. (2002) Sanogo et al. (1999) Galati et al. (1999)
Tinospora cordifolia (Menispermaceae)	Guduchi, giloy (leaf, stem)	(1,4)-alpha-D-glucan (alpha-DG), tinosporine, tinosporide, cordifolide, diterpenoid furanolactone, saponarin, octacosanol	Improve immune system and protect against infections, antipyretic	In diabetes type 1 Polysaccharide from plant act as an immunomodulator and adjuvant Activates human lymphocytes Saponarin-hypoglycaemic activity Chemopreventive-hepatocellular carcinoma Anti-tumour in skin carcinogenesis Antiangiogenic activity Antiosteoporotic agent Immunomodulation for ulcer healing	Patel et al. (2009) Raghu et al. (2009) Koppada et al. (2009) Sengupta et al. (2008b) Dhanasekaran et al. (2009) Chaudhary et al. (2008) Thippeswamy etal. (2008) Kapur et al. (2008) Purandare and Supe (2007)

has been obtained from these plants, some of which led to important drugs that are available on the market today. In last three decades, much of the ethnomedicinal data have been documented and now priorities can be determined as per the usage of these plants. Though drug discovery from natural products is long process, use of modern tools like high-throughput screening techniques and NMR spectroscopy can help in the rapid identification of novel molecules and leads. Adequate and continuous supplies of plant-derived drugs are essential to meet the market demand. Therefore, sustainable use of these plants associated with domestication and cultivation practices can meet the demand. More inputs in biotechnological methods can also help in the enhanced production of these bioactive molecules. Use of cell culture technology in fermenters will be helpful in the production of bioactive molecules where synthesis is not available. Further refinement of existing technology for large-scale micropropagation and establishment of industry will be helpful in continuous supply of plant material. In traditional therapy, where combination of various herbs is given, should be encouraged and the synergism of various bioactive molecules along with bioavailability enhancement should also be carried out for its beneficial health effects. Along with domestication, biological problems like reproductive biology and germplasm conservation need attention.

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Chapter 12
Biotechnological Approaches Towards Micropropagation and Conservation of Cycads and Ephedrales

Manjul Dhiman and Indra Rautela

Abstract Cycads are woody plants, usually trees, which on casual observation resemble palms since many have a stock cylindrical stem bearing a crown of very large, coarse, palm-like leaves. The cycads also attract a great deal of scientific attention because they have retained the ciliated sperms. Cycads, the only surviving representative of the class Cycadophyta, are facing extinction from their natural habitats. In order to conserve these "living fossils", immediate steps are needed to propagate them. *Ephedra* is the only genus belonging to the family Ephedraceae. It comprises nearly 68 species widely distributed in the arid regions of New World and Old World. The major active ingredients of *Ephedra* are alkaloids and are referred to as ephedrine type alkaloids. *Ephedra* has been used for more than 5000 years in China and India to treat various ailments. It has also been an ingredient in many dietary supplements and used for weight loss, increased energy, and enhanced athletic performance. Its excess use as medicine has intensified the pressure of landscapes bearing these species. This has necessitated bioprospection and active planning to ensure safe conservation of the existing gene pool and sustainable utilization of this land resource. Ex-situ conservation of germplasm can be achieved using biotechnological approaches such as tissue culture techniques. Hence it is important that biotechnological tools be employed for micropropagation of selected plants and for establishment of germplasm banks. Present review describes various biotechnological aspects related to conservation of Cycads and *Ephedra*.

Keywords Cycads · *Cycas* · *Zamia* · Ephedrales · *Ephedra* · In vitro culture · Micropropagation

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12.1 Introduction

Gymnosperms, comprising mostly evergreen trees and shrubs, constitute a highly fascinating group of plants. The elegant habit and wide range of shapes of the conifers bestow on them year round appeal. The timber and other forest products obtained from them enhance their economic value considerably. This very factor has led to the continuous and excessive exploitation of natural stands of coniferous forests. Scientists have put in great deal of effort in using tissue culture technology for raising these plants. The remaining orders of gymnosperms have, however, attracted much less attention.

The cycads and *Ephedra* differ strikingly from the conifers both in habit and habitat. They are not valuable as timber, but are important for their aesthetic and medicinal value. The presence of ciliate sperms (primitive feature) in cycads and double fertilization (advanced feature) in *Ephedra* add further to their distinct identity. It is now being realized that great attention has been paid to conifer tissue culture but the other gymnosperms have remained somewhat neglected. Another dimension to this aspect is added by realization that the natural habitats of gymnosperms inhabiting tropical and warm temperate areas are undergoing changes at a rapid pace. This has led to a steady decline in their natural population and very few plants can be located in the wild. Under such circumstances, propagation by natural means is not only very slow and inefficient; it also does not ensure large scale multiplication for the continuation/survival of the population.

Tissue culture techniques offer a suitable alternative to traditional method of multiplication by resorting to micropropagation and then re-introduction of plants into affected areas. Ex-situ conservation of germplasm can be achieved by tissue culture techniques. Plant tissue culture technique offers a wonderful tool for conserving plant species where the seed production is negligible, and also for the endangered or threatened plants where population are less and seed propagation is a lengthy process. Hence it is important that biotechnological tools be employed for micropropagation of selected plants and for establishment of germplasm banks. In order to carry out large scale propagation and re-introduction of endangered species, one method that appears feasible is to micropropagate through tissue culture. Both somatic and reproductive parts of the plant can be used as source material.

12.2 Cycads

Cycads, the only surviving representative of the class Cycadophyta, are facing extinction from their natural habitats. Of the 11 genera and about 182 species (Stevenson and Osborne 1993) that are found today, more than half are categorized as “endangered” or “rare” by international Union for Conservation of Nature and Natural Resources (Gilbert 1984).The cycads also attract a great deal of scientific attention because they have retained the ciliate sperms. This feature, which is a characteristic of lower plants, together with the restricted distribution of some

cycads species, points towards the antiquity of the group. Cycads which were once common in the mid Mesozoic, are now present only as relics of the past and are aptly referred to as the "Dinosaurs of the Plant Kingdom".

The cycads are present both in Western and Eastern Hemispheres. Five, namely *Ceratozamia, Chigua, Dioon, Microcycas* and *Zamia* belong to the New World and the remaining six genera viz. *Bowenia, Cycas, Encephalartos, Lepidozamia, Macrozamia* and *Stangeria* belong to the Old World. In India, only *Cycas* is recorded and four species grow wild, namely *C. beddomei, C. circinalis, C. pentinata* and *C. rumphii*. Besides these, *C. revoluta* and *C. siamensis* are commonly cultivated in the gardens (see Bhatnagar and Moitra 1996).

In many parts of the world, cycads such as *Cycas, Zamia* and *Macrozamia* are used as a source of starch either from seed kernels or from stem pith. Young unfolded leaves of some *Cycas* species (*C. circinalis, C. pectinata* and *C.siamensis*) are cooked and eaten. The seed of many cycads are liked by rodents, baboons and other wild animals including elephants. Some *Cycas* species have medicinal value also. The stem of *C. circinalis* is used as remedy for general debility and rheumatism and its leaf is used as cure for flatulence. The male cone of *C. beddomei* forms a major ingredient of some rejuvenating tonics (Ahmedullah and Nayar 1986). Many cycad species are excellent decorative specimens and have great ornamental value.

Cycads propagate either through seeds or asexually by means of adventitious shoots. Nearly all cycads are slow growing and require a long period of growth before they reach the stage of reproduction. The plants are strictly dioecious and, in Nature, a particular stand may be dominated by plants of either sex. The distance between male and female plants is important as this influences pollination. Sometime the pollinating agents themselves may be missing and seed formation is thus low. Another disadvantage faced by cycads is the long time gap between pollination and fertilization, followed by a prolonged period (many months) of embryo development. Even the few seeds that do mature may be eaten by the animals or, if they fail to find favorable conditions, they lose their viability soon.

Population pressures, reduction in forest area, slash and burn agriculture, overexploitation for horticultural purposes and, at times, environmental conditions have led to a drastic reduction in natural stands of cycads all over the world. In order to conserve these "living fossils", immediate steps are needed to propagate them. These include not only awareness strategies, but also sustained efforts of re-establishment of saplings at the impoverished sites. This obviously requires large-scale multiplication which is not very feasible by the conventional methods. There is an urgent need to conserve and protect these beautiful plants of the bygone era (Webb and Osborne 1989; Pant 1996).

Regeneration from somatic tissue of gymnosperms has been a major obstacle. In cycads, availability of readily usable meristematic tissue is highly restricted. Since most of the cycads have usually unbranched stems, they have only one shoot apex and no axillary buds; therefore, non-meristematic tissues from mature plants serve as the source material for *in vitro* culture studies and micropropagation of these plants. In this context, leaf tissues offer the most promising prospect. Following is a resume of published literature related to *in vitro* culture studies in cycads using different explants (see Tables 12.1, 12.2 and 12.3).

Table 12.1 Resume on embryo culture studies in cycads

Species	Culture medium	Adjuvants	Response	Reference
Zamia integrifolia	WM	Amino acids	Plantlet	Brown (1966)
Z. integrifolia	WM	Auxin, Kn	Callus, adventive embryo, "pseudobulbil"	Norstog and Rhamstine (1967)
Z. floridana	WM + MS	–	Root elongation and nodulation	Webb (1982a, d)
Z. pumila	MS	NA,BAP	Callus, root, shoot, embryo like structure	Webb et al. (1983)
Macrozamia diplomera	WM + MS	–	Root elongation and nodulation	Webb and De Jesus (1982)
Zamia pumila	Norstog'J' Medium	IAA	Suspensor growth and development	Monnier and Norstog (1984)
Cycas revoluta	SH	2,4-D,BAP, Kn	Callus, plantlets	Rinaldi and Leva (1990)
Ceratozamia mexicana *C.hildae* *Zamia pumila* *Z. furfuracea* *Z. fischeri*	MS + B_5	2,4-D,Kn CH, amino acid	Callus, shoot, root and somatic embryo	Chavez et al. (1992b, c)
Cycas revoluta	SH	2,4-D,2ip, BAP,NAA	Shoot, root	Rinaldi and Leva (1995)
cycas revoluta	MS	2,4-D, NAA, Kn	Callus, cycasin formation	Tadera et al. (1995)
Encephalartos cycadifolius *E. dyerianus* *E.natalensis*	MS + B_5	2,4-D, Kn, CH, amino acid	Callus, shoot, somatic embryo	Jager and van Staden (1996a, b)
C. circinalis	MS	2,4-D, BAP	Organogenesis	Dhiman et al. (2000)

12.2.1 In Vitro *Culture Studies Using Embryo as Explant*

In vitro germination and growth of *Zamia pumila* embryos in Knop's solution were studied by Brown (1966). He observed a faster development of *Zamia* embryos *in vitro* than *in vivo*. Embryoids were produced from cultured mature embryos of *Z. pumila* on White's Medium having glutamine and alanine (Norstog 1965). Later, Norstog and Rhamstine (1967) cultured proembryos of *Z. integrifolia* on different culture media containing a relatively low concentration of auxin and kinetin. Proembryos formed callus, which on transfer to modified White's medium showed "pseudobulbils" and adventive embryos. However, plantlets were not formed.

Webb and his co-workers studied *in vitro* culture responses of embryos of different cycads. Webb (1982a) stated that both megagametophyte and cotyledons are important for primary and secondary root production of *Z. floridana* embryos in White's culture medium. Root elongation and nodulation were observed in embryos

Table 12.2 Resume on megagametophyte culture in cycads

Species	Culture medium	Adjuvants	Response	Reference
Cycas thourasii	Soil	–	Root	Du Chartre (1888)
Zamia floridana	WS, HM	–	Root, bud	La Rue (1948)
Cycas revoluta	Sterile moist sand	–	Root, bud,	La Rue (1950, 1954)
Zamina floridana	Agar + sugar	–	Root, bud pseudobulbil	La Rue (1954)
Zamia integrifolia	WM	2,4-D,Kn amino acid	Callus, root, leaf, embryoid	Norstog (1965)
Zamia integrifolia *Cycas revoluta*	WM, LS	2,4-D	Callus, pseudo-bulbil, embryoid	Norstog and Rhamstine (1967)
Ceratozamia mexicana *Cycas revoluta* *Encephalartos umbeluziensis*	WM, LS	2,4-D,Kn	Callus, pseu-dobulbil root, embryo	De Luca et al. (1979)
Cycas revoluta	WM	2,4-D,Kn	Coralloid root	De Luca and Sabato (1980)
Encephalartos villosus	MS + B_5	2,4-D,Kn	Callus	Laliberte et al. (1983)
Ceratozamia hildae *C. mexicana* *Zamia fischeri* *Z. furfuracea* *Z. pumila*	MS + B_5	2,4-D, Kn, CH, amino acids	Callus, shoot, root, somatic embryo	Chavez et al. (1992b, c)
C. circinalis *Zamia integrifolia*	MS	2,4-D,NAA,Kn	Callus, nodular structure	Dhiman et al. (1998b)

Table 12.3 Resume of *in vitro* studies on cycads using somatic tissue as explants

Species	Medium	Adjuvant	Response	Reference
Cycas revoluta	MS	2,4-D,Kn,GA_3	Callus	Brown and Teas (1966)
Encephalartos sp.	MS	NAA, Kn	Callus	Koelman and Small (1982)
Stangeria eriopus	SH	2,4-D, Kn	Callus, leaf	Osborne and van Staden (1987)
Ceratozamia mexicana	MS + B_5	2,4-D, Kn	Callus, somatic embryo	Chavez et al. (1992a)
Ceratozamia hildae	MS + B_5	2,4-D, Kn	Callus, somatic embryo	Litz et al. (1995a)
Cycas revoluta *C. rumphii* *Zamia furfuracea*	MS	2,4-D,BAP, Kn	Callus, somatic embryo	Dhiman et al. (1998a)

culture of *Z. floridana* (Webb 1982d) and *Macrozamia diplomera* (Webb and De Jesus 1982) when these were kept in light. Friable and compact calli were produced when *Z. pumila* embryos were cultured on MS medium supplemented with NAA and/or BAP (Webb et al. 1983). Friable callus formed only roots, whereas compact nodular callus differentiated roots, shoots and embryo-like structures.

Immature embryos of *Z. pumila* were cultured and their development was studied by Monnier and Norstog (1984). Initially, only growth of the suspensor was observed. After 2 months of culture, the suspensor stopped growing and a dicotyledonous embryo was formed. Addition of IAA, CM or "endosperm extract" of the seed to the medium did not improve *in vitro* embryo development.

Chavez et al. (1992c) described the induction of friable callus from zygotic embryos of *Z. furfuracea*, *Z. pumila* and *Z. fischeri* cultured on modified B_5 medium containing various amino acids, 2,4-D and Kn. Callus from *Z. pumila* and *Z. furfuracea* was embryogenic and produced somatic embryos; callus from *Z. pumila* regenerated shoots also. Chavez et al. (1992b) also reported somatic embryo formation in callus obtained from mature zygotic embryos of two cycads, *Ceratozamia hildae* and *C. mexicana*. These somatic embryos germinated *in vitro* but did not form plantlets.

Rinaldi and Leva (1990, 1995) reported callus formation and organogenesis in *Cycas revoluta* embryo explants, cultured on SH medium. Shoots regeneration occurred on medium containing 2, 4-D and BAP. Root formation was induced by addition of NAA to the medium. Embryos of *Cycas revoluta* were reared *in vitro* for biochemical studies (Tadera et al. 1995). They observed cycasin formation in the callus grown on MS medium having NAA and Kn.

Somatic embryogenesis and organogenesis in embryo cultures have been reported in *Encephalartos cycadifolius, E. dyerianus* and *E. natalensis* (Jager and van Staden 1996a, b). Embryo explants of all the three species, when grown on modified B_5 medium containing 2, 4-D and Kn, showed callusing and somatic embryogenesis. *E. cycadifolius* somatic embryos matured and germinated. Shoot formation also occurred in callus obtained from *E.dyerianus* and *E. natalensis* embryos.

Dhiman et al. (1998a, 2000) reported callusing followed by organogenesis and formation of bulbils from hypocotyls segment of embryo of *Cycas circinalis*. The embryo of *C. circinalis* were cultured onto MS medium supplemented with 2, 4-D or NAA alone or along with BAP/Kn. The bulbils developed onto medium containing 1 μM 2, 4-D with 2 μM BAP. The bulbils however, could not develop into plantlet.

12.2.2 In Vitro *Culture Studies Using Megagametophyte as Explant*

Duchartre (1888) reported for the first time regeneration in megagametophytes of *Cycas thourasii.* Roots were produced from megagametophytes placed in soil. Root and shoot differentiation was obtained by La Rue (1948, 1950, 1954) in

megagametophyte cultures of *Cycas revoluta* and *Zamia pumila*. In *Cycas revoluta* (La Rue 1950, 1954), root regeneration occurred in moist sand devoid of minerals and carbohydrates. Rhizogenesis was most frequent where the gametophyte touched the wet sand. IAA in lanolin was used to improve rooting.

Zamia floridana female gametophyte was cultured in White's solution having 0.7–1 % agar (La Rue 1948, 1954). In addition to roots and shoots, detachable "pseudobulbils" also developed. These were parenchymatous but, upon subculture, they produced embryo-like structure. While regeneration was seen all over the megagametophyte surface, it was profuse in the vicinity of archegonia. Organogenesis was optimal when megagametophytes were cultured at the time of fertilization. Extremely immature gametophytes did not survive in culture. Post fertilization gametophytes were not regenerative.

La Rue (1954) also observed surface nodules of periderm and sectors of tracheids. Organogenesis and plant development was rare with a frequency of less than 1 %. Furthermore, regeneration took about a year or longer. Plantlets were formed, but these failed to develop further.

Norstog (1965) grew *Z. pumila* female gametophyte on White's medium supplemented with IAA, 2,4-D, Kn and various amino acids. Some callusing occurred on White's basal medium. Best results were obtained with medium having Kn, 2,4-D, adenine sulphate, alanine, asparagine and glutamine. On modified White's medium, 68 % of explants formed callus after 2 months, and after 5 months 24 % formed leaves and another 35 % developed leaves and roots. Organs so formed elongated after subculture on hormone-free medium. Cytological studies showed haploid root tips. In a few cases, "embryoids" also developed resembling zygotic embryos but these were not examined microscopically. Subsequently, Norstog and Rhamstine (1967) induced *Z. pumila* and *Cycas circinalis* megagametophytes to form callus on White's basal medium and LS medium having high level of 2,4-D. Formation of "pseudobulbils" occurred when callus was transferred to a hormone free medium. "Pseudobulbils" formed pink coloured adventive embryos which developed into minute plantlets.

Female gametophytes of certain other cycads have been cultured by De Luca et al. (1979, 1980) and De Luca and Sabato (1980). On to medium supplemented with glutamine, asparagine and alanine, the megagametophytes of *Ceratozamia mexicana* produced adventive embryos and roots. Spherical outgrowths or "pseudobulbils" differentiated from megagametophytes of *Cycas revoluta*. Later, these were shown to be outgrowths of coralloid roots (De Luca and Sabato 1980). Coralloid roots regenerated from *Macrozamia communis* megagametophytes cultured on White's basal medium having 2, 4-D and Kn (De Luca et al. 1980). No organogenesis was reported from *Encephalartos umbeluziensis* (De Luca et al. 1979).

Rivera Rosa (see Webb and Osborne 1989) showed that regeneration was possible from whole, half and quarter megagametophytes of *Z. pumila*. Mature gametophyte explants were cultured on MS medium containing different concentrations of NAA and BAP. Friable as well as nodular callus gave rise to dicotyledonous, bipolar embryo-like structures which failed to germinate or develop further. When friable calli were transferred to a hormone-free medium, shoot differentiation and

regeneration of pink embryo- like structure or embryoids occurred. These embryoids germinated but grew no further.

