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

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**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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M.R. Ahuja, K.G. Ramawat (eds.), *Biotechnology and Biodiversity,*
Sustainable Development and Biodiversity 4, DOI 10.1007/978-3-319-09381-9_14

## 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-

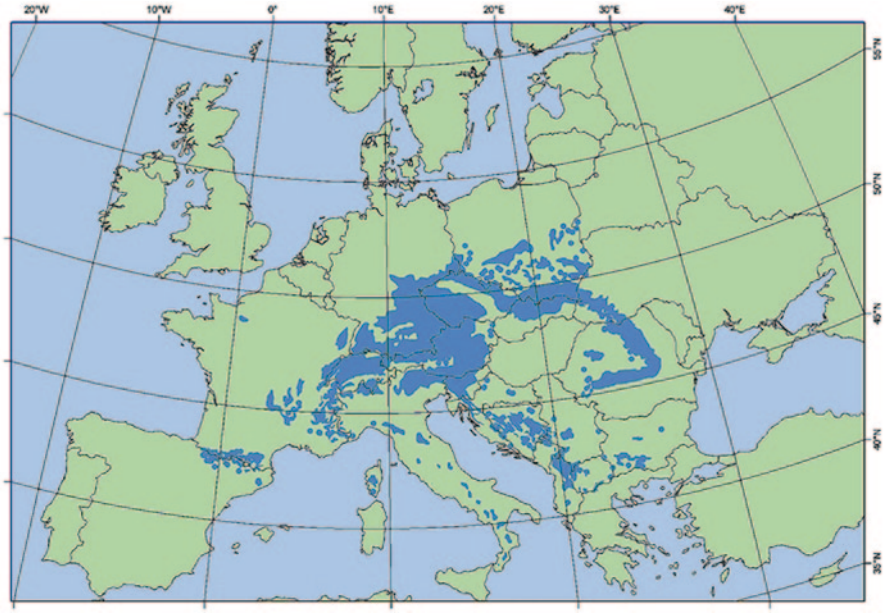


**Fig. 14.1** Distribution map of silver fir (*Abies alba*). EUFORGEN 2009. (http://www.euforgen.org)

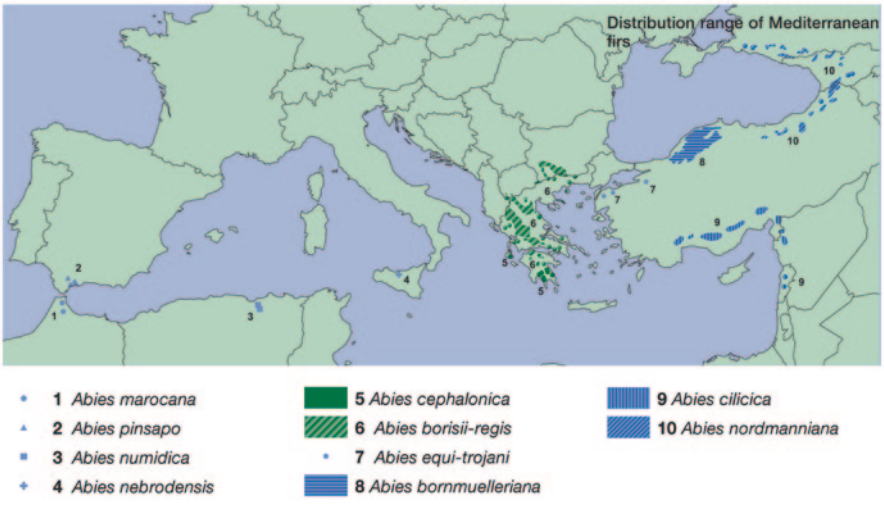


**Fig. 14.2** Distribution range of Mediterranean firs. (Alizoti et al. 2011)

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.*

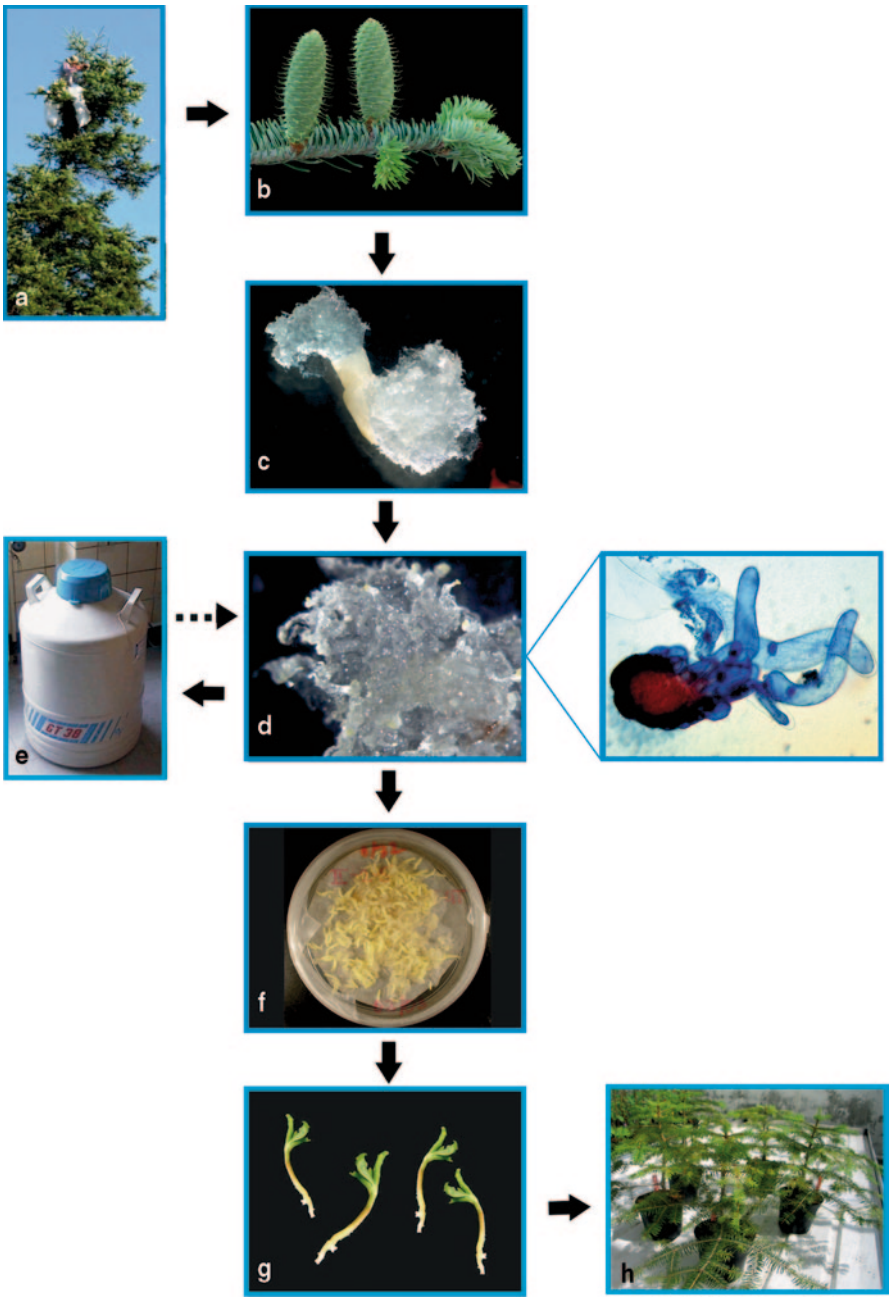


**Fig 14.3** Somatic embryogenesis of *Abies cephalonica*. **a** Elite tree of *A. cephalonica*. **b** Developing green cone shortly after meiosis. **c** Initiation of somatic embryogenesis using immature embryos and proliferation of embryogenic cell mass. **d** Proliferating embryogenic cell mass and 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<br>0.2 M sucrose/24 h<br>0.4 M sucrose/24 h<br>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<br>0.2 M sucrose/ 24 h<br>0.4 M sucrose/24 h<br>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<br>0.4 M sucrose/24 h<br>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<br>0.4 sorbitol/24 h<br>Samples placed on a 120 rpm rotary shaker<br>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<br>A. alba × A. numidica one cell line | 0.4 M or 0.8 M sorbitol applied for 24, 48 or 72 h<br>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.

**Acknowledgment** Authors thank the EUFORGEN as the source of information for downloading the distribution maps from http://www.euforgen.org/distribution_maps.html.

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