Fate of forest tree biotechnology facing climate change

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Abstract

Woody plants have been cultured *in vitro* since the 1930s. After that time much progress has been made in the culture of tissues, organs, cells, and protoplasts in tree species. Tree biotechnology has been making strides in clonal propagation by organogenesis and somatic embryogenesis. These regeneration studies have paved the way for gene transfer in forest trees. Transgenics from a number of forest tree species carrying a variety of recombinant genes that code for herbicide tolerance, pest resistance, lignin modification, increased woody biomass, and flowering control have been produced by *Agrobacterium*-mediated and biolistic methods, and some of them are undergoing confined field trials. Although relatively stable transgenic clones have been produced by genetic transformation in trees using organogenesis or somatic embryogenesis, there were also unintended unstable genetic events. In order to overcome the problems of randomness of transgene integration and instability reported in *Agrobacterium*-mediated or biolistically transformed plants, site-specific transgene insertion strategies involving clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) platform offer prospects for precise genome editing in plants. Nevertheless, it is important to monitor phenotypic and genetic stability of clonal material, not just under greenhouse conditions, but also under natural field conditions. Genetically modified poplars have been commercialized in China, and eucalypts and loblolly pine are expected to be released for commercial deployment in USA. Clonal forestry and transgenic forestry have to cope with rapid global climate changes in the future. Climate change is impacting species distributions and is a significant threat to biodiversity. Therefore, it is important to deploy strategies that will assist the survival and evolution of forest tree species facing rapid climate change. Assisted migration (managed relocation) and biotechnological approaches offer prospects for adaptation of forest trees to climate change.

Keywords: *Biotechnology, Clonal propagation, Genetic engineering, Transgenic trees, Genetic stability, Transgene containment, Climate change, Climate mitigation and Adaptation*

Introduction

Theoretical basis for cell culture and concept of totipotency in plant cells were first formulated by Gottlieb Haberlandt in 1902. It was almost three decades later when Roger Gautheret in 1934 was the first to culture pieces of cambium cut from trees (*Salix caprea*, and *Acer pseudoplatinus*, and *Sambucus nigra*) in culture media. Initially, he did not expect much success, but two months later when he examined his culture, he noticed callus growth on them. Unfortunately, Gautheret's cultures ceased to grow after 6 months, because of some deficiency in the culture medium. Six years later, Gautheret (1940) was able to obtain bud formation from cambial tissues of *Ulmus campestris* L. and *Populus nigra* L. A decade later, Ball (1950) was able to induce buds on callus cultures of *Sequoia sempervirens*. Winton (1968) was the first to achieve plantlet formation from callus cultures of triploid quaking aspen. That was the beginning of clonal plant production in angiosperm forest trees by tissue culture technology. In a sense, it was the beginning of vegetative propagation by micropropagation in forest trees. Vegetative clonal propagation by organogenesis, that is, development of organs from plant tissues *in vitro*, has been successfully exploited in a number of angiosperm forest tree species (Bonga and Durzan, 1987; Ahuja, 1988a, 1991, 1993a, Ahuja and Libby, 1993a, 1993b; Ahuja et al., 1996; Pardos et al., 1994; Ramawat et al. 2014). And field trials have been conducted in a number of forest tree species to ascertain the phenotypic and genetic fidelity of clonal trees.

Although clonal propagation by organogenesis has often been difficult in conifers because of recalcitrance and maturation state of the trees (Bonga et al., 2010; Bonga, 2012), there