Female gametophytes of a rare Cuban cycad, *Microcycas calocom*a were cultured on MS medium supplemented with NAA and CW (Pena et al. 1982). Explants from young ovules formed callus and roots. With an increase in NAA concentration, callus become more compact, and root formation was not observed. Upon transfer to medium with reduced NAA levels, rhizogenesis occurred. In medium with little or on NAA, formation of pink "pseudobulbils" was also observed. Female gametophytes of *Encephalartos villosus* callused when culture on white's medium containing 2,4-D and Kn (Laliberte et al. 1983). Surface nodules were formed but there was no further organogenesis or embryogenesis. Tracheids differentiated in the callus.

Using modified B_5 medium having MS minor salts, vitamins, 2,4-D and Kn, Chavez et al. (1992b) reported callus formation and subsequent root and shoot developed from the female gametophytes of *Ceratozamia hildae* and *C. mexicana*. Shoots of both the species elongated on hormone-free medium but attempts to stimulate rooting in adventitious shoots failed. Plants were not recovered from culture female gametophyte of any of the species. In a subsequent paper, Chavez et al. (1992c) reported induction of haploid embryogenic callus from explanted megagametophytes of *Zamia fischeri*, *Z. furfuracea* and *Z. pumila*. Embryoids differentiated on modified B_5 medium supplemented with 2, 4-D and Kn. The earlier stages of somatic embryo development began with globular masses of callus. Subsequently, a suspensor developed from the base of each haploid proembryo. Haploid embryos germinated but no plantlets were formed. Female gametophyte culture of *Cycas circinalis* and *Zamia integrifolia* was also reported by Dhiman et al. (1998a, 2000). They reported callus induction followed by formation of nodular structure when female gametophyte segment were cultured onto MS medium containing 2, 4-D and Kn (*Z. integrifolia*) or NAA and Kn (*C. circinalis*).

12.2.3 In Vitro *Culture Studies Using Seeding Explants*

Webb and his associates have extensively studied the root elongation and nodulation in seeding of cycads under culture conditions (Webb 1981a, b, 1982b, c; Webb et al. 1984). When germinating seeds of different cycads were cultured on modified White's basal medium in dark, a typical tap root system developed. Exposure to light induction nodulation and apogeotropism in the regeneration roots (Webb 1981a, b, 1982b; Webb et al. 1984). However, seeding of *Dioon edule* failed to nodulate *in vitro* and light induced callus formation in primary and secondary roots (Webb 1982b, 1984). Seedling cultures of *Macrozamia* grown in dark formed apogeotropic nodules at the junction of the primary root and shoot. In light, laterals developed along the primary tap root and were converted into coralloid roots (Webb 1983).

12.2.4 In Vitro *Culture Studies Using Leaf as Explants*

Callus is reported from rachis of *Cycas revoluta* on MS medium supplemented with 2, 4-D, Kn and GA_3 (Brown and Teas 1966). Chavez et al. (1992a) and Litz et al. (1995a) induced somatic embryogenesis in callus derived from the leaf (pinnae) of *Ceratozamia mexicana* and *C. hildae*, respectively. Callus was obtained on modified MS minor salts and organics supplemented with 2,4-D and Kn. Somatic embryos germinated on hormone-free medium but failed to develop into plantlets. Histological studies of somatic embryo (Chavez et al. 1995) confirmed their embryo status.

Petiolar and rachis portion of young leaves from adult plants of *Zamia furfuracea* were induced *in vitro* for somatic embryogenesis by Dhiman et al. (1998). They reported single to polycotyledous somatic embryos from callus obtained from leaf explants onto MS medium containing 2,4-D/Kn with Kn or BAP. The somatic embryos germinated in basal medium but could not develop into plantlets.

12.2.5 In Vitro *Culture Studies Using Root as Explants*

Callus formation from root explants of nine species of *Encephalartos* was reported by Koeleman and Small (1982). Callus initiation was slow and took 4–6 months. Subsequent organization development was not possible. Successful regeneration from primary roots of *Stangeria eriopus* was reported by Osborne and van Staden in 1987. They cultured root segments on SH medium having 2,4-D and Kn. Callus was produced which developed into small green meristematic zones followed by emergence and expansion of a typical circinnate leaf.

12.2.6 Somatic Embryogenesis

Somatic embryo development from embryogenic cultures of cycads remains inefficient, as it does not respond to standard dormancy-inducing stimuli. Because of this, cultures cannot be synchronized, and certain developmental events, i.e. the free nuclear stage and the early stages of embryogenesis are difficult to identify in these cultures (Litz et al. 1995b). Somatic embryogeny has been reported in cycads using megagametophytes and embryos in *Zamia* (Chavez et al. 1992c) and *Ceratozamia* (Chavez et al. 1992b) and leaves in *Ceratozamia* (Chavez et al. 1992a; Litz et al. 1995a) and *Zamia furfuracea* (Dhiman et al. 1998b).

Usually, there is a long time gap between induction and development of somatic embryos in cycads. This varies between 15–18 months as reported by various workers (Chavez et al. 1992a, 10, c; Litz et al. 1995a). It is not known whether this long period is essential for somatic embryogenesis in cycads. Ball et al. (1978) also noted that organogenesis from leaves of *Sequoia sempervirens* required a "prohibitory

long time". Therefore, time needed for somatic embryos and their subsequent development is biologically determined.

The number of cotyledons in cycads is variable even within a species. Chamberlain (1935) reports that usually there are two cotyledons, but number may vary between 1–6. In *Ceratozamia,* De Luca et al. (1979) indicated that the somatic embryo seems to have only a single cotyledon. Chavez et al. (1992c) also showed that *C. mexicana* had only one cotyledon. They also reported that somatic proembryo of *C. hildae* underwent successive cleavage divisions resulting in somatic embryo having many cotyledons (Chavez et al. 1992b). They stated that single cotyledony may result from either fused cotyledons or cotyledons may be surrounded by a 'coleoptiles like' sheath, thus appearing to be monocotyledonous. In *Zamia furfuracea* also, (Dhiman et al. 1998b) number of cotyledons varied between one to as many as eight, single cotyledon being predominant. Saxton (1910) observed that *in vivo* developed embryo of the cycads *Encephalartos* could sometimes be branched and that each branch could bear an equally developed embryo. Thus, polycotyledonous somatic embryo may be explained in term of the fact that branching may occur in early development of a proembryo, resulting in many cotyledons.

Colour of the somatic embryo is also a unique feature in the cycads. Light pink colouration is characteristic feature of certain cycads embryos. It is creamy white to light pink in colour (Litz et al. 1995a). In *Zamia furfuracea* also, somatic embryo were initially pinkish in colour; however some were green also (Dhiman et al. 1998b).

12.3 Ephedrales

There are about 68 species of *Ephedra* (Sharma and Dhiman 2010) spreading worldwide, in Europe, temperate Asia, South America and Afghanistan to Bhutan (2400–5000 m) adapted to semiarid and desert environment. These are widely distributed in both Eastern as well as Western Hemisphere.

In India, Ephedra is represented by nine species (Sahni 1990) namely *E. foliata*, *E. gerardiana, E. intermedia, E. nebrodensis*, *E. regeliana*, *E. saxatilis, E. pachyclada* and *E. przewalskii*. Recently, Sharma and Uniyal (2008) and Sharma et al. (2010), discovered few new species viz. *E. sumlingensis*, *E. kardangensis* and *E. khurikensis* from Sumling (district Spiti), Kardang (district Lahul) and Khurik (district Spiti) respectively in Himachal Pradesh. Medicinally important species of Ephedra includes *E. gerardiana, E. nebrodensis, E. saxatilis, E. sinica* and *E. monosperma.*

Earlier reports indicate that the ancient Aryans discovered *Ephedra* or Soma plant as an energizer-cum-euphoriant. Use of *Ephedra* juice for longevity was a part of ancient Indian Aryans custom mentioned in the Rigveda (the oldest of sacred Sanskrit Vedas) and followed even by ancient Romans (Mahdihassan 1981; Mahdihassan and Mehdi 1989). In China, the *Ephedra* species have been dispensed in Traditional Chinese Medicines (TCM) for at least 5000 years (Morton 1977) and is popularly known as Ma Huang. In TCM, dried stems of *Ephedra* species are

used to alleviate symptoms caused by common cold, influenza, asthma, bronchitis, nasal congestion and hay fever. This was also used for a treatment of arthritis, fever, hives, lack of perspiration, headache, aching joint and bones, wheezing and low blood pressure (Leung and Foster 1996). Traditionally *Ephedra* was made as a fermented drink and was used ceremonially by Vedic and Zoroastrian priests. In 2700 BC, Shen Mung, the first Chinese herbalist used the dried roots and stem as a decongestant to treat asthma, cold, coughs, fever, hay fever and headache. The American Indians used the root for flushing kidneys, internal bleeding and purifying the blood. The young stems are best if eaten raw, though older stem can be used to make a medicinal tea that is known as Mormon tea. Herbal extracts of certain species of *Ephedra* are known as stimulating beverage to the tribal in America and in Indo-China.

In Asian medicine, *Ephedra* is recommended for colds and flu, fever, chills, edema, hyperhidrosis, nasal congestion, bone pains, coughing and wheezing. It is also used as a lack of perspiration. Ephedrine is a potent bronchodilator that, in appropriate doses, can be administered safely along with therapeutic doses of theophylline without the fear of progressive tolerance or toxicity (Soni et al. 2004). In western medicine, ephedrine is used for the treatment of nasal congestion due to hay fever, allergic rhinitis, asthma and common cold. Also, ephedrine salts are prescribed in the form of nasal sprays to relieve congestion and swelling. When injected subcutaneously, ephedrine prevents hypotension during anesthesia. Orally, it has been used in certain forms of epilepsy, nocturnal enuresis, myasthenia gravis and urticaria accompanying edema. Pseudoephedrine, taken orally, is an effective nasal decongestant (Morton 1977).

Recently, a new usage of *Ephedra* different from traditional direction had been widespread in the United States. Focusing on thermogenic and lypolytic effects of *Ephedra,* dietary supplements containing *Ephedra* extracts have been commercially promoted and are used for weight reduction and energy enhancement (Josefson 1996).

The major active ingredients of *Ephedra* are alkaloids that constitute 0.5–2.5 % of total mass, and are referred to as ephedrine type alkaloids. The six optically active alkaloids that have been isolated from *Ephedra* species are (−)—ephedrine, (+)—pseudoephedrine, (−)—*N*-methylephedrine, (+)—*N*-methylpseudoephedrine, (−)—norephedrine, (+)—norpseudoephedrine. Ephedrine is the major isomer comprising of 30–90 % of total alkaloid fraction accompanied by psuedoephedrine, with trace amount of other ephedrine type alkaloids (Blumenthal and King 1995).

Tissue culture has become a popular method for vegetative propagation of several plants. The most significant advantage offered by this aseptic method of clonal propagation, popularly called ‘micropropagation’, over the conventional methods is that in a relatively short time and space a large number of plants can be produced starting from a single individual. There are various reports of *in vitro* work on *Ephedra* using various explants with an aim of organogenic induction and micropropagation. Following is a resume of *in vitro* culture work related to different species of *Ephedra* (see Tables 12.4, 12.5 and 12.6).

Table 12.4 *In vitro* studies in *Ephedra* using stem segment as explant

Species	Medium	Adjuvants	Response	Reference
Ephedra sp.	White medium	2, 4-D, NAA	Callus, auxin oxidase activity	Straus and Gerding (1963)
E. foliata	MS	2, 4-D	Callus, alkaloid production	Khanna and Uddin (1976)
E. foliata	MS	2, 4-D	Callus	Uddin (1977)
E. andina *E. distachya* *E. equisetina* *E. fragilis* *E. gerardiana* *E. major* *E. minima* *E. saxatilis*	MS	2, 4-D, NAA, Kn	Callus, alkaloid production	O'Dowd et al. (1993)
E. fragilis *E. saxatilis*	MS	2, 4-D, BAP IBA, Kn	Callus, adventitious shoot root plantlets transplantation	O'Dowd and Richardson (1993a, b)
E. fragilis *E. major*	MS	NAA, IAA	Roots, tumours by *A. rhizogenes*	O'Dowd and Richardson (1994)
E. gerardiana	MS	Kn, BAP, IBA	Shoots, multiple shoots roots, alkaloid production	Watanabe et al. (1996)
E. foliata	MS	2, 4-D, Kn/BAP	Somatic embryogenesis shoot bud production	Dhiman and Sharma (2010)
E. gerardiana	MS	2, 4-D, NAA, Kn	Callus	Garla et al. (2011)
E. alata	MS	2, 4-D, Kn	Callus	Hegazi and El-Lamey (2011)
E. gerardiana	MS	TDZ	Callus, shoot buds, root	Sharma et al. (2012)
E. foliata	MS	BA, IAA, Kn, IBA	Shoot bud, plantlets ex vitro rooting transplantation	Lodha et al. (2014)

Table 12.5 *In vitro* studies in *Ephedra* using seedling/embryonal explant

Species	Medium	Adjuvants	Response	Reference
E. gerardiana.	LS	2, 4-D	Callus	Nishi (1974)
E. foliata *E. gerardiana*	MS	NAA, Kn	Callus, embryoid root, shoot	Ramawat and Arya (1976)
E. gerardiana *E. foliata*	MS	NAA, Kn	Callus	Ramawat and Arya (1977)
E. gerardiana *E. foliata*	MS	NAA, Kn, BAP IBA	Callus, ephedrine	Ramawat and Arya (1979a, b, c)
E. gerardiana *E. foliata*	MS	Different Nitrogen sources	Callus	Ramawat and Arya (1979d)
E. fragilis	MS	BAP	Callus, shoot bud	O'Dowd and Richardson (1993a)
E. foliata	MS	2, 4-D, BAP, Kn	Callus, shoot bud somatic embryos	Dhiman et al. (1998a)

Table 12.6 *In vitro* studies in *Ephedra foliata* using female gametophyte as explant

Medium	Adjuvants	Response	Reference
WM	CM, 2, 4-D Kn	Callus	Sankhla et al. (1967a)
MS	CM, 2, 4-D Kn	Callus, root, shoot	Konar and Singh (1979)
MS	CM, 2, 4-D, Kn NAA, BAP	Callus, root, shoot, plantlet	Singh and Konar (1981)
MS	CM, 2, 4-D, Kn NAA, BAP	Callus, root, shoot, plantlet	Singh et al. (1981)
MS	CM, 2, 4-D, Kn NAA, BAP	Callus, root, shoot, plantlet	Bhatnagar and Singh (1984)

12.3.1 In Vitro *Culture Studies Using Stem Segment as Explant*

Initial study on callus culture in *Ephedra* goes dates back to 1963 in which Straus and Gerding explained IAA oxidase activity in isolated callus culture from stem of an unknown species of *Ephedra*. They observed that *Ephedra* tissue produces IAA oxidase in cultures which destroy IAA but not 2, 4-D or NAA. Work on tissue culture of *Ephedra* stem segment mainly deals with studies on alkaloid content of the callus. Khanna and Uddin (1976) extracted a compound from *E. foliata* stem callus and identified it as ephedrine. Uddin (1977) studied the presence of different amino acids in *E. foliata* suspension cultures. Callus was obtained from stem pieces in MS medium supplemented with 2, 4-D. He stated that total amino acid contents increase with the age of the culture. They reported a higher concentration of glutamic acid and arginine as compared to leucine and serine.

O'Dowd et al. (1993) observed the effect of various plant growth regulators on callus production from stem explant in many species of *Ephedra* viz. *E. andina*, *E. distachya*, *E. equisetina*, *E. fragilis, E. gerardiana, E. intermedia, E. major, E. minima* and *E. saxatilis*. All species produced callus on MS medium supplemented with Kn and 2, 4-D or NAA. Neither IAA nor IBA induced any significant callus formation. Suspension cultures of callus were also established. Trace quantities of l-ephedrine and d-pseudoephedrine were produced in suspension cultures of all the species except *E. distachya, E. fragilis* and *E. saxatilis*.

O'Dowd and Richardson (1993a) cultured internodal portions of many species of *Ephedra* on MS medium supplemented with 2, 4-D and BAP. *E. fragilis* produced green callus and adventitious buds. However, *E. andina*, *E. distachya*, *E. gerardiana*, *E. gerardiana* var. *sikkimensis* and *E. saxatilis* formed green nodular structures with roots.

In vitro micropropagation of 11 species of *Ephedra* was carried out by O'Dowd and Richardson (1993b). *E. fragilis* nodal explants were cultured on MS medium supplemented with 0.05 µM IBA and 0.05 µM Kn, Zeatin or BAP. At high concentrations (3.75–5 µM) of cytokinin, multiple shoots were produced. Substituting IBA with 2,4-D caused callus formation and distorted shoot growth. Shoots from nodal explants of ten other species were also obtained on 0.05 µM IBA and 0.05 µM Kn. Rooting of shoots was observed on medium containing 1–5 µM IBA. Plantlets, thus obtained, were successfully transplanted to pots.

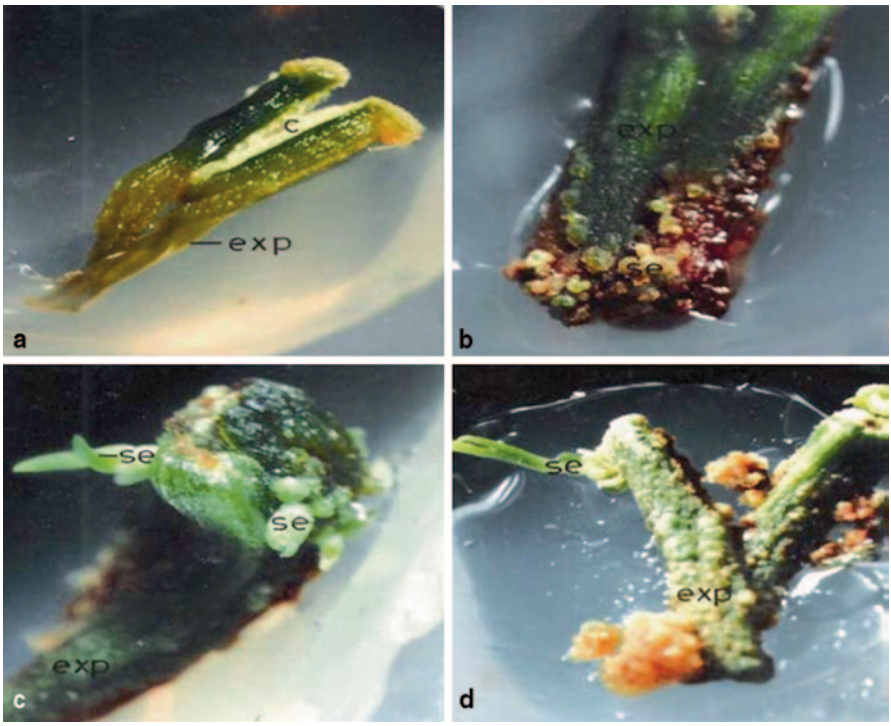

Fig. 12.1 **a** Two-week-old explants on BM+2 μM 2,4-D+2 μM Kn showing callus initiation from injured surface of the explants. **b** Three-week-old explants on BM+2 μM 2,4-D+2 μM Kn showing somatic embryos initiation from cut surface of the explants. **c** Four-week-old explants on BM+2 μM 2,4-D+8 μM Kn showing direct formation of somatic embryos. **d** Same as c on BM+2 μM 2,4-D+10 μM Kn. (From Dhiman and Sharma 2010)

O'Dowd and Richardson (1994) studied the *in vitro* infection of *Agrobacterium rhizogenes* on *Ephedra* tissues to examine alkaloid production in any resultant tumours and roots. Vertically inoculated stem portions of *E. fragilis* and *E. major* grown on MS medium devoid of any growth regulators produced roots upon infection. However, horizontally placed stem explants were most successfully infected. Tumours were formed in 47% wound sites of *E. fragilis* with half of them giving rise to roots. *E. equisetina* had the highest rate of tumour and root formation. *E. gerardiana*, *E. minima* and *E. minima* hybrids had high rate of tumour production but low root production. Slow growing *in vitro* cultured tumors of *E. fragilis* contained up to 0.01% l-ephedrine but alkaloid was not detected in faster growing isolates.

Watanabe et al. in 1996 cultured young shoots of *Ephedra gerardiana* onto MS and WP medium having Kn or BAP respectively. They reported axillary and adventitious bud production. Shoots were further grown for rooting onto half WP medium without hormones. The regenerated plantlets were analysed for alkaloid contents.

Dhiman and Sharma (2010) reported somatic embryogenesis and plant regeneration from internodal segment cultures in *E. foliata*. They observed direct (Fig. 12.1a–d) and indirect (Fig. 12.2a–b) somatic embryogenesis onto MS media

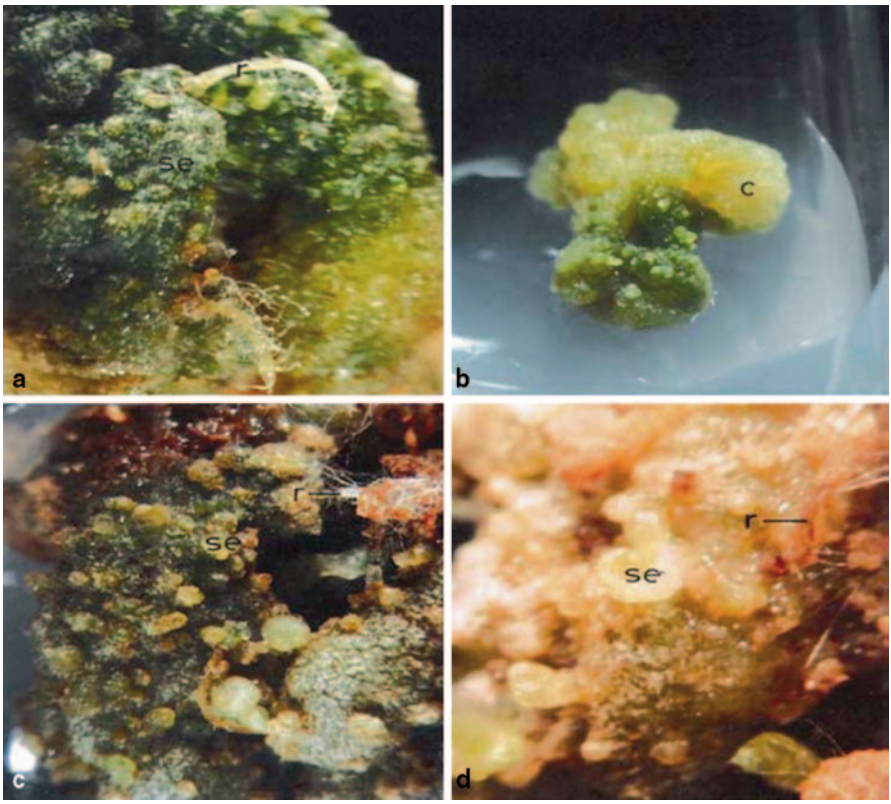

Fig. 12.2 **a** Four-week-old culture on BM + 5 µM 2,4-D + 5 µM Kn showing compact green callus with initiation of somatic embryos and roots. **b** Four-week-old culture on BM + 5 µM 2,4-D + 8 µM Kn showing callus with initiation of somatic embryos. **c**, **d** 30 and 45 days old cultures, respectively on BM + 8 µM 2,4-D + 8 µM Kn showing initiation and further development of somatic embryos. Roots are also visible. (From Dhiman and Sharma 2010)

containing 2, 4-D and Kinetin. The somatic embryo showed germination in basal medium and plants thus regenerated were transferred to pots. Histological studies confirmed the somatic embryogenesis.

Garla et al. in 2011 cultured nodal segment of *E. gerardiana* and reported callus formation followed by shoot bud production however, root induction or plantlet production was not achieved. Hegazi and El-Lamey (2011) studied ephedrine content in callus obtained from stem segment culture of *E. alata*. They reported excess ephedrine content in 2, 4-D and Kn derived callus as compared to stem of both wild & cultured plants.

Sharma et al. (2012) cultured nodal and internodal segments of *E. gerardiana* onto MS medium containing Thidiazuron. The internodal segments showed callusing followed by somatic embryogenesis onto lower concentration of TDZ. However, nodal segment exhibited direct and indirect shoot bud production onto different TDZ supplemented medium (Fig. 12.3a–i).

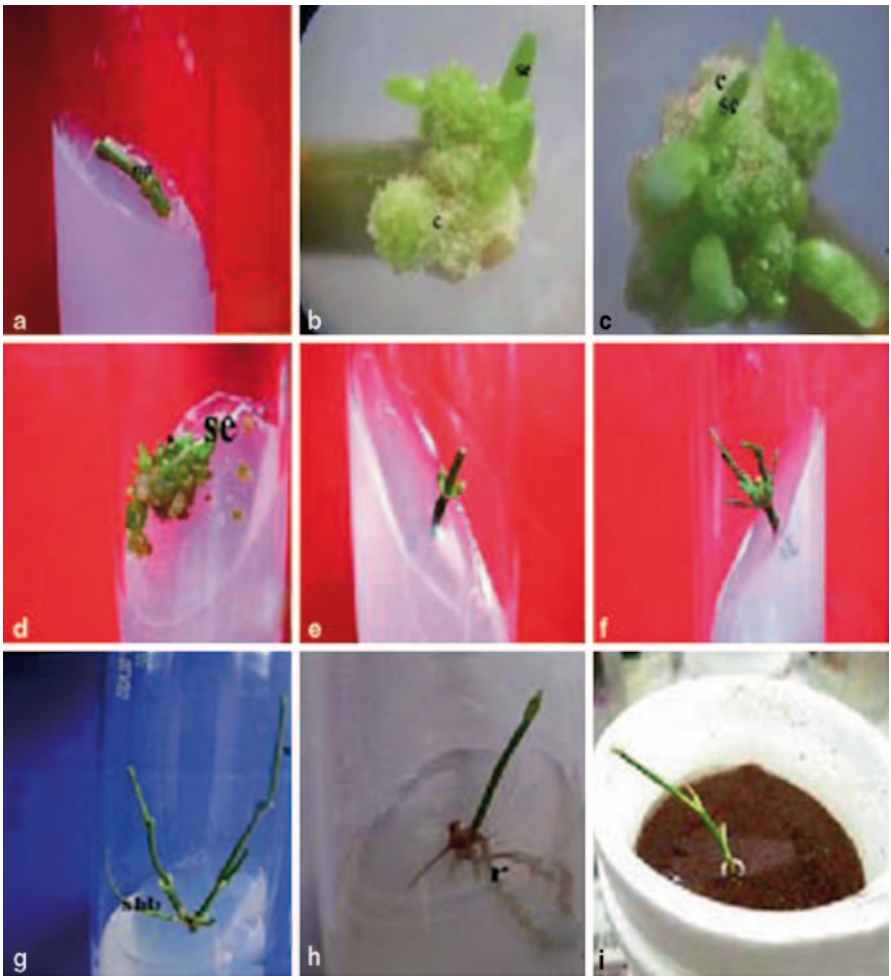

Fig. 12.3 Somatic embryogenesis, shoot bud formation and plant regeneration in *Ephedra gerardiana*. **a** Internodal explant showing callus initiation on to MS + 1 μM TDZ after 2 weeks of culture. **b**, **c** Embryoid formation from callus obtained on to MS + 0.5 μM TDZ after 4 weeks. **d** Somatic embryos obtained from same as c after 2 weeks of transfer onto basal medium. **e** Induction of shoot buds from nodal segment cultured on to MS + 0.5 μM TDZ after 1 week of culture. **f** Same as e, after 6 weeks. **g** Elongation of shoot on to MS after 3 weeks of transfer. **h** Rooting of shoots on to one fourth MS + 20 μM IBA after 2 weeks. **i** Transplanted plants in plastic pots. (From Sharma et al. 2012)

In vitro propagation protocol of female *E. foliata* has been proposed by Lodha et al. (2014). They reported shootbuds formation followed by *ex vitro* rooting using IBA treatment to the base of the microshoots. Plantlets thus produced were transferred to black polybags followed by nursery beds with a survival rate of 70% of acclimatized plants.

12.3.2 In Vitro *Culture Studies Using Embryonal/ Seedling Explants*

There are various reports of *in vitro* work on *Ephedra* using seedling explant. Callus induction from hypocotyls segments of *E. gerardiana* and subsequent differentiation of tissues was reported by Ramawat and Arya (1976). Green to yellowish brown callus was formed on MS medium containing Kn and NAA. Embryoids were produced on medium supplemented with Kn and NAA but they did not differentiated into plantlets.

Ramawat and Arya (1977, 1979d) studied the effect of various sugars and nitrogen sources on callus growth of *E. gerardiana* and *E. foliata*. Sucrose supported best callus growth in both the species followed by glucose, maltose and fructose. Various nitrogen sources such as ammonium nitrate, potassium nitrate, ammonium sulphate, calcium nitrate, ammonium citrate and urea were added to the medium. On a single source of nitrogen, callus failed to grow while on a mixture of nitrate and ammonium nitrogen, the callus growth in both the species was satisfactory. Protein content of *E. gerardiana* callus was nearly four times higher than that of *E. foliata*, irrespective of nitrogen source.

Ramawat and Arya (1979a, b, c) also studied the alkaloid contents and ephedrine production in callus culture of *Ephedra*. They found that the callus derived from *E. foliata* was devoid of alkaloid. However, 8 week-old callus of *E. gerardiana* yielded 0.17% alkaloid on MS + Kn + NAA. Moreover, there was an increase in ephedrine content (0.3%) in callus subculture on MS + Kn and IBA. They noticed a decline in ephedrine level (0.13%) when medium was supplemented with 2,4-D. They also observed the effect of some precursor amino acids (phenylalanine, methionine and glycine) on ephedrine production in *E. gerardiana* callus cultures and found that these amino acids increase the alkaloid contents as compared to control. Maximum yield (0.6%) was obtained from callus grown in a medium supplemented with 4 µM IBA and 0.1 g/l phenylalanine.

O'Dowd and Richardson (1993a) obtained adventitious shoot bud primordial formation from germinating seeds of *E. fragilis* onto MS medium supplemented with 2, 4-D and BAP. When these shoot bud primordia were transferred to medium containing 0.05 µM IBA and 0.05 µM Kn, only 5% grew into shoots. However, they could not obtain plantlets.

Dhiman et al. (2010) reported somatic embryogenesis (Fig. 12.4a–c) from half embryonal segments of *E. foliata* grown onto MS medium containing 2, 4-D and Kn/BAP. The somatic embryos germinated on the basal medium to form 'emblings'. The plants thus produced were transferred to pots containing sterilized mixture of coarse sand and garden soil (1:1). During the process of gradual hardening, nearly 70% plants survived (Fig. 12.5a–c).

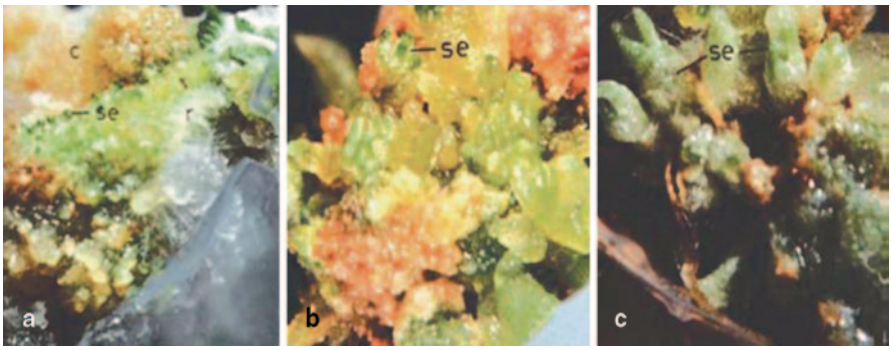

Fig. 12.4 **a** Induction of somatic embryos from half embryo explants of *Ephedra foliata* cultured onto BM+2 μM 2, 4-D+2 μM Kn after 5 weeks of culture. Roots and callus are also visible (×2.4). **b** Somatic embryos onto BM+5 μM 2, 4-D+5 μM BAP after 45 days of culture (×3.4). **c** Somatic embryos produced onto BM+5 μM 2, 4-D+8 μM BAP after 45 days of culture (×3.3). (c-callus, se-somatic embryos) (From Dhiman et al. 2010)

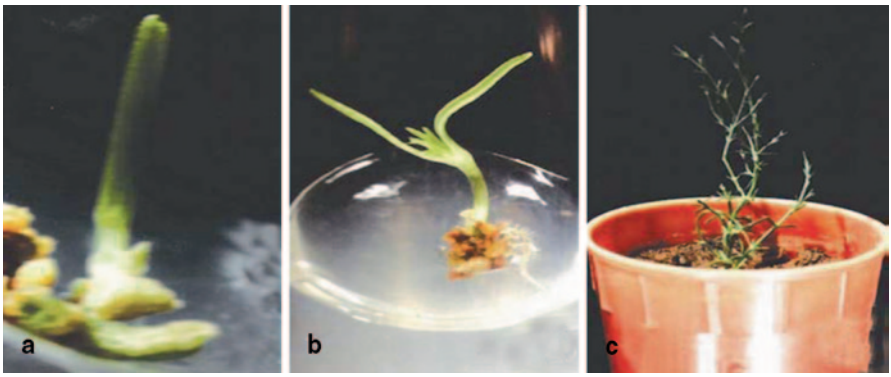

Fig. 12.5 **a**, **b** Different stages of germination of somatic embryos after 1 and 3 weeks of transfer, respectively onto BM (2A×2.7, 2B×2.0). C. Regenerated plant after 6 weeks of transplantation (×0.69). (From Dhiman et al. 2010)

12.3.3 In Vitro *Culture Studies Using Female Gametophyte as Explant*

Sankhla et al. (1967a) reported callus formation from the female gametophyte of *E. foliata* on White's basal medium containing 2,4-D and Kn. Callus could not be maintained and it did not undergo morphogenesis. A considerable amount of work has been done onto the regeneration potentialities of female gametophyte of *E. foliata* (Konar and Singh 1979; Singh et al. 1981; Singh and Konar 1981; Bhatnagar and Singh 1984). They obtained haploid callus, roots, shoot buds and plantlets from mature and immature female gametophytes cultured on MS medium with 2% sucrose and 10% CM. It was found that the age of the explant, culture conditions

and a subtle balance of auxins (2, 4-D and NAA) and cytokinins (Kn and BAP) could make the female gametophyte of *E. foliata* a plastic system for morphogenic potential in general and induction of haploid roots and shoots in particular. Female gametophytes at archegonial stage were more regenerative than at mature embryo stage in terms of percentage of root and shoot bud regeneration as well as maximum shoot production per explant. When 2, 4-D (9 µM) and Kn (9.3 µM) were added to the medium, the explant showed maximum percentage of shoot bud regeneration (75%). The regeneration of roots was dependent upon the presence of NAA (0.27–21.48 µM), while BAP (0.22–2.22 µM) enhanced the root promotion effect of NAA. However, at higher concentrations of BAP (4.4–26.8 µM), both roots and shoots were formed (Bhatnagar and Singh 1984).

12.3.4 Somatic Embryogenesis

The somatic embryogenesis in coniferous gymnosperms has opened up various vistas of plant improvement including large scale production of emblings for micropropagation, protoplast work, cryopreservation, artificial seed production and most important genetic transformation (Jain et al. 1995; Bhatnagar and Moitra 1996). A great deal of progress has been made in this regard for conifer reforestation (Tautorus et al. 1991; Gupta et al. 1993; Attree and Fowke 1993). Somatic embryogenesis and automated micropropagation have a vast potential for rapid multiplication of desired genotypes. Studies on somatic embryogenesis of non coniferous gymnosperms are highly limited. Dhiman et al. (2010) reported organogenesis and somatic embryogenesis from half embryonal segments of *E. foliata* grown onto MS medium containing 2,4-D and Kn/BAP. Somatic embryogenesis and shoot bud production from internodal explant of *E. foliata* cultured on MS medium containing various concentrations of 2,4-D and Kn/BAP was also reported by Dhiman and Sharma (2010). They observed direct and indirect somatic embryogenesis besides shoot bud production. The somatic embryos exhibit germination onto basal medium.

12.4 Conclusion

The cycads are trees resembling palms and have manoxylic wood which is of no commercial value. The antiquity of cycads is indicated by the presence of ciliate sperms and their restricted distribution. These plants usually propagate through seeds or asexually by adventitious shoots or “bulbils”. Nearly all cycads are slow growing and require a long period of growth before reaching the stage of reproduction vegetative by or sexual means. Aptly referred as “Living fossils” and “Dinosaurs of plant kingdom”, the cycads have survived up to the present age. The presence of the poisonous glycosides, cycasin and macrozamin (Tadera et al. 1985a; Yagi and Tadera 1987) in cycad tissues may be a potential deterrent to the

consumption of these plants and helped in the long term survival of this plant group (Fosberg 1964). Cycasin (Methylazoxymethanol glycoside) is known as a carcinogenic agent, neutroxic and some studies also report its interference with germination and growth of seeds (Kobayashi et al. 1980; Tadera et al. 1982). Cycasin is also reported to alter the *in vitro* growth and development of some molds as well as insect larvae (Kobayashi et al. 1980; Tadera et al. 1985b, 1987). Biosynthesis of cycasin in callus culture of *Cycas revoluta* has been reported by Tadera et al. (1995).

If cycasin is effective against microbes and insect, it could be screened as a potential tool for biological control. To obtain the chemical, either destruction of the plants would have to be carried out or the other option is it could be produced by mass scale callus cultures. The latter is dependent on techniques of tissue culture. Cycad tissue grown *in vitro* are extremely slow growing and mimic the slow growth in Nature.

Ephedra is one of the source of the alkaloids mainly l-ephedrine and d-pseudoephedrine. Callus culture can be possibly used for ephedrine biosynthesis in reactors. It may also be feasible to screen *Ephedra* populations and then clonally propagate the elite plants.

There are, successive losses in population number and changes in distribution ranges over a period of time, this has led to the listing of *Ephedra gerardiana*, a high alkaloid containing species as an endangered species. The plant is categorized as IV ranked plant of cold desert zone based upon knowledge, cultivation prospect and marketing. Therefore, micropropagation of *E. gerardiana* & re-introduction of elite germplasm into its natural habitat can be an aid for conservation of endangered medicinal germplasm.

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Chapter 13
Genetic Resources and Biodiversity Conservation in Nigeria Through Biotechnology Approaches

Justin U. Ogbu

Abstract The chapter presented a treatise on plant genetic resources (PGR) and biodiversity conservation in Nigeria *vis-a-vis* the relevance of biotechnological approaches. It showed synopsis of plant resources base of the world at global and Tropical African perspectives, and the shrinking diversity of present day agroecosystems. Attempts were made to review floristic and economic crops diversity of the country as well as the concerns for the current spiral depletion and loss of vital national plant genetic resources. Biotechnology has been recognized as a versatile tool for biodiversity conservation, management and use. It offers range of applications to improve the understanding and management of genetic resources for food and agriculture. It has been proven that modern biotechnologies can help to counteract trends of genetic erosion in all food and agriculture sectors. Biotechnology procedures in conservation and management of PGR were clearly discussed, while institutional framework for conservation of national PGR was highlighted.

Keywords Conservation biotechnology · Extinction · Genetic resources · Nigeria · Plant diversity

13.1 Introduction

Modern production and marketing of agricultural and horticultural crops depend to great extent on a limited number of almost genetically uniform varieties that deliver expected uniform food produce/products. With this prevailing approach becoming wide spread across various parts of the world, genetic diversity is endangered. The loss of genetic resources has resulted in major concerns about future food production and nutrition security. Thus, the vulnerability of present day convectional crop production systems towards pests, diseases and climate change makes the issues of genetic resources and biological diversity conservation very pertinent as well as urgent.