have been a number of reports on the plantlet regeneration by organogenesis in conifers. The first successful attempt to clonally propagate a conifer by organogenesis was in *Pinus palustris* by Sommer et al., (1975). Subsequently clonal propagation by organogenesis was achieved in *Pseudotsuga menziesii* (Gupta and Durzan, 1987), *Pinus* spp. (Diego et al., 2008, 2010; Cortizo et al., 2009; Bello-Bello et al., 2012; de Oliveira et al 2012), *Larix deciduas* (Ewald 2007), and *Sequoia sempervirens* (Boulay, 1978; Arnaud et al., 1993; Ahuja, 1996, 2017; Korban and Sul, 2007). However, somatic embryogenesis has been more successfully exploited for the clonal propagation of a large number of conifer tree species using embryonal tissues and other explants. The first successful reports on somatic embryogenesis appeared in 1985, using immature embryos in *Picea abies* (Hakman et al., 1985; Chalupa, 1985), and *Larix deciduas* (Nagmani and Bonga, 1985). After these reports, there has been an explosion of somatic embryogenesis in a large number of conifers, and angiosperm trees (Jain and Gupta 2005; Park et al. 2010; Park and Bonga, 2012, 2014; Park et al., 2016; Bonga, 2016; Guan et al., 2016; Trontin et al., 2016; Tikkinin et al., 2018; Bonga et al., 2018). Although somatic embryogenesis has been obtained for many conifer species, it has achieved an industrial level of propagation with only a few (Park et al., 2016). However, clonal propagation from mature trees *in vitro* still remains a problem in some forest tree species, mainly in conifers; but in angiosperms micropropagation has been achieved from mature trees. Therefore, rejuvenation of organs/tissues from recalcitrant mature trees is a necessary prerequisite for clonal propagation *in vitro*, and has been accomplished in some tree species. Subsequently much progress has been made in the culture of tissues, organs, cells, and protoplasts in tree species. Tree Biotechnology has been making rapid progress in clonal propagation by organogenesis and somatic embryogenesis (Bonga and Durzan, 1987; Ahuja, 1993a; Park et al., 2016). Genetic control of differentiation and genetic stability of clones produced by organogenesis and somatic embryogenesis are important aspects of *in vitro* regeneration that need to be addressed in a clonal forestry program. Although there are limitations in cost-effective production of clonal propagules by organogenesis and somatic embryogenesis in forest trees, constructive and realistic biotech approaches are underway to overcome the limitations for large scale plantations of micropropagated clonal trees.

In this review, I shall discuss tree biotechnology and climate change in the framework of clonal propagation, genetic engineering in trees, and clonal forestry and genetically modified trees facing climate change. I shall briefly describe that some of the tree clones are ancient, and have been there for thousands of years (so-called Methuselah), and then discuss clonal forestry by vegetative propagation (macropropagation), and micropropagation. This will be followed by genetic transformation and confined field trials of transgenic trees, and finally I will discuss strategies for mitigation of global warming and adaptation of clonal forestry and transgenic forestry to cope with climate change.

Ancient and potentially immortal clones

Some of the forest trees have long life spans of thousands of years, and have become Methuselah trees. The oldest living forest trees in the world are the bristlecone pines (*Pinus longaeva*) first described to be 4845 years old (Schulman, 1958), but later revised to be 5062 years old by 2012 (www.rmtrr.org/oldlist.htm). The family Cupressaceae has also some of the oldest living conifers. These include 3622 years old alerce ($2n=4x=44$) (*Fitzroya cupressoides*), 3266 years old giant sequoia ($2n=22$) (*Sequoiadendron giganteum*), and 2200 years old hehaploid coast redwood ($2n=6x=66$) (*Sequoia sempervirens*) (see www.rmtrr.org/oldlist.htm). Whereas in *Sequoia sempervirens* vegetative propagation occurs naturally from stump sprouts and burls (O'Hara et al., 2017), other Methuselahs in this group do not normally clonally propagate in nature by vegetative propagation. However, there are tree species that have produced ancient clones that are thousands of years older than these Methuselahs.

Natural clones of some forest trees have been around for thousands of years, and in a sense, clones of such trees may be considered potentially immortal! There are a number of plant species in which ancient clonal colonies have survived for thousands of years. One of the oldest living clone is known as "Pando" (a Latin name meaning "I spread"). This ancient clone of quaking aspen (*Populus tremuloides*) belongs to the family Salicaceae, and its age has been estimated to be around 80,000 to 1,000,000 years. The Pando clonal colony is located in the Fish Lake National Forest, north of Bryce Canyon National Park in central Utah. Vegetative propagation occurs in aspens from root suckers (or basal sprouts). Pando's clonal colony is world's largest and massive known organism, and has about 47,000 tree trunks, each with a normal complement of leaves and branches. Pando covers 43 hectares (106 acres) of the forest area (Grant et. al., 1992; Grant, 1993; Mitton and Grant, 1996). Pando originated from a male tree and its clonal colony shares the common root system, and the same genetic constitution (DeWoody et al., 2008); and Pando most likely is a triploid ($2n=3x=57$) clone (Mock et al. 2008; DeRose et al., 2015). Triploidy is widespread in aspens, and the three genomes in triploids compared to two in diploids aspens, may offer distinct advantages (heterozygosity, improved growth, and reproduction by vegetative means) for the survival of diversity in Pando genets in different environmental conditions (DeRose et al., 2015; Greer et al., 2017).