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13.2 Defining Terms and Scope in the Context

FAO defined biological diversity (commonly abbreviated as biodiversity) as the total variability within and among species of all living organisms—animals, plants and microorganisms—and their habitats (FAO/SDRR 2006). Biodiversity is usually described at three levels, namely, genetic, species and ecosystem diversity. Genetic diversity is the total genetic information contained in the genes of individual organism. This type of diversity can be characterized at the molecular, species, population or ecosystem level. Much attention has been paid to genetic diversity due to its direct applications in plant breeding programme and crop production, as well as for evolutionary studies (Purves et al. 2004). Species diversity describes the variety of living organisms. Such level of diversity is as a result of the relation between the species' richness and their relative abundance (that is, number of species and number of individuals of each species respectively) in a specified area (Ricklefs and Miller 1999). Lastly, ecosystem diversity relates to the numerous diversity of habitats and biotic communities as well as to the variety of ecological processes within ecosystems. Since biodiversity can occur at any level of classification, erosion can also happen at any level of classification. Such depletion or extinction at any given level of ecological organisation can fatally affect the quality and continuity of life at other levels, although the erosion of biodiversity at species level is the most commonly used method of assessing biodiversity loss. Species diversity in natural habitat is higher in warm and rainy zones (the tropics) and diminishes inversely with latitude and altitude across the earth. Thus, the richest zones of the world as per biodiversity are the tropical rain forests, which cover about 7 % of the earth surface (CBD 2008; Purves et al. 2004; FAO 2011a).

For FAO, genetic resources comprise of the genetic materials of plants, animals and other organisms that are of value for present and future generation of humans. Genetic resources for agriculture and food production are the basis of world food and nutritional security, and directly or indirectly support the livelihoods of every human on earth (FAO 2011a). Plant genetic resources (PGR) *per se*, refers to the genetic materials of plants, including modern cultivars, landraces and wild relatives of crop plants, of value as a resource for present and future generation of people (Fraleigh 2006; Singh et al. 2006). PGR have been the basis of genetic improvement of crop species in order to overcome challenges of crop production as well as to increase productivity and quality of crop yield. They provide insurance to meet future challenges posed by changes in the environment, diseases, new pest insurgence and marketing opportunities among others. Besides many crop cultivars have significant cultural and religious value for their holders in certain tribal communities. Therefore, PGR constitute a vital segment of earth biodiversity in general and agrobiodiversity in particular (Dhillon and Saxena 2003; LEISA 2004). As genetic resource, the PGR may be of reproductive or vegetative propagule such as embryos, seeds, shoots, tissues, cells, pollen, DNA molecule etc, containing the functional unit of heredity in addition to corresponding information and knowledge about their uses that can be applied in crop improvement programme and other product

development. The categories of PGR range from landraces and farmers' varieties, absolute cultivars, modern cultivars, breeding lines and genetic stocks, wild relative, weedy races and potential domesticate species, exotic and indigenous species (Engels and Visser 2006; Ruane and Sonnino 2006; Sharma 2007; FAO 2011a).

Biotechnology has been described (according to Convention on Biological Diversity—CBD) as any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use. Following FAO statement on biotechnology, a narrower sense of interpretation has also been added which described biotechnology to include other range of different molecular technologies such as gene manipulation and gene transfer, DNA typing and cloning of plants and animals (FAO/SDRR 2006; Speedy 2007). Biotechnology has been recognized as a veritable and vital tool for biodiversity conservation, management and use. It offers range of applications to improve the understanding and management of genetic resources for food and agriculture. It has been proven that modern biotechnologies can help to counteract trends of genetic erosion in all food and agriculture sectors (Ruane and Sonnino 2006).

13.3 Overview of Global and Tropical Africa Plant Resources

It has been reported that approximately 13,000,000–14,000,000 plant species have flourished on earth's biota; out of which 1,750,000 species have been somewhat described, while the number of higher plants worldwide were estimated to be 300,000–400,000 species (Dhillon and Saxena 2003; FAO 2011a). Similar reports by Engels and Visser (2006) and Spore (2010) indicated an approximate number of edible plant species to be 75,000; actual number of plant species used for food and agriculture to be 7000; whereas commercially important plant species exploited worldwide were placed at 150 species. According to Plant Resources of Tropical Africa (PROTA) annual report 25,000 plant species have been estimated to be used by mankind for sundry purposes in the tropics, out of which 7000 species are found in south east Asia, 11,000 species in the Latin America, while 7000 species are grown in the tropical Africa (PROTA 2005).

Like Asia and the Americas, the continent of Africa is blessed with a rich tropical flora. Many of the 5000 or so plants that evolved within Africa's forests, savannas and deserts yield valuable fruits, vegetables, spices and medicines for the people. Generally stating, Africa has as many of these plant resources home to its vast array of landscape just as tropical Asia or America (Grubben and Denton 2005; Van der Vossen and Mkamilo 2007). This fact, however, is something one would never guess by looking in produce markets or college textbooks on agriculture, horticulture and silviculture. Today, American, Asian and European crop species dominate tropical fruit and vegetable production worldwide, including within Africa itself. The reasons may not be far-fetched. Most Africa's native edible garden and orchard species have not, by and large, been brought up to their potential in terms of

quality, production, value addition and availability. Geographically speaking, few have moved beyond Africa's shores.

According to WCMC (2002) and CBD (2008), the Guinean forests of West Africa is one of the 25 biodiversity hotspots of the world designated by Conservation International, which included the belt of tropical moist broadleaf forests along the coast of West Africa, running from Sierra Leone and Guinea in the west to the Sanaga river of Cameroon in the east. The Dahomey Gap, a region of savanna and dry forest in Togo and Benin, divides the Guinean forests into the Upper Guinean forests and Lower Guinean forests. The Upper Guinean forests extend from Sierra Leone and Guinea in the west through Liberia, Cote d'Ivoire, and Ghana to Togo in the east. The Lower Guinean forests extend east from Benin through Nigeria and Cameroon. The Lower Guinean forests also extend south past the Sanaga River, the southern boundary of the hotspot, into southern Cameroon, Equatorial Guinea, Gabon, Republic of the Congo, Cabinda, and Democratic Republic of the Congo (Conservation International 2013).

13.4 Floristic and Economic Plant Diversity of Nigeria

Nigeria is one of the largest countries in West Africa, and has a land area of approximately 91.07 million ha. Nigeria's territorial area spanned from latitude 4°14'N to 13°48'N and from longitude 2°42'E to 14°40'E. The country is a physically, climatically and biologically diverse one. Essentially the country encompasses three major ecological regions, *viz*: a humid tropical forest region, a sub-humid region with highland and a semi-arid region; with annual rainfall ranging from 250 mm in the Sahelian north to over 3000 mm in the southern coastal areas. The country's climate is largely tropical humid, characterised by high humidity in south, high temperatures and intense heat in the north. In some areas north of the country (Kano, Kaduna, Bauchi, Plateau and other similar states), the harmattan wind from Saharan desert results in a mild cold dry winter, and thus permits the growth of winter crops such as wheat and cold loving vegetables crops during the cool harmattan period between December and February. The natural vegetation varies from rainforest to savanna. Nigeria is also endowed with substantial biological resources. These include 68 million ha of arable land (but barely 32 million ha are annually cultivated), and fresh water resources covering 12 million ha. Land use patterns in the country shows that cropland takes 34% of total land area, pasture takes 23%, forest 16%, rivers/lakes/reservoirs 13% and others 14% (Shaib et al. 1997; Akoroda 2010).

Reports by Federal Environmental Protection Agency (FEPA) (Adejuwon 2000), showed that the floristic diversity in Nigeria comprised of 4903 species of angiosperms, 32 species gymnosperms, 155 pteridophytes, 80 species bryophytes, 784 species algae, 3423 species fungi and more than 500 species virus. According to the reports also, 20 species of plants had become extinct since 1950, 431 species are endangered, 45 species are classified as rare, 20 species are vulnerable, while 305 species are endemic. All these are of PGR conservation concern to the country

considering the unprecedented rates of occurrence in comparison with normal natural history rates. The causes of these gradual but steady lost of native natural bioresources in general and PGR in particular have been identified to include overexploitation, massive deforestation and desertification, paucity of institutional frame work to engage in deliberate conservation of PGR of relevant to food, agriculture/forestry, inadvertent emphasis by most national agricultural research institutes on more introduced exotic crop species/varieties to the neglect of own useful indigenous plants among other factors.

Nigeria has large number of underdeveloped (but useful) indigenous plant resources spread across her diverse agroecosystems from the humid south to the Sahel savanna region of the north. In line with this assertion, Ndubizu (1990) stated that there were about 95 fruit species belonging to 32 botanical families which are home to the flora of Nigeria. This is beside the array of traditional vegetables, medicinal plants, spices and condiment plants, field crops and forest trees that are almost been overshadowed by exotic species commonly cultivated alongside with these local species (Okigbo 1980; Keay 1989; Etukudo 2000; Olowu and Atu 2001; Adebooye et al. 2003; Ogbu et al. 2007, 2011; Akoroda 2010). However, many of these local species have not been substantially developed up to commercial scale cultivation, exploitation and deliberate conservation. Undoubtedly, quite a few of them, if any, have been so developed to attract appreciable research attention and or industrial investment. Akoroda (2010) reported that out of the about 70 economic crop species grown across Nigerian arable lands, 35 food crop species account for most of the foodstuff that the people consume daily.

13.5 Aspects of Plant Diversity Depletion and Extinction

Over the several millennia of human existence on earth, Plant Genetic Resources (PGR) had constituted the basis of development and sustainability of agricultural production systems. After 10,000 years of sedentary agriculture and the discovery of about 75,000 varieties of edible plants, close to 7000 identified species have been used in agriculture for food and fodder (Dhillon and Saxena 2003). However, today, less than 2 % of these are recognized as economically relevant at regional, national or global levels (FAO 1996). Currently, only 30 cultivated plant species provide 90 % of all the human food obtained from plants, while 12 plant and 14 animal species together provide 70 % of the world human diet (Spore 2010). Three crops namely rice, wheat and maize, make up the basic food for two third of the world population (Jaramillo et al. 2011; Bioversity International 2012).

Extinction of genetic resources and PGR in particular, has been a naturally occurring phenomenon over millions of years, without any human involvement. However, due to unprecedented human activities in the past few scores of years and their effect on the environment, species and ecosystems have become increasingly threatened in an alarming way, thereby undermining the basis required for sustainable development. According to a recent CTA report on agrobiodiversity, 75 % of all

known crops have disappeared in the past century (Spore 2010). On the other hand, the United Nations FAO has projected that unless the spiral loss of genetic diversity is controlled, about 60,000 plant species (quarter of the world plant capital) might be lost by 2025 (WCMC 2002).

13.6 Institutional Framework for Conservation of National Plant Genetic Resources

Plant genetic resources systems typically comprised of acquisition, maintenance, characterisation and evaluation of genetic resources; in addition to deliberate strategic conservation and documentation of the viability and genetic integrity of such materials in order to facilitate their use by providing access to samples of the materials and associated information. The collection, characterization, evaluation, conservation and use of PGR are enormous tasks especially in a country like Nigeria, which has a vast heritage of these bioresources. A large number of agronomic, horticultural and plantation plants are grown in the country due to different agro-climatic factors, socioeconomic cultural needs. There is a wide diversity in these crops with respect to mode of reproduction (seed vs. vegetative propagation), seed storage behaviour (orthodox vs. recalcitrant), growth habit (annual vs. perennial), adaptation, uses (as staple food crops, fruit, vegetables, ornamentals, plantation crops, medicinal and aromatic plants), agro-technology and commercial value (like cash crops, staple food crop, minor and under-utilized).

Keeping in view the enormity and diversity of the task involved, networking approach is essentially required among the relevant National Agricultural Research System (NARS) with plant based research and development mandates in the country (Table 13.1). Such network should include National Horticultural Research Institute—NIHORT, National Root Crops Research Institute—NRCRI [cocoyam,

Table 13.1 Some National Agricultural Research Institutes with plant based mandates and similar institutions relevant to PGR conservation

NIFOR—Nigerian Institute for Oil Palm Research, Benin City
NIHORT—Nigerian Institute of Horticultural Research, Ibadan
NRCRI—National Root Crops Research Institute, Umudike
NCCRI—National Cereal Crops Research Institute, Badegi
CRIN—Cocoa Research Institute of Nigeria, Ibadan
FRIN—Forestry Research Institute of Nigeria, Ibadan
IAR—Institute of Agricultural Research, Samarru Zaria
IART—Institute of Agricultural Research and Training, Ibadan
RRIN—Rubber Research Institute of Nigeria, Benin City
NACGRAB—National Centre for Genetic Resources and Biotechnology, Ibadan
NABDA—National Biotechnology Development Agency, Abuja
NPQS—Nigerian Plant Quarantine Services, Moor Plantation Ibadan
NISLT—Nigerian Institute of Science Laboratory Technology, Ibadan

yam, potato, sweet potato, ginger, turmeric], Nigeria Institute for Oil Palm Research—NIFOR [coconut, oil palm, date, ornamental palms], National Centre for Genetic Resources and Biotechnology (NACGRAB), Forestry Research Institute of Nigeria (FRIN) [for inputs on conservation/domestication of certain important indigenous fruit trees, aromatic and medicinal plants, as well as the forest trees] and other organization that share similar interest in PGR and biodiversity management.

With reference to organisations in the Nigeria that have plant biotechnology activities (including conservation biotechnology of plant genetic resources), there are several of them ranging from government owned, CGIAR owned and privately owned institutions (Table 13.2).

13.7 Biotechnology Strategies for Conservation of Plant Genetic Resources

It been advocated by experts that the best conservation approach to conservation of PGR is an integrated one which combines conventional *in situ* and *ex situ* approaches with the modern biotechnological application to achieve a holistic management of the PGR. The concept of integrated conservation of plant species is described by Falk (1987) and Oldfield and Newton (2012), who noted the need for multiple conservation approaches to be employed. Given the variety and complexity of threats to biodiversity, a single approach, such as seed bank, legal protection for species or the acquisition of land for field genebank, is unlikely to be successful. Essentially, modern conservation biotechnology approach is an off shoot of *ex situ* conservation techniques, although it is presently been given special detail attention because of its versatility, economy of space and wide range applications. However, it should be stated at this point that conservation biotechnology should not be seen as a substitute to conventional conservation techniques, but a complementary tool.

Conservation biotechnology studies the use and management of biodiversity present in natural and manipulated ecosystems (including agroecosystem) by biotechnological applications in order to guarantee their renewal, conservation and productivity, thereby providing benefits and opportunities for present generation and posterity. There are wide range of technologies offered in conservation biotechnology for use in plant genetic resources and biodiversity management for food and agriculture (Mandal 2003; FAO 2011b). These included *in vitro* conservation, cryopreservation, DNA banking, micropropagation and molecular marker technology (Fig. 13.1). The various biotechnology tools can be used to achieve plant genetic resources objectives such as conservation and clonal multiplication, characterisation and identification, as well as evaluation, selection, enhancement and distribution among others (Table 13.3).

Table 13.2 Institutions involved in plant genetic resources biotechnology in Nigeria

Institutions/funding agent	Main activities
International Institute of Tropical Agriculture (IITA)—Tissue Culture Laboratory: CGIAR funded	Conservation, micropropagation and distribution of banana/plantain, cassava, and yam; training
National Centre for Genetic Resources and Biotechnology (NACGRAB): Federal Government	Research, training and consultancy on PGR and their *in vitro* multiplication and conservation
National Root Crops Research Institute (NRCRI)—Plant Tissue Culture and Molecular Biology Laboratory: Federal Government	Micropropagation of root and tuber crops; training and extensions
Nigerian Institute of Horticultural Research (NIHORT)—Tissue Culture Laboratory: Federal Government	Micropropagation and distribution of improved horticultural crops planting materials
Institute of Agricultural Research and Training (IAR&T)—Tissue Culture Laboratory: Federal Government	Tissue culture of some crops including cassava, fluted pumpkin and yams
Forestry Research Institute of Nigeria (FRIN)—Biotechnology unit: Federal Government	Development and distribution of improved vegetatively propagated cuttings of economic forest species
Univeristy of Ibadan (UI)—Department of Agronomy: Federal Government	Protocol development for *in vitro* propagation of selected crops
Nigerian Institute for Oil Palm Research (NIFOR)—Tissue Culture Laboratory: Federal Government	Development of improved planting materials of mandate research crops and provision of extension and consultant services
University of Jos (UJ)—Tissue Culture Laboratory—Federal Government	Development of protocol for *in vitro* propagation of selected crops
Ahmadu Bello University (ABU)—Biotechnology Centre: Federal Government	Protocol development for *in vitro* propagation of selected crops
Sheda Science and Technology Complex (SHESTCO)—Biotechnology Advanced Laboratory: Federal Government and International Donor agencies	Development of protocols for genetic transformation of some indigenous plants; Micropropagation and distribution of acacias banana/plantain, cassava, pineapple
Jigawa Research Institute (JRI)—Tissue Culture Laboratory: State Government	Production and distribution of improved planting materials of banana/plantain, cactus, date palm, pineapple and sugarcane; Agricultural extension services
Biocrops Biotechnology Limited (BIOCROPS): Private funding	Contract micropropagation of planting materials of plantation crops
Molecular Bioscience Limited (BIOSCIENCE): Private funding	Micropropagation of plantation crops and medicinal plants
University of Nigeria, Nsukka (UNN)—Tissue Culture Laboratory: Federal Government	Protocol development for *in vitro* propagation of selected crops
University of Maiduguri (UNIMAID): Federal Government	As above
Univeristy of Port Harcourt (UNIPORT): Federal Government	As above

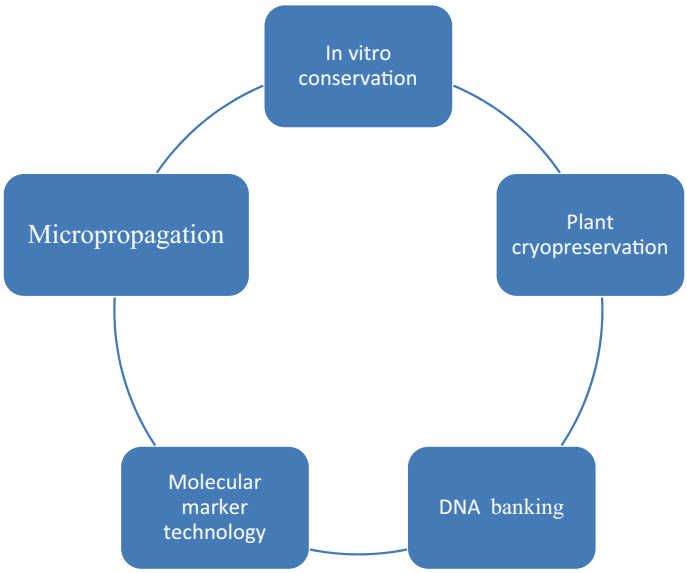

Fig. 13.1 Biotechnology tools for conservation and use of PGR

Table 13.3 Biotechnology strategies for conservation and management of PGR

Targets	Applications	Relevant techniques
Characterisation/ identification	Protein analysis, DNA analysis, diversity assessment	Genetic fingerprinting, isozymes, AFLP, RAPD, RFPD, SSR, etc.
Conservation/ multiplication	Clonal multiplication, artificial seed production, distribution of materials, exchange of materials, plant health screening	Cryopreservation, DNA genebank, in vitro conservation, micropropagation, virus indexing
Evaluation/ enhancement	Genetic marker, genetic drift	Another culture, embryo rescue, genetic map, QTL mapping

13.7.1 In Vitro *Conservation and Cryopreservation Techniques*

Efficient conservation of genetic resources in case of vegetatively propagated plants and recalcitrant seed species has been hampered due to problems faced during application of their conventional method of *ex situ* conservation in field genebank. To tackle these challenges, *in vitro* techniques have been increasingly used for conservation and its related activities like collecting and exchange of germplasm of these problem species. *In vitro* meristem culture technique offers the possibility of eliminating viruses and thus, exchange of virus-free germplasm. *In vitro* slow/ normal growth techniques offer up to medium-term storage option, avoiding risk of losses of germplasm on field genebank due to insects, nematodes, disease attacks

and natural disasters. It is commonly use for vegetatively propagated species, non-orthodox seeded species and wild species which produce little or no seeds.

While cryopreservation at ultra-low temperature, usually that of liquid nitrogen (−196 °C), is the only option currently available for the long-term conservation of these PGR avoiding exogenous contamination, requiring small space and minimum maintenance. At this very low temperature, all metabolic activities of cell cease, and theoretically the cell or tissue can be stored for an indefinitely period. Both *in vitro* conservation and cryopreservation techniques use tissue culture principles for conservation (Roca et al. 1989; Reed 1993; Mandal 2003).