Another ancient forest tree clone belongs to a disjunct population of Palmer's oak (*Quercus palmeri*), belonging to the family Fagaceae, is located in the Jurupa mountains of Riverside county in California. Based on annual ring counts from a number of stems, the age of this clone was estimated to be around 13,000 years. This ancient oak species presumably spouted from a single acorn during Pleistocene, and has continued to clone for the last thousands of years (May et al., 2009). This ancient clone shows morphological homogeneity, allozyme polymorphism, and fire induced sprouting all support the hypothesis that Palmer's oak is a single ancient clone (May et al. 2009). Widespread fruit abortion suggests that Palmer's oak may be an ancient triploid clone. The current status of this

species is categorized as threatened, and may become extinct in the near future.

Although spruces do not normally reproduce by vegetative means, there is an exceptional Norway spruce (*Picea abies*), belonging to the family *Pinaceae*, that also seems to be an ancient clone. This Norway spruce clone was nick-named by Leif Kullman (2008) as “Old Tjikko” after his late dog (Siberian Husky) and considered to be many thousand years old. This ancient Norway spruce clone is about 5 meters (16 ft) tall, and is located in the Fulufjället Mountains of Dalarna Province of Sweden. This ancient clone has survived for thousands of years due to vegetative cloning. The present visible tree is relatively young, around 600 years old, but it is a part of a much older root system. The trunk of this clone may live up to 600 or more years, and when it dies, another trunk grows back in its place from the old root system by a process of layering. According to Kullman, heavy snow can bring branches close to earth, and new shoots can sprout from the contact point. Fossil wood pieces and cones have been recovered from this ancient clone, and their carbon dating suggests its age to be around 9,500 years, and they have the same genetic constitution as the root system (Olberg and Kullman, 2011).

Global warming is playing roulette with forest tree refugia from northern Hemisphere as they are moving poleward for genetic adaptation. However, how well will these refugia adapt to their new environment remains largely unknown. Although ancient clones are uncommon in the members of the family *Pinaceae*, more recently ancient clone has been found in larch (*Larix* spp). Larches are native to most of the temperate regions of northern hemisphere. Larches are among the dominant trees in the boreal forests of Siberia and Canada. One of the refugia is Dahurian larch (*Larix gmelinii*) is growing at a favorable site in the southern Taimyr Peninsula located in northcentral Siberia. Although larches can reach an age up to 609 years in Siberia when growing as upright trees (Abaimov, 2010), and up to a 1000 years for North American species (*Larix lyallii*) (Arno and Habeck, 1972). Other individuals from the family *Pinaceae* attain ages of thousands of years. But most of them are not ancient clones.

Kruse et al., (2020) analyzed 16 nuclear microsatellite markers in 194 larch individuals to determine their genotypes. The age of the individual genotypes was determined by counted the tree rings. Their findings suggest that majority of the individuals in their study belong to genetically identical groups, and the predominant mode of reproduction was clonal as evidenced by short distance spreading of ramets. Clonal propagation was achieved by lateral growth/layering of spreading branches at the ground level. Based on tree rings the age it was determined that the clones were at least 2,200 years old, and these larch clones have survived the harsh climate of Tundra for hundreds and thousands of years (Kruse et al., 2020).

King's Holly (*Lomatia tasmanica*) is a small tree usually between 2-4 meters tall, but sometimes reaching a height of 6-8 meters, is a native shrub-tree of Tasmania. It belongs to the family *Proteaceae*. The population of *Lomatia tasmanica* occurs over a distance of 1.2 km, and consists of several hundred stems. Genetic studies, based on allozyme analysis, indicate

that *Lomatia tasmanica* is an ancient clone and possibly one of the oldest living plants approximately 43,000 years old (Lynch et al., 1998). The chromosome counts in the somatic cells indicate that *Lomatia* is a triploid ($2n=3x=33$), compared to four other of *Lomatia* which are diploid ($2n=22$). The entire population of this species may be composed of one genet, as fruit production has not been observed. *Lomatia* regenerates continuously by root suckering (Lynch and Balmer, 2004). It has been postulated that *Lomatia tasmanica* is a hybrid originating by hybridization between *Lomatia polymorpha* and *Lomatia tinctoria* (Lynch et al., 1998).

In a changing world environment, many species are under considerable pressure to evolve, or face the extinction risk. clonal plants would be at a disadvantage, because of their narrow genetic base and genetic homogeneity, limited mobility, and capacity for adaptation. So how have these ancient clones survived and outlived the previous environmental changes, and persisted over the millennia. One possible hypothesis suggests that gene regulation through epigenetic mechanisms (changes) might provide the optimum phenotypic variation for clonal spread, adaptation and survival in a changing climate (Douhoniokoff and Dodd, 2015; Dodd and Douhoniokoff, 2016). Another possibility may be the presence of triploidy in certain plant species that can propagate in nature by asexual methods. The advantage of triploidy lies in its heterozygosity, that buffers the effect of gene redundancy on mutations, and triploids generally exhibit vigorous vegetative growth. And in certain cases, as in the ancient aspen clone Pando, Palmer's oak clone, and King's Holly clone, their survival is seemingly further facilitated by vegetative propagation.

Regeneration and clonal forestry

Clonal forestry involves production and use of vegetative propagules by vegetative propagation. Clonal propagation has been achieved by macropropagation or micropropagation. Vegetative propagation by macropropagation involves traditional vegetative propagation techniques, while micropropagation is carried out by tissue culture technology.

Macropropagation

In the past several decades serious consideration has been given to genetic improvement of forest tree species. This involves hybridization between superior individuals and then establishment of seed orchards. In order to achieve genetic gain in forest tree species, it would be necessary to cross breed elite trees for at least a few generations, each requiring anywhere from 10 to 50 years. One way to capture genetic gain more readily in the same generation is to clonally propagate selected genotypes by vegetative means. In this respect, clonal forestry has certain distinct advantages over zygotic forestry. Clonal forestry involves efficient management and increase of genetic gain by selection and maintenance of additive and non-additive gene effects. On the other hand, through sexual reproduction only additive gene effects can be utilized; the non-additive gene effects arising from interaction of genes are not normally transmitted through generative reproduction (Ahuja and Libby, 1993a). The non-additive gene effects can give rise

to exceptional individuals within superior families (in out-crossed plants, such as forest trees), and these can be captured along with additive gene effects by vegetative propagation, or by crossing homozygous individuals. Clonal forestry is not new. Vegetative propagation has been part of the evolutionary history of herbaceous plants and forest trees, and ancient clones have existed for thousands of years.

Micropropagation

Regeneration of forest trees has been routinely achieved by organogenesis and somatic embryogenesis. Genetic control of regeneration and morphological and genetic stability of regenerants by organogenesis and somatic embryogenesis have been investigated in a number of forest tree species.

In vitro regeneration potential of tissues depends on several parameters. These include tissue source (axillary bud, nodal segment, cotyledon, zygotic embryo, shoot tip, bud meristem), physiology, developmental stage, time of the year explants cultured, age of the donor and genotype of the donor trees, and media composition and *in vitro* environment.

The use of regeneration technology in forestry offers prospects for: (1) manipulation of the mature state of tissues, (2) germplasm preservation by long-term *in vitro* cultures, and cryopreservation, and (3) somaclonal variation for isolation of useful genotypes, and (4) gene transfer and production of transgenic trees.