The realization of the potential of *in vitro* conservation came about in the early 1970s, at a time when the storage of microbial cultures was a routine procedure. Since then, tissue culture techniques have been applied to more than 1000 plant species. Subsequently, the technique has progressed from mere speculation to development, and today it is routinely being used for conservation of vegetatively propagated crops and perennial species (Tyagi and Yusuf 2003). The art and science of plant tissue culture is based on devising media for each genotype/species that would elicit the optimal response in terms of growth rate of the explants. However, when tissue techniques are employed for conservation, the aim is to devise a medium that would decrease the growth rate of explants to the minimum, thereby increasing the subculture intervals. Slow growth techniques have been developed for medium-term conservation of crop species (Engelmann and Drew 1998; Sarkar and Naik 1999). The various methods used to achieve this include the following: use of growth retardants, use of minimal growth media, use of osmotic regulators, reduction in oxygen concentration, size and type of culture vessels, type of enclosures, maintenance under reduced temperature and for reduced light intensity and combination of more than one treatment. Explants used for *in vitro* conservation must be of right type as well as physiological stage. The apical and auxiliary meristems of very small size are the preferred explants for *in vitro* storage. In fact, organized explants have proved better than unorganized tissues, in terms of genetic stability of the germplasm (Mandal 2003; Reed et al. 2004; Chaudhury and Vasil 1993).

13.7.2 Cryopreservation

Cryopreservation refers to the non-lethal storage of biological tissues at ultra-low temperature, usually that of liquid nitrogen (LN) which is −196 °C. Currently, it is the only option available for the long-term conservation of germplasm of vegetatively propagated and recalcitrant seed species. Due to storage at the temperature of the vapor phase (−150 to −180 °C) or liquid phase (−196 °C) of LN, cell divisions and metabolic activities are arrested and thus, plant material can be stored for unlimited periods of time. Conservation of germplasm using cryogenic approach required very limited space; the plant material stored is protected from exogenous contamination and needs very limited maintenance. It causes no change in viability, vigor and genetic make up of the conserved materials. It also eliminates the need

to test stored materials frequently, thus making storage cost-effective. There have been several reviews on the use of cryopreservation for storage of plant materials (Kartha and Engelmann 1994; Engelmann and Takagi 2000; Towill and Bajaj 2002; Chaudhury 2002; Mandal 2003; Reed et al. 2004). The choice of material for cryogenic storage will depend on the plant species as well as the objectives of storage. For conservation of PGR, the explants can include shoot apices, auxiliary buds, dormant buds, somatic embryos, seeds, zygotic embryos, embryonic axes or pollens. Cryopreserved explants (but pollen) should eventually regenerate whole plants to be used and therefore, regeneration protocols need to be clearly defined prior to embarking on cryopreservation. Regenerated plants should also maintain genetic integrity of the starting material. The various techniques currently under investigation or in use include: the classical freezing method, encapsulation-dehydration, vitrification, encapsulation–vitrification, desiccation, pre-growth droplet freezing, and pre-growth desiccation (Mandal 2003; Panis 2007).

13.7.3 DNA Bank/Library

DNA storage is a relatively new technique that is rapidly gaining recognition. Several DNA libraries are being established which provide an easy access for scientists. DNA from the nucleus, mitochondria and chloroplasts are now routinely extracted and immobilized into nitrocellulose sheets where the DNA can be probed with numerous cloned genes. With the development of PCR (polymerase chain reaction), one can now routinely amplify specific genes or oligonucleolides from the entire mixture of genomic DNA. This approach, according to Engelmann et al. (2003), is relatively easy and of low cost. It is particularly useful for the conservation of specific genes and it allows easy access to specific material. The exchange of germplasm through DNA sequences is safe since infestations with pathogens can be simply avoided. Note however, that entire plant cannot be regenerated from conserved DNA (Reed et al. 2004; Guimaraes et al. 2007).

13.7.4 Micropropagation

Micropropagation is a popular technique applied majorly in the regeneration and or clonal multiplication of plant materials under aseptic and controlled environment using miniature plant tissues. This technique is already used in the commercial production of improved virus free planting materials of some economically important crops for distribution to farmers in many parts of the world. It has also been used to rescue endangered rare plant species from extinction due to over exploitation, poor natural regeneration and unfavorable environmental conditions. Clonal multiplication is a pre-requisite for conserving germplasm *in vitro*. The benefits of micropropagation in relation to conventional propagation methods include: multiplication of unique/difficult-to-maintain plant genotypes, potentially unlimited multiplication

of selected plant species line, elimination of pathogens from seedlings, lesser requirement of space, time and labour, season-independent, ease of transportation, ease of exchange of plant germplasm and less quarantine restrictions. Various approaches used in this biotechnology include but not limited to these only: *in vitro* micrografting, artificial seed production, somatic embryogenesis, adventitious regeneration, single node culture, meristems culture, anther culture, and axillary branching (Speedy 2007; Sonnino et al. 2009).

13.7.5 Molecular Markers

Molecular markers biotechnology comprises the use of identifiable DNA sequences found at specific location of the genome and transmitted by Mendelian laws of inheritance from one generation to another. The technology relies mostly on DNA assay, and there are various kinds of molecular marker systems available for use at present, such as amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphism (RFLP), microsatellites and single nucleotide polymorphisms (SNPs). The different marker systems may vary in aspects, for instance in their sophistication and basic technical requirements, the amount of labour, money and time needed, as well as the number of genetic markers that can be detected throughout the genome. FAO in Guimaraes et al. (2007) has fairly treated in detail the molecular marker biotechnology currently used in PGR as well as livestock and fisheries genetic resources conservation, management and exploitation.

Molecular markers are very versatile and can be used to achieve a variety of PGR purposes. Thus, they are used to characterised and conserve genetic resources, for example in the estimation of genetic relationships between populations within a species, measuring genetic diversity of accessions, verifying genetic identity of individuals among provenances; to identify duplicate accessions in crop genebanks; to carry out biological studies of pollen movement and seed dispersal in forest tree populations. They are also used in disease diagnosis to characterise and detect pathogens in crops and forest trees (Ruane and Sonnino 2006; FAO 2011b). Moreover they are used widely in modern crop breeding programme through the so-called marker-assisted selection (MAS).

13.8 Prospects and Challenges

Conservation and management of PGR for food and agricultural production via biotechnology procedure in developing countries including, Nigeria, at present prove to be a knotty issue among the policy makers and ruling class as the immediate economic gain for such venture is not always readily felt. Often times the hype and hot debate about genetically modified (GM) crops/food have tend to becloud the

potential rich benefits from non-transgenic biotechnologies that can be exploited to boost food production, enhance nutritional security as well as conserve biodiversity without compromising wholesomeness of the environment now and in the future. Granted that the initial capital outlays for acquiring and establishing functional plant biotechnology laboratory with full complement of personnel and materials resources for conservation of PGR may be rather enormous, in the long run such investment mostly pays off. Moreover in view of the vagaries of climate change phenomenon and human induced environmental degradation, *vis a vis* the unprecedented negative impacts on biodiversity in general and PGR in particular, the need for biotechnology approach to conservation of biological resources cannot be regarded as optional or expensive. Already few institutions in the country (as earlier mentioned) are engaged in some conservation biotechnological activities of certain economic crops like banana/plantain, cassava, ginger, yams and others. However, there is need for the institutions to expand their scope to accommodate more indigenous plant species which are actually cherished by the local people, although such plants are not commercially grown in large scale production systems. The issue of biodiversity is essentially summed up thus: value it, conserve and use it, or regard it as common and loss it for good.

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Chapter 14
Biotechnology Tools for Conservation of the Biodiversity of European and Mediterranean *Abies* Species

Jana Krajňáková, Dušan Gömöry and Hely Häggman

Abstract The review underlines the importance of European and Mediterranean firs (*Abies* sp.) in European forests, their geographical distribution, ecological and economical values. The present status of endangerment is given as well as the importance of genetic conservation of these species is illustrated by results from population genetics studies. Moreover, the current status of *in situ* and *ex situ* conservation methods is discussed and a special attention is paid to the role of biotechnological methods (*in vitro* regeneration system and cryopreservation) in their *ex situ* conservation. Among *in vitro* methods till now, only somatic embryogenesis proved to be promising and five species (*A. alba*, *A. cephalonica*, *A. cilicica*, *A. nordmanniana*, *A. numidica* and several hybrids) were regenerated. Based on the success of regeneration method, the slow cooling cryopreservation protocols for three *Abies* species (*A. alba*, *A. cephalonica*, *A. nordmanianna*) and their hybrids were developed. The biotechnology approaches have confirmed their place in the toolbox of conservation methods of firs. Transfer of the experience gained in widespread species and development of reliable procedures for somatic embryogenesis and cryopreservation for the endemics remain tasks for the future.

Keywords Gene pools · Ex situ conservation · Cryopreservation · Somatic embryogenesis · Genetic fidelity · Greek fir · Silver fir

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14.1 Introduction

According to the United Nations Food and Agriculture Organization (FAO 2013), the world forest area is slightly more than 4 billion ha and its importance as a carbon sink is enormous. In Europe, forests represent almost half of the land surface (102 million ha, which amount to 25 % of the world total), of which 65 % are conifers. Over the last 20 years, the forest area has expanded in all European regions and has gained 0.8 million ha in each year (Forest Europe 2011). European forests sequester increasing amounts of carbon in tree biomass, between 2005 and 2010, about 870 million t of CO_2 have been removed annually from the atmosphere by photosynthesis and tree biomass growth in European countries. This corresponds to about 10 % of the greenhouse gas emissions in 2008 of these countries (Forest Europe 2011). Moreover, increasing population numbers in combination with accelerated climate change including weather extremes (Nellemann et al. 2009) are predicted to increase the need for more wood production. Wood is the world's only large scale renewable, sustainable and environmentally friendly raw material and more systematic use of its potential needs to be made at the global level, if the aim of achieving true sustainability for the world is to be met (Sutton 2013).

In forested landscapes, trees play also essential roles in ecosystem structure and functioning. They mediate energy and material flows and are associated with processes such as water and nutrient cycling, biomass production, soil formation etc. Genetic diversity, which is closely associated with adaptability and population stability, is an inevitable prerequisite for fulfilling these functions (Pimm 1984; Johnson et al. 1996; Lefèvre et al. 2013). In spite of positive data about the increasing forest area in Europe, about a fifth of all trees are damaged or dead and 11 million ha (or 1 %) of Europe's forests are affected by forest damage, most frequently caused by insects and diseases, followed by wildlife and grazing (Forest Europe 2011).

Currently, the IUCN Red list includes 6277 tree species that are threatened with extinction in the wild (http://www.iucnredlist.org). Of these, 1002 tree species are recorded as Critically Endangered, the most threatened category for species based on the risk of extinction (Oldfield 2009) indicating an urgent need for germplasm conservation. Fulfilling the commitments adopted within the Global Strategy for Plant Conservation (Convention on Biological Diversity 2010), especially preservation and sustainable use of genetic resources, requires elaboration and application of a wide spectrum of tools for *in situ* and *ex situ* conservation. Biotechnological approaches can substantially contribute to the success of such efforts.

14.2 European and Mediterranean *Abies* Species

14.2.1 Biology, Ecology and Geographical Distribution of European and Mediterranean Firs

Euro-Mediterranean firs (the genus *Abies* Mill.) belong to ecologically and commercially most important tree genera in Europe (Table 14.1). Fir forests represent a major component of Central European, Alpine and Mediterranean mountain forests. Their distribution ranges from 6°W to 44°E in longitude, from 35°N to 52°N in latitude and from 135 to 2900 m in altitude (Alizoti et al. 2011) (Fig. 14.1 *Abies alba*, Fig. 14.2 Mediterranean fir species).

Like in the other tree species in Europe, the history of firs has been turbulent and left profound traces in their species diversity and genetic structures. Glacial/interglacial climatic cycles during the Pleistocene provoked large retreats and expansions of species' ranges. Mediterranean Sea bordering Europe from the south largely prevented southward migration; this obstacle drove several tree genera to local extinction (e.g., *Pseudotsuga, Cryptomeria, Sequoia, Taxodium*; Martinetto 2001; Svenning 2003). At the species level, the consequences are manifested in reduced species diversity. Only four fir species have survived in Europe until recent times (*A. alba, A. cephalonica, A. pinsapo*, and *A. nebrodensis*).

Greek fir (*Abies cephalonica* Loudon) is endemic to Greece, where it grows between 400 and 1800 (2000) m a.s.l. on a variety of parent rocks such as limestones, dolomites, serpentines, sandstones, and schist with soil pH ranging from 5 to 8 (Panetsos 1975). At present, the population of Greek fir is considered stable. On the other hand, the remaining two fir species are truly rare. Spanish fir (*A. pinsapo* Boiss.) range covers only 1200 ha in southwestern Spain (Arista 1995), on dolomitic and serpentine soils at elevations between 1000 and 1600 m. Its population decreases. Climate change associated with increasing incidence of wildfires, pests and diseases might under circumstances drive the species to extinction. The single existing natural population of the Sicilian fir (*A. nebrodensis* Mattei) is extremely small, consisting of 29 adult trees only (Alizoti et al. 2011), and grows on a single limestone site in Sicily at elevations around 1500 m. Although population size is stable and genetic diversity is surprisingly high, the species is logically considered critically endangered.

Abies alba Mill., silver fir, is the only widespread and abundant species of the genus *Abies* in Europe. Longitudinally, the range spans between the Central Massive in France and the Eastern Carpathians in Romania. Isolated occurrences can be found even more westwards, in the Pyrenees and Normandy. Latitudinally, silver fir is distributed between the Dinaric Mountains and central Poland. Again, isolated

Table 14.1 List of the European and Mediterranean *Abies* species, threats to genetic diversity and information about in situ and ex situ conservation

Scientific name	Common name	Category according to IUCN Red List of threatened species	*In situ* conservation stands[a]	*Ex situ* conservation	
				Stands/seed orchard	Tissue culture system
Section *Abies*					
A. alba Mill.	Silver fir	Least concern	36.315 ha[b]	Conservation stands 307 ha	Yes, SE
A. nebrodensis (Lojac) Mattei	Sicilian fir	Critically endangered	–	One seed orchard	No
A. cephalonica Loudon	Greek fir	Least concern	1.210 ha[b]	Conservation stands 6 ha	Yes, SE
A. borisii-regis Mattf.	Bulgarian fir	Least concern	456 ha[b]	–	No
A. nordmanniana (Steven) Spach.	Nordmann fir, Caucasian fir	Least concern	unknown	–	Yes, SE
A. bornmuelleriana Mattf. (*A. nordmanniana* ssp. *bornmuelleriana*)	Bithynian fir	Endangered	213 ha[b]	–	No
A. equi-trojani Coode and Cullen (*A. nordmanniana* ssp. *equi-trojani*)	Turkish fir, Kazdaghi fir	Endangered	293 ha[b] 24.374 ha[c]	–	No
Section *Piceaster*					
A. pinsapo Boiss.	Spanish fir	Endangered	100 ha[b]	–	No
A. marocana Trabut (*A. pinsapo* ssp. *marocana*)	Moroccan fir	Critically endangered	–	Seven ex situ stands	No
A. cilicica (Ant. and Kotschy) Carrière	Taurus fir, Cilicia fir	Near threatened	69 ha[b]	–	Yes, SE
A. numidica de Lannoy ex Carrière	Algerian Fir	Critically endangered	–	–	Yes, SE

[a] Specific conservation measures beyond nature conservation[b] Dynamic gene conservation units fulfilling the minimum criteria of Euforgen (http://portal.eufgis.org)[c] Multispecies Gene Management Zones (Ozturk et al. 2010)

populations are scattered along the northeastern range limit (Poland, Ukraine) and the southern part of the range (Apennine and Balkan peninsulas) is highly fragmented (Wolf 2003).

Silver fir forms pure stands, but more frequently it can be found in mixed stands with European beech and Norway spruce, in the south with pines and oaks. It toler-

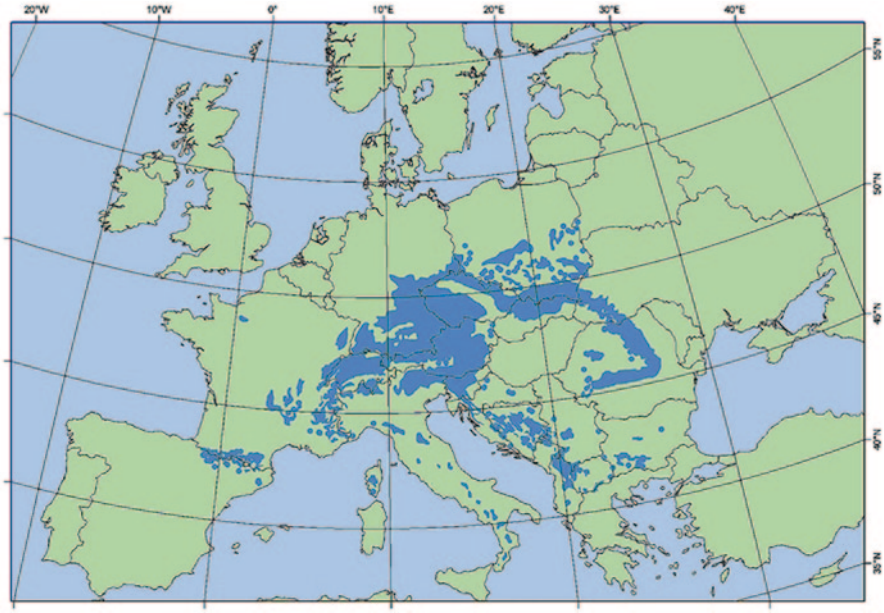

Fig. 14.1 Distribution map of silver fir (*Abies alba*). EUFORGEN 2009. (http://www.euforgen.org)

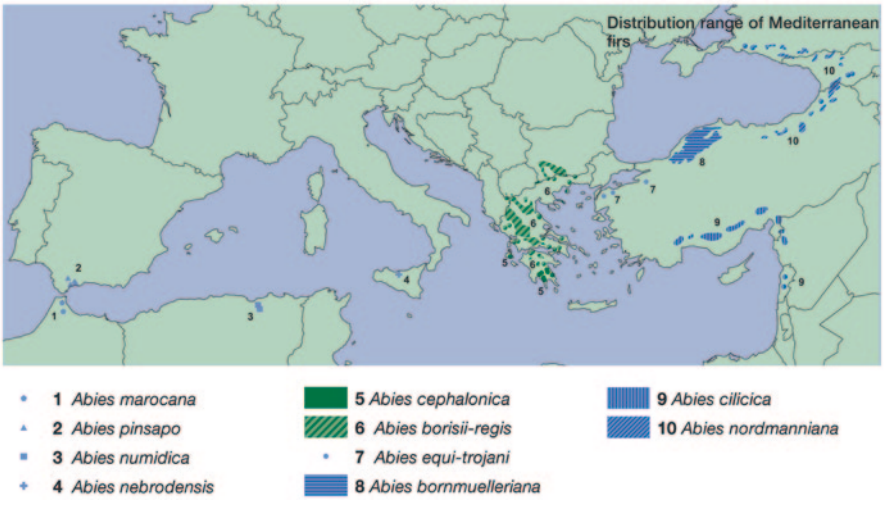

Fig. 14.2 Distribution range of Mediterranean firs. (Alizoti et al. 2011)

ates a wide range of soil conditions. Consequently, it can be found over a variety of parent rocks, covered by soils with varying textures, nutrient levels and pH, avoiding both waterlogged and dry soils. Nevertheless, the best growth and competition ability of silver fir can be expected on deep, nutrient-rich, fine- to medium-textured

and well-drained soils. Climatic niche of silver fir is also broad. The species is cold-hardy, but sensitive to winter desiccation, late and early frosts, and water deficit during shoot elongation (Hansen and Larsen 2004). Silver fir is very shade tolerant, especially in young age. Although it is generally considered a typical climax species, silver fir is able to colonize pioneer pine forests and even open lands.

In addition to Europe, other fir species occur around the Mediterranean. *A. nordmanniana* Spach is distributed in eastern Turkey and the Caucasus. In spite of a fragmented range its population is stable and not endangered. Two subspecies, *A. equi trojani* Coode and Cullen and *A. bornmuelleriana* Mattf. (sometimes considered separate species or, alternatively, hybrids *A. nordmanniana* × *A. cephalonica*), grow in western and northern Turkey, respectively, the former having a very limited area of occupancy of 164 km^2. *A. cilicica* de Lannoy occurs in the Turkish Taurus Mts., Syria and Lebanon on an area of almost 3400 km^2. Although its range is not small, population size decreases and especially Syrian and Lebanonian local populations are threatened. Both African fir species, *Abies numidica* Carrière (Kabylian Mts. in Algeria), and *A. marocana* Trabut (sometimes considered a subspecies of *A. pinsapo*; Rif Mts. in Morocco) have extremely small areas of occupancy (1 and 28 km^2, respectively), and are critically endangered.

14.2.2 Economical Importance and Use of Firs

The interest of foresters, nature conservationists, landscape ecologists etc. in *Abies* species is driven mainly by the commercial and ecological importance of the genus. Silver fir is the most productive native tree species of European forests. Although the maximum dimensions do not reach those of its North American counterparts, they are still impressive—the maximum height was recorded in the Peručica virgin forest in Bosnia and amounted 65 m (Leibundgut 1976). However, heights over 60 m were measured in several reserves over East Europe—Mionší, Biogradsko jezero, Žofín, Dobroč and elsewhere (Holeksa et al. 2009). Fir also contributes to ecological stabilization of forest communities, as it possesses a better stability against wind throw and is more resistant to fungal pathogens than, e.g., Norway spruce (Hansen and Larsen 2004).

Fir species are of high economic importance both for timber (construction wood, furniture, pulp production, fuel wood etc.) and for non-wood forest products (turpentine and Christmas trees). The bark, buds and cones may contain a large amount of fine, highly resinous turpentine. Fresh oleoresin is mainly used for pharmaceutical purposes.

Because of their fragrance, colour, good form and exceptionally long leaf retention after being cut, most of the firs are used as ornamental trees and are grown in plantations for Christmas trees (e.g., *A. borisii-regis, A. cephalonica* and *A. nordmanniana*).

This is true also for hybrids—the genus *Abies* was object of intensive hybridization studies, and several artificial hybrids, including *A. alba* × *A. cephalonica* were found promising and exceeded pure species in growth (Kormuťák and Vooková

2001; Kobliha et al. 2013). They have thus a potential also for forestry, but their primary field of use is greenery and Christmas tree production.

14.2.3 History and Genetic Variation of Mediterranean Firs

Genetic structures of the extant fir populations in Europe have largely been determined by historical factors. As mentioned above, Pleistocene climatic fluctuations severely reduced population sizes of all temperate species. Refugial population of rare fir species (*A. pinsapo, A. nebrodensis*) did not expand; either due to decreased vitality caused by inbreeding and lowered genetic variation, or because they remained trapped in islands of favorable environments surrounded by dry highlands or by sea. Almost nothing is known about the population development of fir species South and East of the Mediterranean Sea in the postglacial period; nevertheless, these regions have been less influenced by the glaciation, so that local fir populations may have persisted since the Tertiary. Holocene warming may, however, have contributed to the contraction of ranges of *A. numidica, A. cilicica* or *A. marocana* and fragmentation of *A. nordmanniana*. For *A. cephalonica*, Fady and Conkle (1993) concluded that the divergence between *A. alba* and this species occurred quite recently, at the beginning of the last glaciation. The reconstruction of the Holocene history of *A. cephalonica* is difficult because the pollen of different *Abies* species cannot be distinguished in the fossil pollen record (Terhürne-Berson et al. 2004). Nevertheless, as the range of *A. cephalonica* is located in southern Balkans, which served as an important refugial area during the Holocene, population sizes, distribution and genetic structures of this species probably have not changed substantially.

The history of *A. alba* is more complicated, as this species recurrently succeeded to colonize Europe during the warm phases of the Pleistocene, and during the Eemian interglacial it even covered larger area than the current range (Terhürne-Berson et al. 2004). Pollen and macrofossils (mainly charcoal) documented that cryptic Pleniglacial refugia of silver fir were localized as far north as in Hungary or Moravia (Willis et al. 2000; Terhürne-Berson et al. 2004). Nevertheless, main refugial areas were situated more in the south. The analysis of maternally inherited mitochondrial DNA revealed two genetic lineages of silver fir, one distributed in western and central Europe, the other in southern Balkans and Eastern Carpathians (Liepelt et al. 2002). A synthesis of paleobotanical and genetic data by Liepelt et al. (2009) suggested that the effective refugia for the western lineage could have been localized in northern Apennines and possibly Maritime Alps, those for the eastern lineage in southeastern Balkans. Nevertheless, some regional silver fir populations have originated from local minor refugia, e.g. those in the Pyrenees or southern Italy.

Not much information is available about the past of *Abies* species in Asia Minor and Africa. Genetic diversity of conifers in the Mediterranean is relatively high compared with other regions of the world (Fady-Welterlen 2005). The rear-edge

populations are frequently highly differentiated and contain many private alleles (Petit et al. 2005; Awad et al. 2014). Most rear-edge populations did not substantially contribute to postglacial recolonization, but rather reacted to climate fluctuations by altitudinal range shifts (Hampe and Petit 2005). Traces of such local extinction/expansion cycles can still be recognized in gene pools of *A. cilicica* (Awad et al. 2014).

During postglacial recolonization, genetic lineages met and formed broad hybrid zones on both sides of the Danube plain (Gömöry et al. 2012). However, natural hybridization of firs is not limited to the intraspecific level. Mediterranean firs (at least those within the section *Abies*) intercross easily. Fir in northern Greece, distinguished by growth vigour and capable of massive colonization of open areas, shows intermediate traits between *A. alba* and *A. cephalonica* and was classified as a separate taxon *A. borisii-regis* Mattf. Phylogeny of this taxon is still unclear, but genetic analyses generally support the hypothesis of its hybridogenous origin (Fady et al. 1992; Scaltsoyiannes et al. 1999). Two further taxa, *A. equi-trojani* Asch. and *A. bornmuelleriana* Mattf. occurring in Turkey, are also suspected to be hybrids, in this case between *A. nordmanniana* and *A. cephalonica*.

14.2.4 Threats to Abies *Gene Pools*

Genetic inventories of rare Mediterranean firs indicate that in spite of restricted ranges and small population sizes they possess genetic variation levels comparable to the other European conifers; this is true even for extremely endemic *A. nebrodensis* (Scaltsoyiannes et al. 1999; Parducci et al. 2001; Hansen et al. 2005; Terrab et al. 2007). The widespread *A. alba* has long been considered less variable than other conifers because of its low morphological variation. However, neutral marker studies did not confirm this (Konnert and Bergmann 1995; Liepelt et al. 2009). Unfortunately, adaptive markers for *Abies* are still under development (Mosca et al. 2012a, b; Roschanski et al. 2013) and no range-wide mapping of genetic variation has been performed yet. Nevertheless, as fir populations occupy a very broad range of ecological conditions, they may be extremely diverse in their adaptive potential. Common-garden experiments and laboratory tests showed strong differentiation in mortality, growth, ecophysiology and biochemical traits among populations descended from different parts of the distribution area (Mayer et al. 1982; Larsen and Mekić 1991; Wolf 2003). Silver fir is known to suffer from a periodically appearing syndrome of "silver fir decline", associated with physiological damage, needle cast and reduced increment. The aetiology of this syndrome is largely unknown, air pollution and lack of genetic variation being most often suggested as causes (Larsen 1986). At present, fir populations mostly recover (Bošeľa et al. 2014), but the regress is likely to reoccur, as long-term fluctuations in health state were observed in the past. What is important, the decline is restricted to populations originated from the northern-Apennine refugium. Neither the Balkan lineage, nor the populations from Calabria, Central Massive or Pyrenees seem to be affected (Larsen 1986). The

effects of selective pressures on silver fir gene pools have also been demonstrated in association with climate (Bergmann and Gregorius 1993) or pollution (Longauer et al. 2001). This underlines the significance of genetic variation for adaptive properties of fir populations.

Climatic niche offers much broader distribution of silver fir than the realized spatial range (Tinner et al. 2013), which, in addition to interspecific competition, is an indication of strong direct or indirect human pressures. First of all, the area of forests as such has steadily decreased since the Neolithic, as they were converted into agricultural land (mainly pastures and meadows in the case of fir forests). Moreover, since the eighteenth century, natural mixed forests have largely been being replaced by commercial conifer monocultures in many European countries. Improper silvicultural systems associated with clear cutting or shelterwood cutting with rapid canopy opening were also unfavourable for fir (Mayer 1984). Among indirect influences, game browsing is one of the most important limiting factors for silver fir regeneration. Current game management practices in many parts of Europe often support high stocks of red deer, which heavily damages fir juveniles. Last but not least, fir is susceptible to industrial pollution. The composition of pollutants changes, sulphur dioxide, which was a serious problem in Central Europe in 1970s and 1980s, was replaced by tropospheric ozone, but as a whole, air pollution remains a serious threat at least locally.

It is difficult to predict the future of firs under the ongoing climate change. Arguing by the extent of fundamental climatic niche based on the comparison of past climates and past distribution of fir during the Holocene and the Eemian, Tinner et al. (2013) suggested that silver fir may profit from changing climate almost all over the range. On the other hand, their study does not take into account potential genetic differentiation in the past and the complexity of the phenomenon of climate change, which is not necessarily limited to altering overall levels of temperatures and precipitations. Drought stress and increased incidence of wildfires are generally considered the cardinal problem linked to climate change, as most climate scenarios predict increasing temperatures and prolonged drought periods, resulting in increased continentality in much of Europe. However, the effects of climate change are not restricted to drought. Elevated-temperature events during winter may induce winter desiccation associated with xylem cavitation and needle loss, which may decrease productivity of fir forests. Heritable features of tree architecture such as crown shape or branching form result from evolutionary adaptation to snow pressure and occurrence of hoarfrost and ice (Geburek et al. 2008). Changed winter precipitation patterns in terms of a shift of wet and heavy snow towards higher altitudes may bring excessive damage. Vegetative phenology (budburst, shoot growth cessation, frost hardening etc.) results from evolutionary tradeoffs between the length of the growing season and the risk of frost damage. A part of circum-annual ontogenetic rhythms is internally regulated and proceed almost regardless of external signals, however, climate-associated environmental signals (chilling, thermal accumulation) play essential role in the timing of growth and reproduction (Konnert et al. 2014). Changed temperature distribution over the year may confuse the temporal course of life processes and lead to important economical losses.

In spite of the protection in national parks and reserves, overharvesting and grazing remain the main threats for rare fir species in southern Europe, Asia Minor and North Africa. Unfavorable consequences of climate change, such as drought and wildfires are expected to be even more pronounced and thus more risky for the persistence of fir populations in this area than in central or northern Europe (Alizoti et al. 2011).

14.3 *Abies* Conservation Strategies

Generally, the germplasm conservation of European and Mediterranean firs, like in other forest trees, includes both *in situ* and *ex situ* strategies. In the case of widely distributed and wind-pollinated species, the principal method is to establish gene-reserve forests that include a considerable proportion of the genetic diversity within a species and, in this way, to ensure the continuous evolution of the species (Geburek and Turok 2005). *Ex situ* strategies, such as clonal field repositories, seed orchards, and seed banks based on desiccated orthodox seeds belong to the group of classical conservation approaches. Tissue culture techniques, *in vitro* collections, and cryopreservation are regarded as biotechnology based approaches (Pence 2014). Thus, the biotechnology based approaches and the cryopreservation of tree material has generally been considered as a complementary system for existing *in situ* and *ex situ* conservation practises (Blakesley et al. 1996; Häggman et al. 2008; Li and Pritchard 2009).

14.3.1 Classical In Situ *and* Ex Situ *Conservation Strategies*

The importance of genetic conservation of European and Mediterranean firs was recognized at the national level as well as by international institutions such as FAO and Bioversity International. In the frame of the EUFORGEN Conifers Network, "Technical guidelines for genetic conservation and use" of *A. alba* (Wolf 2003) and Mediterranean firs (Alizoti et al. 2011) were elaborated.

As fir populations at the southern edge of the distribution are potentially most threatened by climate change, they deserve special attention. Marginal populations may harbor specific genes, which may prove to be a relevant pre-disposition during future adaptation processes. Dynamic *in situ* conservation with emphasis on marginal and genetically distinct populations is the preferred way to prevent extinction and to sustain the evolutionary potential, taking also into account that local populations are regarded as the functional units of ecosystems (Alizoti et al. 2011). Gene reserves as the basic type of conservation units in forest trees have been established in practically all European countries and many of them contain *Abies* species (Koskela et al. 2013; Lefèvre et al. 2013). Nevertheless, attention has always focused on

silver fir. The area of *in situ* gene conservation units meeting the newly defined pan-European minimum requirements for dynamic gene conservation units (Koskela et al. 2013) is over 38,000 ha for firs (cf. http://portal.eufgis.org).

The rate of the environmental change may exceed the capacity of genetic systems of population to adapt through natural selection and gene flow or to disperse into more favourable habitats. Assisted migration or *ex situ* conservation aimed at safeguarding populations which are in danger of physical destruction or genetic deterioration become viable options under such conditions (Konnert et al. 2014). Conservation measures include establishing conservation stands, seed orchards, clonal archives or storing genetic material in gene banks (Skrøppa 2005). At present, there are 307 ha of *ex situ* conservation stands for *A. alba* and 6 ha for *A. cephalonica* (cf. http://portal.eufgis.org). In addition, all Mediterranean species are represented on numerous experimental sites such as provenance or progeny tests, and are also conserved in many botanical gardens throughout Europe.

As firs have orthodox seeds, they can be stored over longer period (5 years) after decreasing water content to 5–10% with only a minor loss of viability (Bonner 2008) and seeds as stored in the national seed banks. On the other hand, the cryostorage of *A. alba* seeds was also successfully tested nearly 30 years ago (Ahuja 1986), but till now, this method has not been vigorously involved in seed storage banks, as the seed preparation and cooling procedures are complicated (Chmielarz 2008). Therefore, practical application is limited to few seed banks (e.g., the Kostrzyca Forest Gene Bank in Poland; http://www.lbg.jgora.pl).

Ex situ conservation may also be driven by the effort of preserving specific genotypes, including products of breeding. However, not all measures mentioned above are applicable in the case of firs. In such cases, non-conventional biotechnological solutions including cryopreservation and tissue culture techniques may become the primary method of choice (Blakesley et al. 1996; Li and Pritchard 2009).

14.3.2 Biotechnology Tools as Ex Situ *Conservation Strategy for* Abies *Species*

In vitro conservation and cryopreservation are the most specialized form of *ex situ* conservation of genetic resources and the detailed gene bank standards for *in vitro* culture, slow growth storage, and cryopreservation were published by FAO (2013) recently. Engelmann (2011) recognizes three possibilities of biotechnological applications for *ex situ* conservation: (i) *in vitro* cultures, (ii) slow growth storage and (iii) cryopreservation.

The recent biorepositories or banks are mostly established by using *in vitro* produced plant material and they are depended on the success of *in vitro* propagation techniques which have been used for particular species (Pence 2014). In some specific cases, like isolated embryos or dormant buds, the *in vitro* methods may only be applied at the recovery stage.

14.3.2.1 Tissue Culture Techniques

Generally, *Abies* species are considered recalcitrant for vegetative propagation. Even *ex vitro* methods were either unsuccessful or limited by very strong plagiotropism (Blazich and Hinesley 1994). Rooting problem is associated with tree maturation phase and age-related developmental process (Nielsen et al. 2008; Bonga et al. 2010).

In *Abies* species, like in a majority of coniferous species, the applications of using *in vitro* biotechnology propagation methods by axillary and adventitious buds are hampered by low multiplication rates, difficulties in rooting, and high production costs due to multiple manual operations required during propagation. Of the *in vitro* methods, somatic embryogenesis has proved to be the most promising method for regeneration of all *Abies* species (reviewed by Vooková and Kormuťák 2007, 2014).

Somatic embryogenesis (SE) is a cloning technique based on tissue culture whereby genetically identical copies of a genotype are produced in unlimited numbers (Park 2013). A key advantage of SE over other vegetative propagation methods is that the embryogenic clonal lines can be cryostored in liquid nitrogen, while corresponding trees are tested in the field (Park 2002; Nehra et al. 2005; Bettinger et al. 2009; Whetten and Kellison 2010; Park 2013). The ability to maintain donor tissue juvenility throughout cryopreservation represents an advantage over propagation programs based on rooted cuttings (Grossnickle et al. 1996), and the genotype response supersedes that of systems based on organogenesis (Menzies and Aimers-Halliday 2004).

In *Abies* species, like in other conifers, SE the multi-step regeneration process starts with induction of pro-embryogenic masses, followed by somatic embryo formation, maturation, desiccation and plant regeneration as illustrated for *A. cephalonica* on Fig. 14.3. Despite the fact, that *A. alba* was one of the first coniferous species where the induction of SE was reported (Erdelský and Barančok 1986a, b), and a few studies on the regeneration of silver fir employing SE were published nearly 20 years ago (Chalupa 1991; Hristoforoglu et al. 1995), a standard protocol for propagation by SE on a large scale is still lacking. Till now, out of 11 species belonging to the group of European and Mediterranean firs the successful regeneration via SE was reported for five species; *A. alba* (Chalupa 1991; Hristoforoglu et al. 1995; Vooková and Kormuťák 2009), *A. cephalonica* (Krajňáková et al. 2008), *A. cilicica* (Vooková and Kormuťák 2003), *A. nordmanniana* (Nørgaard 1997), *A. numidica* (Vooková and Kormuťák 2002) and several hybrids: *A. alba* × *A. numidica*, *A. cilicica* × *A. nordmanniana*, *A. nordmanniana* × *A. veitchii* (Salaj et al. 2004; Vooková and Kormuťák 2014).