Although, somaclonal variation in trees is usually undesirable (Tremblay et al., 1999; Breton et al. 2006; Marum et al., 2009), a few useful somaclonal variants have been reported in tree species (Ahuja, 1998; Bairu et al., 2011; Krishna et al., 2016). Some of these useful variants include: height and morphological traits in poplar (Fry et al., 1997) and olive (Leva et al., 2012); disease resistance in poplar (Ostry and Ward, 2003), apple citrus, and mango (Krishna et al., 2016); different growth rates in poplar (Gamburg and Voinikov, 2013); and stress tolerance in radiata pine (Montalbán et al., 2014).

These regeneration studies have paved the way for genetic transformation and production of transgenics carrying a variety of transgenes that code for herbicide tolerance, insect resistance, lignin modification, increased woody biomass, and reproductive manipulation, in a number of different forest tree species. Clonal forestry and transgenic forestry have come of age. However, more research is needed for their application in forestry.

Micropropagation from mature trees

We have employed tissue culture technology for rapid clonal propagation of a number of forest tree species. Our aim was to optimize cost-effective protocols for micropropagation of selected genotypes, whose clones exhibited phenotypic and genetic stability not only in greenhouse, but also under field conditions. The second objective was to find out the ability of the propagules of an introduced species to adapt to a new environment. In both cases phenotypic and genetic fidelity of clones was also an important consideration. Here I shall focus on micropropagation of selected trees of aspens (*Populus tremula*, *P. tremuloides*, and their hybrids), and coast redwood

(*Sequoia sempervirens*) introduced into Germany for its ability to survive under temperate and cold climates. We have employed direct organogenesis for micropropagation of mature trees.

Populus-Aspens

At the Thuenen-Institute of Forest Genetics at Grosshansdorf, Germany, elite genotypes had been selected in European aspen (2n=38) (*Populus tremula*) and quaking aspen (2n=38) (*P. tremuloides*), and their hybrids (2n=38) following controlled crossings between European aspen and quaking aspen (*P. tremula* x *P. tremuloides*) (Melchior, 1985). The hybrid aspens have exhibited good form and growth under field trials (von Wuehlisch and Ahuja, unpublished). Aspens are difficult to propagate vegetatively by woody stems cuttings, because of lack of preformed root primordials. On the other hand, poplars can be easily propagated by stem cuttings (hardwood cuttings), which root readily in soil or peat-perlite substrate. However, aspens can be vegetatively propagated by root suckers, grafting, and juvenile green shoots. Because of cost factors, propagation by grafting, green shoots, and root suckers had limited commercial applicability in the aspen regeneration programs. For this reason, aspens have so far been mostly propagated by seed (zygotic forestry). However, tissue culture technology offered prospects of rapid large scale micropropagation of aspens. We (Ahuja and Muhs, 1985) undertook clonal propagation by organ culture of selected mature aspen and hybrid aspen genotypes by direct organogenesis by tissue culture technology.

Dormant buds from 17 to 40 years old aspen and hybrid aspen were collected during winter months and stored at 0°C. Bud meristems from more than 100 aspen and hybrid aspen clones were cultured in Aspen Culture Medium (ACM) (Ahuja, 1983), which is a slightly modified version of Woody Plant Medium (WPM) (Lloyd and McCown (1981) for their regenerative potential. Initially, a 4-step micropropagation method (Ahuja, 1983) was employed for clonal propagation of aspens (Fig. 2, 4-step method). This method consisted of: (1) Bud break and conditioning of bud meristems in ACM-1 (ACM + 20 mg/l adenine sulfate + 0.5 mg/l BA), (2) growth proliferation in ACM-2 (ACM + 0.2 mg/l BA + 0.02 mg/l NAA) (Fig. 1 A), (3) rooting of microshoots in ACM-3 (ACM + 0.5 mg/l IBA + 0.1 mg/l NAA) (Fig. 1 B), and (4) transfer of plantlets derived from ACM-3 to pots (after sloughing off agar from the roots) for hardening and acclimatization in controlled chambers and greenhouse (Ahuja 1983, 1986, 1987).