Embryogenic cultures of *Abies* species have been derived in majority of cases from immature zygotic embryos but also mature embryos were successfully used (Hristoforoglu et al. 1995; Salaj and Salaj 2003; Nawrot-Chorabik 2008). Besides pure species also embryogenic cultures of interspecific hybrids have been derived from immature (*A. alba* × *A. alba*, *A. alba* × *A. nordmanniana*, Gajdošová et al. 1995; *A. alba* × *A. cephalonica*, *A. alba* × *A. numidica*, Salajová et al. 1996; *A. cilicica* × *A. nordmanniana,* Vooková and Kormuťák 2003) and mature (*A. alba* × *A.*

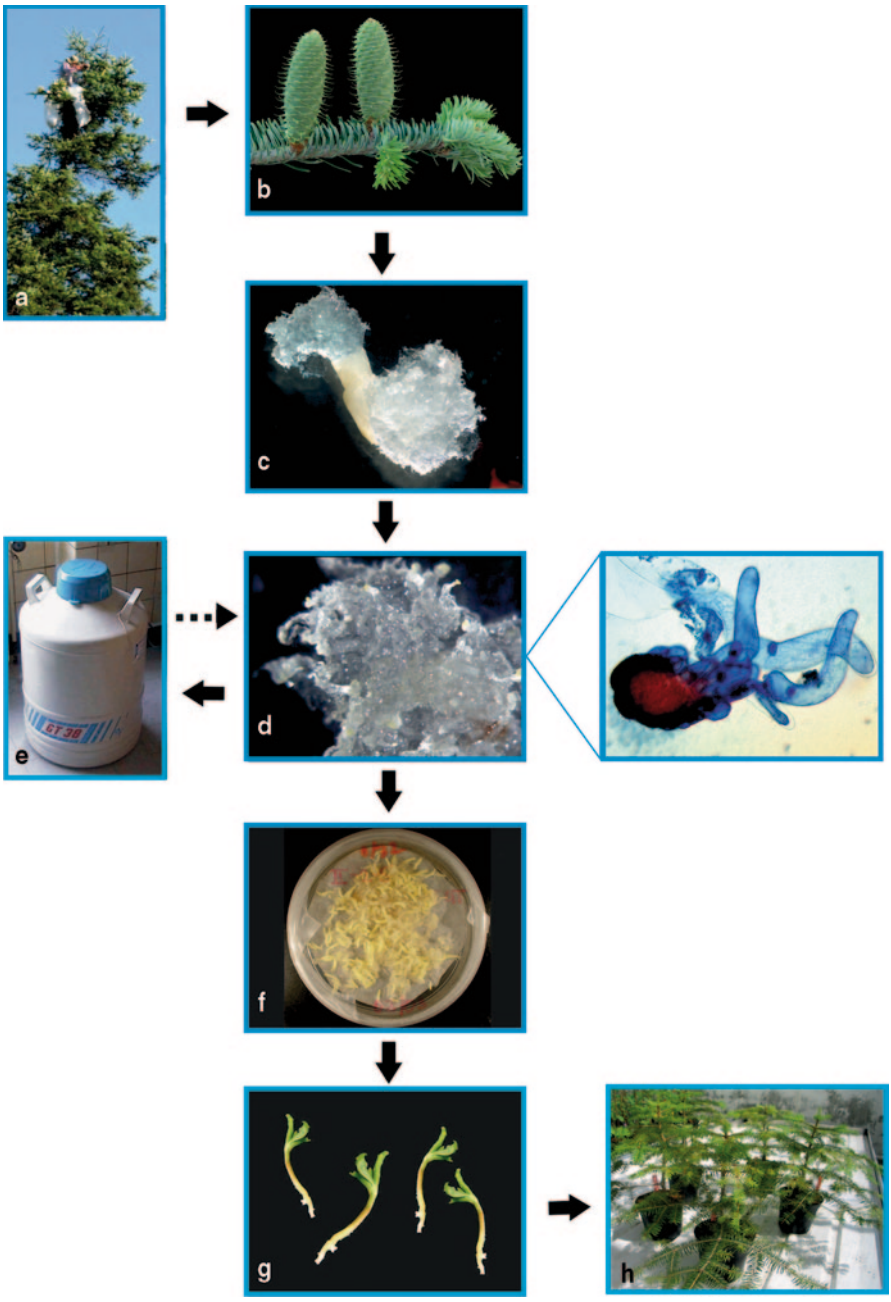

Fig 14.3 Somatic embryogenesis of *Abies cephalonica*. **a** Elite tree of *A. cephalonica*. **b** Developing green cone shortly after meiosis. **c** Initiation of somatic embryogenesis using immature embryos and proliferation of embryogenic cell mass. **d** Proliferating embryogenic cell mass and detail of proembryogenic cell masses after staining with acetocarmine and Evan's blue. **e** Option for cryopreservation of the germplasm. **f** Maturation of somatic embryos. **g** Conversion of somatic embryo plants. **h** Experimental field trail

cephalonica, Salaj and Salaj 2003) zygotic embryos. Secondary or repetitive SE from cotyledon explants of *A. alba* × *A. cephalonica* and *A. alba* × *A. numidica* somatic embryos was reported by Salajová and Salaj (2001) and for *A. numidica* by Vooková et al. (2003).

Induction and proliferation of several *Abies* species differ from most other genera of the *Pinaceae*, because they can be achieved with cytokinin as the sole plant growth regulator in the tissue culture medium (Nørgaard and Krogstrup 1995), although the embryogenic cultures of *A. alba* proliferated on a medium supplemented with auxin (Vondráková et al. 2011). Maturation of fir somatic embryos is promoted by abscisic acid and maltose is the preferable carbohydrate. The addition of polyethylene glycol promoted the development of somatic embryos (Nørgaard 1997; Salajová et al. 2004; Krajňáková et al. 2009). For germination, well-developed cotyledonary somatic embryos are selected and subjected to a partial desiccation treatment for 3 weeks (Nørgaard et al. 1997; Vooková et al. 1998).

Despite positive achievements, the bottlenecks in *Abies* species, like in most conifers, are the low initiation rate, uneven maturation of embryos, problems in rooting and germination phases. This is due to poor understanding of embryo development and therefore inability to develop proper SE methods for practical purposes. Exception is only SE of *A. nordmanniana* were technology has been used already tested in large scale. In Denmark, within the last two years, from 400 different embryogenic cell lines around 20,000 plantlets were produced which will go into field trials in 2014 and 2015. The expectation is to continue with production of 20,000 plants in the following year but only with 5–10 elite, most productive genotypes (Jens Find, personal communication).

14.3.2.2 Cryopreservation

Cryopreservation for conservation purposes allows for storage of valuable seed (some recalcitrant seeds), pollen, shoot tips, meristems, axillary and dormant buds, embryogenic axes, zygotic or somatic embryos, genetically modified lines, callus, or cell cultures depending on the species (Engelmann 2011; Pijut et al. 2011). Engelmann (2011) divided the cryopreservation techniques into two main categories (i) classical, based on slow cooling down to a defined prefreezing temperature, followed by rapid immersion in liquid nitrogen and (ii) new—vitrification based procedures (seven different identified).

The first reports on cryopreservation of conifers were published in the late 1980s, the target species being *Picea abies* (L.) H. Karst, *Pinus taeda* L. (Gupta et al. 1987), and *Picea glauca* (Moench) Voss (Kartha et al. 1988). Since that time the number of target species has increased rapidly and the most cryopreserved species belong to the genera of *Pinus*, *Picea*, *Larix*, *Pseudotsuga* and *Abies*. Nowadays, the cryopreservation technology plays an important part in gene conservation, biodiversity, and in maintaining juvenility (Park 2013; Pence 2014).

As SE has become the most preferable propagation method of coniferous species, the majority of cryopreservation protocols for coniferous species deal with actively proliferating embryogenic cell masses. The most common cryopreservation

protocol for the embryonic cultures of conifers is the classical slow-cooling and fast-thawing one (as reviewed by Häggman et al. 2000; Lambardi et al. 2008). Successful cryopreservation relies on the removal of freezable water in order to avoid damage from ice crystallization and on the stabilization of membranes and molecular structure of the cells to avoid damage from the loss of water (Benson 2008). Preculturing embryogenic cell masses, somatic embryos or *in vitro* shoot tissues with treatments such as cold, increased sugars, or ABA can also work to increase survival through cryopreservation, presumably by triggering natural desiccation-adaptive physiology (Kushnarenko et al. 2009). However, even with preculturing, most plant tissues require the application of further cryoprotective procedures to remove water and stabilize tissues to maintain viability through LN exposure.

The "slow-cooling" method requires the use of a controlled-freezing apparatus to lower the temperature in a constant and controlled way, at rates of 0.1–1.0 °C per min. When temperatures reach −35 °C or −40 °C, the samples are plunged into LN. During the slow freezing, as intercellular water freezes, water moves out of the cells into the intercellular spaces, slowly dehydrating the cells. Limitations of the slow-cooling method include the expense of the equipment and the amount of LN needed. Mr. Frosty and similar products provide a less expensive alternative for slow cooling (Pence 2014). Cryovials containing samples in a bath of isopropanol are kept in the freezer at −80 °C (cooling rate of the samples being 1 °C per min). Thereafter the samples are transferred to LN (−196 °C). For thawing and regrowth of embryogenic cell masses, the cryovials are rapidly thawed in water bath at 37 °C for 1–2 min. Cryoprotectants are removed from the thawed embryogenic cellular masses by gradual elution. The regrowth of culture is obtained and followed on semi-solid proliferation medium for 4–6 weeks depending on species and cell line.

In order to overcome some of the limitations of the slow cooling method, Sakai et al. (1990) reported a different approach, known as vitrification, which combined rapid freezing with cryoprotectants to cause the formation of glass, rather than crystals, within the tissues. For vitrification, tissues are cryoprotected using more concentrated cryoprotectant solutions, the most widely used being PVS2, a mixture of 30% glycerol, 15% ethylene glycol, 15% DMSO, and 0.4 M sucrose. Till now, there are only a few reports where embryogenic cultures of *Picea mariana* (Mill.) B.S.P. and *Picea sitchensis* (Bong.) Carr. have been cryopreserved successfully by vitrification (Touchell et al. 2002; Gale et al. 2008). Recently, vitrification method based on a pregrowth-dehydration method was successfully applied to cryopreservation of *Picea omorica* (Pančič) Purk. and *Picea abies* embryogenic cell lines (Hazubska-Przybyl et al. 2010, 2013) without using cryoprotectants. Other approaches of elimination of toxic cryoprotectants, such as DMSO, have used the desiccation tolerance of somatic embryos in preparation for cryostorage and have also been successful (Bomal and Tremblay 2000; Kong and von Aderkas 2011).

For the species belonging to the genus *Abies*, the classical, slow cooling cryopreservation procedure has been described only for three *Abies* species: for *A. alba* (Krajňáková et al. 2013) *A. cephalonica* (Aronen et al. 1999), *A. nordmanniana* (Nørgaard et al. 1993; Misson et al. 2006), and some fir hybrids (Salaj et al. 2010) (Table 14.2). As preculture treatment, the culturing of embryogenic cell masses

Table 14.2 Cryopreservation protocols used for ex situ conservation and based on existing SE protocols of European and Mediterranean *Abies* species

Species	Preculture	Cryoprotectant	Time in cryostorage	Cryo-method used	Recovery and regeneration	Genetic fidelity tested by genetic markers	References
Abies alba							
12 embryogenic cell lines	Cold hardening for 14 days, 5 °C, dark 0.2 M sucrose/24 h 0.4 M sucrose/24 h 5 °C, dark	5% PGD (polyethylene glycol 6000, glucose, DMSO)	6 years	Programmable controlled-temperature chamber	4 out of 12 cryopreserved cell lines recovered Maturation experiment	No	Krajňáková et al. (2013)
Abies cephalonica							
Eight cell lines	Cold hardening for 14 days, 5 °C, dark 0.2 M sucrose/ 24 h 0.4 M sucrose/24 h 5 °C, dark	5% PGD (polyethylene glycol 6000, glucose, DMSO)	7 days	Programmable controlled-temperature chamber	All cell lines recovered	Yes	Aronen et al. (1999)
Two cell lines	detto	detto	6 years	detto	All cell lines recovered Maturation experiments	Yes	Krajňáková et al. (2011a)
Two cell lines	0.2 M sucrose/24 h 0.4 M sucrose/24 h 5 °C, dark	detto	7 days	Nalgene™, Mr. Frosty	Occurrence of oxidative stress monitored Biochemical parameters used	No	Krajňáková et al. (2011b)
A. nordmanniana							
Five cell lines	0.2 M sorbitol/24 h 0.4 sorbitol/24 h Samples placed on a 120 rpm rotary shaker 24 °C, dark	5% DMSO	2 h	Programmable freezer	All cell lines recovered	No	Nørgaard et al. (1993)

Table 14.2 (continued)

Species	Preculture	Cryoprotectant	Time in cryostorage	Cryo-method used	Recovery and regeneration	Genetic fidelity tested by genetic markers	References
15 cell lines	0.52 M sucrose Samples placed on a 100 rpm rotary shaker 22 °C for 24 h/dark	7.5 % DMSO	1 h	Isopropanol container	All cell lines recovered; recovery rate depended from treatment	No	Misson et al. (2006)
Abies hybrids							
A. alba × A. cephalonica, three cell lines A. alba × A. numidica one cell line	0.4 M or 0.8 M sorbitol applied for 24, 48 or 72 h 24 ± 1 °C, dark	5 % of DMSO	1 h	Nalgene™ Mr. Frosty container	Cell viability Maturation experiment performed	Yes	Salaj et al. (2010)

was done on solid or liquid media with increased concentration of sucrose (0.2 and 0.4 M) or sorbitol (0.2 and 0.4 M) applied for subsequent 24 h. The most common cryoprotectants which were used are 5% PGD (polyethylene glycol 6000, glucose, DMSO) and DMSO reaching the final concentration 7.5% and 5%, respectively. The duration of storage in LN_2 varied from 1 h (Misson et al. 2006; Salaj et al. 2010) till 6 years (Krajňáková et al. 2011a, 2013).

The first reports have evaluated only the recovery after cryopreservation monitored as increase in proliferation rate or as vital staining of embryogenic cell masses (Nørgaard et al. 1993; Aronen et al. 1999). The most recent studies compared also occurrence of oxidative stress (histological localization of H_2O_2) and the biochemical parameters (cellular levels of ATP and glucose-6-phosphate) during each step of cryo-procedure and thawing (Krajňáková et al. 2011b). The evaluation of maturation abilities after cryopreservation was done by Salaj et al. (2010) for fir hybrids and by Krajňáková et al. (2011a, 2013) for *A. cephalonica* and *A. alba*.

However, despite more than 20 years of experience in conifer cryopreservation, including *Abies* species, there are only a limited number of reports on long-term storage. The present scenarios for global forest management and conservation, the need to conserve breeding material during clonal field testing and the consequences of climate change, not only underline the importance of cryopreservation as a safe storage against external threats but also emphasize the significance of the genetic fidelity of cryopreserved material. Long-term cryopreservation of an *Abies* species has only been reported for *A. cephalonica* (Krajňáková et al. 2011a) and *A. alba* (Krajňáková et al. 2013).

The experience and reports on the effects of prolonged storage in liquid nitrogen are still limited, and the genetic fidelity at DNA level of the cryopreserved material has rarely been considered (Aronen et al. 1999; Salaj et al. 2010; Krajňáková et al. 2011a). However, cryopreservation as a cost-effective, low labor- and space-demanding alternative will have an important role for conservation of coniferous tree species, including European and Mediterranean fir species in the near future.

14.4 Concluding Remarks

Despite the fact that five European and Mediterranean fir species and some hybrids were regenerated using somatic embryogenesis technique and the successful cryopreservation protocols were applied to three species, there is still need for further studies. First, the critically endangered and endangered fir species were not subjected to above mentioned studies. Second, the current protocols for regeneration have some limitations and have been applied only to a few embryogenic cell lines. Due to the fact, that *in vitro* cultures are clonally propagated lines, it is important to remember that multiple genotypes of these tissues need to be banked in order to achieve a high level of genetic diversity in the collection. This can dramatically increase the labour and resources needed initially to establish the lines and cryopreserve the tissues, but once the lines are banked, maintenance costs are similar to those of other

cryopreserved materials, such as seeds (Li and Pritchard 2009). Thus, biotechnological approaches have their place in the toolbox of conservation methods of firs.

Biotechnology means for *ex situ* conservation are of specific value in the context of rare endangered species with small local populations like *A. nebrodensis* or *A. numidica*, where populations are small and virtually all trees are worth of being conserved. They also can be useful in the case of small local populations, mainly fragmentary demes on the edges of the distribution range, potentially containing specific alleles. Transfer of the biotechnology experience gained in widespread species and development of reliable procedures for somatic embryogenesis and cryopreservation for the endemics remain, however, the tasks for the future.

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Chapter 15
Conservation of Global Wheat Biodiversity: Factors, Concerns and Approaches

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Abstract Wheat is an important food crop in the world. It is also one of the top three global food crops produced after rice and maize that constitutes an immensely significant role with respect to global food security. Due to finite land resources that can be dedicated to agriculture global wheat production has been consistently dependent on genetic improvement of wheat germplasm across the world. Traditional plant breeding has been an important tool in increasing global food production by producing disease and stress resistant, high yielding and early maturing wheat varieties. However, it is necessary to have a stable and divergent pool of wheat genotypes grown under different environmental conditions and different land races of wheat as genetic feedstock for enhanced genetic improvements. Due to increased global human population, extensive anthropogenic pollution and damages to the vulnerable local ecosystems, existing genotypes and land races of wheat are under constant threat of becoming extinct. Hence it is absolutely necessary to conserve the global wheat biodiversity for securing the future of our food security.

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Recent progress and developments in technological applications including those in the realm of biotechnology have turned out into an essential tool that could be effectively and efficiently utilized for wheat biodiversity conservation. This short review is an attempt to investigate different factors and concerns jeopardizing global wheat biodiversity and pinpoints some potential approaches for its successful conservation.

Keywords Wheat · Biodiversity · Conservation · Genetic · Cultivars · Genotypes · Germplasm · Landraces · Biotechnology

Abbreviations

AFLP	Amplified fragment length polymorphism
CAAS	Chinese Academy of Agricultural Sciences
CIMMYT	International Maize and Wheat Improvement Center
DArT	Diversity arrays technology
FAO	Food and Agriculture Organization
ICARDA	International Center for Agricultural Research in the Dry Areas
ICGR	Institute of Crop Germplasm Resources
NBPGR	National Bureau of Plant Genetic Resources
NIAS	National Institute of Agrobiological Sciences
NSGC	National Small Grains Germplasm Research Facility
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats

15.1 Introduction

Biodiversity is one of the most requisite factors to enhance productivity of agricultural crops, and to maintain better health of functional eco-systems. Balance of ecology and environment can be achieved through appropriate combination of flora and fauna in the eco-systems. Beside the known beneficial plants and organisms to human kinds, other wild forms of living organisms with unknown functions also play crucial role in overall biodiversity in the eco-systems.

Life of plants, animals, human and other microorganisms is most unique feature of this planet (Cardinale et al. 2012). In the most recent era, living organisms including plants, animals and microorganisms are being explored for beneficial activities to human life and human society eventually that can be used as commercial or industrial products or services. In high-tech era, modernization of agriculture production practices, urbanizations and industrialization policies of governments have been challenging biodiversity. Unwanted human actions are the primary factors of diminishing earth's eco-system (Cardinale et al. 2012). Exploration is of immense importance along with conservation for sustainable agriculture system and food production. Limited efforts, however, are being made for conservation

and enhancement of biodiversity especially for major crop species. Cardinale et al. (2012) reviewed results of research experiments of last two decades and revealed impact of loss of biodiversity on functional ecosystems and goods and services supply. Long term research on grassland revealed diverse plant communities tolerate more and recover fully from major biotic and abiotic stresses (Naeem et al. 1994; Tilman and Downing 1994; Zavaleta et al. 2010). Dismantling of eco-systems cause loss of biodiversity and this is a primary concern around the globe. Loss of biodiversity is mainly due to habitat fragmentation and destruction, overexploitation, climate change, deterioration and extinction cascades, invasion by alien species and many other factors (Brook et al. 2008; Dunn et al. 2009; Thomas et al. 2004; Tilman et al. 2001).

Wheat is one of most cereal produced in the world followed by rice and maize. It is a primary source of calories for 1.2 billion people around the globe and thus constitutes a main platform for the global food security. Traditional plant breeding has been an important tool in increasing global food production by producing high yielding, disease resistant cultivars with better agronomic practices. However, genotypic variation is one of prerequisites to improve any trait including grain yield. Therefore, scientific community often rely on land races or wild progenitors when the genetic diversity/variation is not present in the immediate gene pool. Unfortunately many existing genotypes and land races are now being threatened with threat of extinction due to several natural and anthropogenic factors. In this chapter, we have tried to investigate different factors and concerns jeopardizing global wheat biodiversity and pinpoints some potential approaches for its successful conservation.

15.2 Wheat: Biodiversity, Taxonomy and Systematics

The knowledge of the ancestral lineages of wheat and their distribution are the key to the conservation of the current taxa. The wheat is widely distributed from the Arctic Circle to the equator (Curtis 2002). Wheat is divided into two broad categories i.e., spring and winter based on its growth habit. Wheat is also divided into various classes depending on the grain color and texture (Jing-Song et al. 2012). It has been reported that several cultivars are more successful between the latitudes of 30–60° N and 27–40° S (Nuttonson 1955).

The human being in their quest to identify their natural environment has assigned names to wheat plants in their distribution range. First classification of wheat was proposed by Linnaeus in 1753 that was based on phenological characters: *Triticum aestivum* L. for spring wheat and *T. hybernum* L. (nom. rejic.) for winter wheat; in addition he described *T. spelta* and *T. polonicum* based on its morphology. *Triticum aestivum* L., commonly known as wheat, is a species belonging to grass family Poaceae, in the Pooideae subfamily and Triticeae tribe. Later on, different classifications based on morphological characters were proposed. Dumortier (1823) divided the wheat based on the consistency of the spike (fragile vs. not fragile). Bowden

(1959) integrated the two genera: *Triticum* L. and *Aegilops* L. into a single genus (representing 40 *Triticum* species) which was later supported by Morris and Sears (1967). During post second world war period, the older classifications have been strongly opposed based on later studies focusing on cytogenetics (Bowden 1959; Morris and Sears 1967), genetics (MacKey 1966, 2005) and molecular biological evidences (Goncharov 2002; Goncharov et al. 2009). Other important proposals include MacKey's classification of the intrageneric taxonomy of *Triticum* followed by Mansfeld's Encyclopedia of Agricultural and Horticultural Crops based on smaller number of genes regulating morphometric variations among different wheat species As a result, this particular classification system is quite simple comprising of only 10 *Triticum* species and 20 infra-specific taxa (Goncharov 2011).

One of the most recent classifications are the Goncharov (2002) and Goncharov et al. (2009) after the traditional Körnicke-Flaksberger-Dorofeev system comprising of 29 different species represented by five separate sections. However, the taxonomic treatments carried out in wheat usually lack robust criteria to distinguish natural hybrids from artificial hybrids and this has caused confusion in wheat research. The criteria commonly used by plant taxonomists are morphological, anatomical, ecological, geographical, karyological, biochemical, cytogenetic, and molecular genetic methods have also been recently provided. There are many artificial hybrids without assigned names or invalid names which complicates the systematics of *Triticum*. Based on the actual phylogenetic studies, there is evidence that the long evolutionary process of wheat was parallel to that of humans. It is estimated that approximately 3 million years ago, a common ancestor of wheat suffered a divergence and raised ancestral diploid genomes, called A, B, and D (Gill et al. 2004).

According to Golovnina et al. (2007), inter-generic hybridization of genomes (*Triticum* × *Aegilops*) has been fundamental to the process of speciation of wheat. Furthermore, Feldman and Levy (2005) also suggested wheat genome evolution through two major approaches: allopolyploidization with rapid and sporadic genomic modifications. The evolutionary process of wheat was also coupled with domestication. According to Harlan (1992), 1500 years ago humans domesticated wheat, cultivating it over large areas in present-day Iraq, parts of middle-east Asia including Iran, Turkey and Syria.

All the modern polyploid wheat species have originated from the tetraploid wild ancestor (*T. dicoccoides* (Körn. ex Aschers. & Graebn.) Schweinf.)). Nesbitt and Samuel (1996) and Tanno and Willcox (2006) indicated that the wild emmer wheat (*T. dicoccoides*) was possibly the first cereal species domesticated by our human ancestors. However, the exact period of domestication is still highly debated by different scholars. It was subsequently identified into four subspecies for *T. dicoccon* Schrank ex Schübl.: (1) ssp. *maroccanum* Flaksb., (2) ssp. *abyssinicum* Vav., (3) ssp. *europaeum* Vav. = ssp. *dicoccon*, and (4) ssp. *asiaticum* Vav. (Gökgöl 1955; Dorofeev et al. 1979; Szabó and Hammer 1996; Teklu et al. 2007). The process of domestication holds the link to the evolution of bread wheat (Ozkan et al. 2010). According to phylogenetic studies of Golovnina et al. (2007), the genus *Triticum* represents three groups, each including wild and cultivated species: (I) Diploids

(section Monococcon), includes wild species *T. boeoticum* Boiss. and *T. urartu* Thumanjan ex Gandilyan. (II) Tetra- and hexaploids (sections *Dicoccoides* and *Triticum*) includes *T. dicoccoides* and (III) *Timopheevii* (section *Timopheevii*) include *T. araraticum* Jakubz. A recent study of Ozkan et al. (2010) reported that wheat cultivars can be divided into two groups: (1) Tetraploid durum wheat ($2n=28$, BBAA): *T. durum* Desf. (2) Hexaploid bread wheat ($2n=42$, BBAADD): *T. aestivum*. However, Goncharov (2011) mentioned that the approach of the hierarchical classification of wheat species needs further research and analysis; for developing into a comprehensive classification system with integration of data from different related fields of research on wheat taxonomy and systematics.

In spite of significant improvement in the tools and techniques of molecular biology, the comprehensive phylogenetic status of wheat is debated; mostly due to shorter evolutionary period (last 6000–12,000 years) during which most wheat species have developed (Nesbitt 2002). Morrison (1995) and later, Goncharov (2002) argued for a more comprehensive approach including information from phylogenetics, cytogenetics and molecular biology to be corroborated with traditional morphometric data for establishing useful identification keys and thereby devise a more technically sound nomenclature system for the species. Globally the most common species are *T. aestivum* (bread wheat) and *T. turgidum* spp. *durum* (Desf.) Bowden (durum/macaroni wheat).

15.3 Wheat Biodiversity: Challenges

Major grain crops such as wheat, rice, maize, soybean, canola etc. have been losing diversity due to long term monoculture of a few high yielding cultivars. High level of selection pressure for favourable traits is the primary reason for narrow genetic bases for numerous other characters. Single assemblage limits the multi-functionality in a crop eco-system. Uniformity in genetic make-up of varieties in same genetic pool causes reduction of overall performance and stability, at the same time bring in risk of vulnerability to biotic and abiotic stresses (Pecetti et al. 1992; Porceddu et al. 1988). For instant, stress tolerance and high yield can be negatively related and very difficult to maximize simultaneously, either one can be penalized in join functions (Diaz et al. 2004; Grime 1974).

a. Loss of Wheat Biodiversity Biodiversity of *Triticum* species for physiological performance, quickly adapting to biotic and abiotic stresses such as evolutionary adaptation is reduced in elite germplasm compared to landraces and wild relatives. It could be seriously threatened in the crop improvement by future epidemic, global warming and high level of regional droughts. Sustainable performance such as durable diseases and pest resistance, drought and salinity tolerance is highly responsible traits on wider biodiversity. Uniqueness of individuals within population for physiological, biochemical, metabolomic processes govern high biodiversity within species. Biodiversity of crop plants could be directly impacted by the existence of

genetic diversity. It has been known that wheat was domesticated about 10,000 year ago, since then courteous selection and breeding efforts has been significantly eroded genetic diversity (Chatzav et al. 2010; Tanksley and McCouch 1997).

b. Genetic Erosion/Gene Pollution Genetic erosion term referred as the loss of variability of crop production in the areas of domestication and secondary diversification i.e. centre of origin (Tsegaye and Berg 2007). Genetic variability of a crop population is altered in ways that make negative genetic gain over a period. Genetic erosion is one of the most important factors contributing to global wheat biodiversity. de Carvalho et al. (2013) defined genetic erosion as the "*Steady reduction of combination of alleles over time in a defined areas or lasting reduction in richness of common alleles*". Variability is coined to heterogeneity of alleles and genotypes that reflect morphotypes and phenotypes composition. The numbers of crops grown are declining steadily and crop with commercial importance enhancing production areas with highly similar genetic constitution. As a result wild and weedy relatives lose their own genetic make-up over long period of time. The primary cause of genetic erosion is wide distribution of modern cultivars from crop breeding programs (Brush 1995).

Initially domesticated landraces were replaced by the cultivars selected based on conventional breeding programs and recently those cultivars were replaced by modern high-quality homogenous new varieties or hybrid selected by molecular marker assisted selection. Concentrated focus on breeding for crop yield and related traits is highly responsible factors for genetic erosion of major crop species. In genetic diversity analysis, about 20% genetic erosion of local gene pool of Russian origin ancestors observed in 78 spring durum wheat genotypes introduced in Russia between 1929–2004 (Martynov et al. 2005). Gradually reduction in genetic diversity reported in spring bread wheat from early domestication to traditional landrace cultivars to modern breeding varieties and collected germplasm for long term breeding program through 90 SSR markers distributed across the wheat genome (Reif et al. 2005). Similarly, decay in genetic diversity reported in 242 accession of common wheat released in China since 1940s. The study revealed lower genetic diversity found in cultivars released in 1990s compared to 1940s (Tian et al. 2005). In addition to that other gene pools also found similar pattern of decayed in the genetic diversity of wheat cultivars over times (Huang et al. 2007; Smale et al. 2002; Tsegaye and Berg 2007). A study reported a survey based on *in situ* conservation of local varieties and landraces protected by farmers in Ethiopia, results suggested that localized landraces of species *Triticum polonicum* and *T. turgidum* have great genetic erosion. The primary causes of the genetic erosion of landraces of several species of genus *Triticum* in Ethiopia is displacement of landraces by other high yield modern wheat cultivars (Teklu and Hammer 2006).

15.4 Factors Contributing to the Loss of Wheat Biodiversity

Plant breeding continuously selects the favourable allelic combinations in gene pool to improve *per se* performance of elite lines that eventually to be used as parents of new variety development.

a. Changing Genetic Architect of Wheat Populations in Genetic Pools Long term breeding operations change original genetic architect of plants as a result genetic shift observed in improved gene pools in wheat, barley and maize (Donini et al. 2000; Fu 2006; Koebner et al. 2003). Improved gene pools could have desirable allelic composition for traits like yield, quality and agronomy but that may not have durable resistance capacity to diseases and pest, long term sustainability to changing climate and high performance stability.

b. Narrowing Genetic Base for Biotic and Abiotic Stresses Robust and rapid maker assisted selection system for selection of foreground and background genome of elite lines causes elimination of large genomic variations resulting genetic bases of elite germplasm would be narrow for quantitative control of biotic and abiotic stresses in the newly developed cultivars. Most recent concern of this genetic bottleneck has been taken into consideration and breeder perform wide inter-specific or inter-generic crosses to enhance genetic variation within gene pool for sustainable durable resistance to biotic and abiotic stresses in wheat.

c. Vanishing Original Genetic Composition of Wild and Weedy Relatives In the current agriculture system, farmers prefer to grow genetically uniform crop varieties and majority farmers select high yield varieties of same crop because of high revenue. Cultivation of large area with highly genetically uniform varieties creates pressure on original genetic composition of wild and weedy relatives which exist in the surrounding areas. Selection pressure over many generations in wild populations due to pollen drift from cultivated areas causes loss in the genetic diversity in wild and weedy relatives.

d. Insufficient Resources for Germplasm Conservation and Utilization In addition to above mention factors, inadequate resources for germplasm conservation and long term maintenance highly impact on the biodiversity of important crop like wheat. Collection, conservation, research and utilization of wheat germplasm resources play an important role in wheat breeding program to improve production and productivity in the world. More resources need to be allocated for the preservation of germplasm that can be used in current and future breeding program to maintain biodiversity and overall performance of wheat crop. Current collections in the gene banks have limited or no contribution to the modern cultivar development programs for most essential agricultural traits. Crop improvement, is still focused on poor genetic base for all the major crop species including wheat (Tanksley and McCouch 1997).

15.5 Wheat Biodiversity Conservation: Current Status

Wheat is one of the most important cereal crops in term of its production and consumption in the world. Various sub species of genus *Triticum* are cultivated in different regions of the world; therefore genetic diversity is one of the most crucial factors for wheat crop improvement. It has been widely debated that the genetic diversity of major self-pollinating crops such as wheat has suffered with reduction over time due to pure line breeding and selection (Donini et al. 2000; Hoisington et al. 1999). Advance in molecular techniques have been used in molecular marker development for marker assisted selection of foreground and background of genome in elite lines. Numerous types of molecular markers are currently being used for the study of genetic diversity such as Simple Sequence Repeats (SSR), Diversity Arrays Technology (DArT), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) in different crop species to know the status of diversity in different breeding materials and gene pools. Huang et al. (2007) reported existence of genetic diversity among the widely adopted 511 European winter wheat varieties developed by modern plant breeding and suggested that there was no quantitative loss of genetic diversity in different decadal groups of varieties. However within decadal groups very small percentage of variation observed by 42 microsatellite markers. Interestingly, the grain yield related traits such as grain size and shape has progressively increased indicating that instrumental favourable genetic variation in these traits observed however it reduced phenotypic variation over generations (Gegas et al. 2010).

Currently, spring bread wheat area in the developing world has about 77% related to International Maize and Wheat Improvement Center (CIMMYT) wheat (Smale et al. 2002). However, genetic uniformity or diversity does not imply without evidences of molecular analysis. Soleimani et al. (2002) reported significant amount of genetic variation between and within 13 registered modern Canadian durum wheat cultivar by polymorphism of AFLP molecular markers. In modern cultivars, in spite of rigorous genetic selection for pure breeder seeds, genetic variation observed between individuals of same cultivars (Soleimani et al. 2002). Similarly, modern cultivars and landraces developed in different gene pools displayed genetic variation that revealed by polymorphic detection of different molecular markers (Autrique et al. 1996; Bebiakin et al. 1976; Carvalho et al. 2009a; Carvalho et al. 2009b; Carver et al. 1989; Shoaib and Arabi 2006). Genetic diversity evaluated for the 150 accessions of durum wheat collected from worldwide using Single Nucleotide polymorphism (SNP) molecular markers. The SNP diversity analysis study indicated significant loss of gene diversity in terms of landraces as well as older and later released cultivars during initial stages of green revolution, however genetic diversity increased during post green revolution (Ren et al. 2013). In comparative analysis of genetic diversity in geographical regions Middle East showed moderate compared to the North and South Americas and the European regions (Ren et al. 2013).

Several studies reported about the current status of genetic diversity of wheat in different geographical regions of the world based on the molecular marker analysis on old landraces and modern cultivars. Studies based on various molecular marker systems suggested existence of genetic diversity at certain degree. There is no evidence however reported for the existence or loss of physiological, agronomical and overall fitness diversity status since plants undergo numerous biochemical and physiological and metabolomic mechanisms during growth and development. Molecular markers revealed genetic however modification after transcriptional level, protein level and metabolomic level are poorly understood and so their impact on biodiversity and fitness performance of plants.

15.6 Conserving Global Wheat Biodiversity: From a Multi-disciplinary Perspective

Plant germplasms including modern cultivated varieties, landraces, closely and distance wild and weedy relative can be conserved by *ex situ* and *in situ* methods. These methods facilitate management, maintenance and databank that can be available for future use in the breeding programs for crop improvement (Benz 2012).

15.6.1 Ex Situ *Conservation*

The *ex situ* germplasm conservation method is most traditional approach that has been utilized for many crop species especially for the maintenance of landraces and wild relatives. Earlier *ex situ* conservation was assumed to have limited usage, however, germplasm collected in *ex situ* serve primary source for trait improvement in most of the elite breeding materials in breeding programs. *Ex situ* conservation is continuous requirement to preserve neglected landraces, disappearing wild and weedy relative or distinct relative and cultivated wheat species. Such germplasm collected in *ex situ* can be indispensable resources to restore cropping system after major disease epidemic or other disasters. *Ex situ* conservation has played important role in distribution of productive crop varieties and breeding lines to those countries or regions where crop has challenges for producing enough to sustain agriculture production (Benz 2012).

Bettencourt and Konopka (1990) reported that 83,377 out of 529,577 wheat accessions were found to be landraces representing ~15.7% of total germplasm collections maintained in 102 collection centres in 47 countries. Later, Knüpffer (2009) reported 732,262 wheat germplasm accessions stored in 223 germplasm collection centers across the globe. Most recently, Food and Agriculture Organization (FAO) reported a total 856,168 wheat accessions in 229 institutes representing 24% landraces (de Carvalho et al. 2013). Together with conservation of cultivated

bread wheat accessions, *ex situ* conservation of 35 genera of *Triticeae* with ~300 species representing 20% global germplasm collection has also been reported (Knüpffer 2009). Globally, major wheat germplasm collections are (i) CIMMYT (Mexico) representing 111,396 accessions (landraces ~31%), (ii) National Small Grains Germplasm Research Facility (NSGC) in USA maintaining 57,788 accessions (landraces ~57%), (iii) the Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences (ICGR-CAAS) in China maintaining 41,030 accessions, (iv) the National Bureau of Plant Genetic Resources (NBPGR) in India with a total of 35,889 (landraces ~2%), (v) the International Center for Agricultural Research in the Dry Areas (ICARDA) in Syria holding a total of 34,983 (landraces ~75%), and (vi) the National Institute of Agrobiological Sciences (NIAS) in Japan maintains 34,652 accessions (landraces ~4.4%) (de Carvalho et al. 2013).

Nowadays, *ex situ* conservation faces numerous challenges in term of maintenance of large number of accession in various biotic abiotic stresses and changes in different agro-climatic conditions such as global warming. In addition to that, there is a vast collection of valuable data regarding global genetic resources maintained globally in different Genebank. Unfortunately, significant portion of such useful information is not easily accessible to researchers due to lack of infrastructural facilities and lapses in the existing data exchange programs. Particular information about accession such as traits, pedigree, growth and physiological habits are also lack in the Genebank which make collected s and landraces unusable in several existing breeding programs conducted all over the world in wheat growing belts.

15.6.2 In Situ *Conservation*

In situ conservation offers an alternative conservation approach to *ex situ*, in which conservation has high priority than development. Numerous factors involved in promoting the *in situ* conservation are listed below (Brush 1995).

- Fragmentation of land holding in developing world
- Marginal agricultural conditions associated with hill lands
- Heterogeneous soil within farm
- Economic isolation, cultural values of crop to be cultivated
- Conservation of evolutionary events

There are limited resources and effort being allocated for *in situ* conservation of wheat germplasm. However, the European Union projects have the major objective in bringing data from different sources to a common platform to facilitate conservation efforts globally.

Recently, agricultural biotechnology is only being precept as genetically modified crops in the society (Ammann 2005). Biotechnology, however, has numerous applications in the enhancement of crop production, productivity and increase biodiversity of cultivated major crops such as wheat, rice, maize etc. Biotechnology represents several techniques for enhancing genetic diversity in crop through the

introduction of novel genes. Introduction of genes through traditional breeding approaches has many challenges that hindrance expansion of biodiversity which can be overcome through biotechnological approaches. Several biotechnological tools have been employed for wheat biodiversity conservation (Belokurova 2010).

15.6.3 Cryopreservation of Wheat Species

Long term conservation of non-used landraces or wild relative of wheat can be preserved using biotechnological approaches, and those accessions can be used in the future based on requirements. Cryopreservation of wheat suspension culture and subsequent callus regeneration after long time preservation reported for variable retention of important genotypes of different species of wheat (Chen et al. 1985).

15.6.4 Cell and Tissue Culture Techniques

Tissue culture based techniques for plant regeneration via organogenesis or embryogenesis is most common in almost all the crop species. However, it is important to identify suitable culture systems to minimize unwanted genetic variations during preservation and subsequent regeneration. Most likely, regeneration of plantlet from callus culture is associated with somaclonal variation or chromosomal rearrangements that restrict the tissue culture techniques to be used for germplasm conservation. Using explants such as apical meristems have been suggested as one of the most successful approach for avoiding wide genetic variability during conservation process. In addition, preserving both somatic as well as zygotic embryos are also regarded as viable alternatives for successful conservation (Villalobos et al. 1991).

15.6.5 Seed Germplasm Bank

Collection and storage of viable seeds is most common and feasible approach for germplasm conservation. Germplasm of cereal crops including wheat can be stored in two types of collections (i) working collections, and (ii) preservation. Seeds can be storage at near freezing and low humidity through this way viability can be maintained for 10 years or longer (Sachs 2009). Long term storage of wheat seed needs ambient temperature gradient between −10 to −20 °C. Seeds can be efficiently stored for long term purposes after carefully drying the seed and then storing in specialized sealed vessels. Another approach suggested is to vacuum pack the seeds in specially designed aluminum foil envelopes or in storage cans. Seeds can also be stored by using cryogenic methods like application of liquid nitrogen (Walters et al. 2005).

15.7 Conclusion

Wheat represents an important food crop species that is closely related to global food security. It is therefore important to conserve all existing landraces, genotypes and germplasm of different types of wheat (including both wild and cultivated species) to protect the wide global genotypic diversity of this crop. Such genetic variability will be useful for future breeding programs and biotechnological improvements for developing high yielding, disease and stress resistant varieties locally suitable for different agro-climatic regions of the world. Rapid loss of genetic diversity of wheat has been reported from different corners of the world and hence it is important to initiate an efficient and effective integrated global wheat conservation program to conserve diverse species including landraces and germplasm of this valuable food crop. We have explored the current status of wheat biodiversity and identified several factors hampering such diversity, globally. We have also suggested potential measures for wheat conservation from a multi-disciplinary perspective with special emphasis on modern biotechnological approaches.

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