A

B

C

Fig. 1

Microshoot regeneration in Hybrid aspen in the culture medium ACM-2 (A); plantlet regeneration in the culture medium ACM-3 (B), and 25 years-old hybrid aspen trees produced by tissue culture in field trials in northwestern Germany (C)

Subsequently, the 4-step method was simplified so as to reduce the number of steps to make the micropropagation method cost-effective (Fig. 2). First the 4-steps were reduced to three, by eliminating step 1, and accomplishing both meristem conditioning and shoot proliferation on ACM-2, without any appreciable impact on growth and differentiation (Fig. 2, 3-step method). Later step-3 (ACM-3) was also eliminated from the scheme, and microshoots were rooted directly in

Fig. 2

Diagrammatic representation of 4-step method, 3-step method, and 2-step method for micropropagation of aspen clones from mature trees. By simplification of this procedure from 4-step to 2-step protocol, it is possible to reduce costs for clonal propagation (From Ahuja and Muhs, 1985)

once autoclaved soil-free potting mixture under high humidity controlled environment (Ahuja, 1984). Thus, it was possible to evolve a 2-step micropropagation procedure (Fig 2, 2-step method) from the original 4-step method, not only to reduce costs, but also to minimize the exposure of tissues to prolonged cultural conditions for minimizing somaclonal variation. There was a very little or negligible amount of callus formation in our direct organogenesis method for micropropagation. The micropropagated aspens showed phenotypic and genetic stability when grown under greenhouse and field conditions (Ahuja, 1983, 1987, 1993b). We have randomly checked chromosome numbers of some of the micropropagated plants. Aspens have a diploid chromosome number of $2n=38$, and our tested plants had the same genetic diploid constitution (Ahuja, 1984). By employing the 2-step micropropagation method, we regenerated several thousand plantlets from more than 30 selected mature aspen and hybrid aspen clones. Twelve hybrid aspen clones have been approved under the category "Tested Material" in the multiclinal variety 'Grosshansdorf', Germany. We have established a number of field trials for testing micropropagated aspens and hybrid aspen clones, along with zygotic seedlings, and some of these are now more than 25 years old (Fig. 1, C) and are still undergoing phenotypic and genotypic evaluations (von Wuehlisch and Ahuja, unpublished).

Initial data from 48 clones revealed that regeneration of shoot differentiation was achieved in about half (22 of 48) of

the clones tested (Ahuja, 1983). Subsequently, additional data on 70 aspen clones also revealed that half (37 of 70) tested showed good microshoot differentiation (Ahuja, 1984). Similarly, there was a 1:1 segregation in 39 clones (17 of 39) for shoot differentiation vs. no differentiation in the female and male hybrid aspen clones (Ahuja, 1993b). Genetic control of microshoot differentiation by organogenesis and somatic embryogenesis in tissue cultures were also observed in both angiosperm (Wann and Einspahr, 1986; Coleman and Ernst, 1989; Coritzo et al., 2009; Erst et al., 2014), and conifer (Klimaszewska et al., 2007; Trontin et al., 2016) tree species.

Coast redwood (*Sequoia sempervirens*)

Coast redwood (*Sequoia sempervirens*) is an interesting and unique conifer. *Sequoia* is a long-lived conifer belonging to the family *Taxodiaceae*, (now included under *Cupressaceae*). It has the world's tallest trees, one measuring ~113 m in height (Koch et al. 2004). However, recently another *Sequoia* tree called Hyperion, which measured ~115 m in height, has become the tallest tree in the world (Sillett et al., 2015). *Sequoia* is the only conifer that is hexaploid ($2n=6x=66$), and origin of its enigmatic polyploidy still remains unresolved. It is not known when the polyploidy *Sequoia* evolved from its diploid ancestors, and which really are its putative progenitors (Stebbins, 1948; Ahuja and Neale, 2002; Ahuja, 2009). *Sequoia* reproduces by both sexual (seed) and vegetative methods (from basal sprouts and

A

B

C

D

Fig. 3

Micropropagation of *Sequoia sempervirens* showing microshoots (A), and rooted plantlet (B). Greenhouse grown micropropagated plants (C), and more than 25 years old frost-tolerant *Sequoia* trees (photograph by Dr. Georg von Wuehlisch) in the arboretum (D) of Thuenen-Institute of Forest Genetics, Grosshansdorf, Germany

burls) to maintain heterozygosity and adaptability for survival. It is a resilient conifer that presumably originated as a polyploid from its putative progenitors most likely during Cretaceous and Tertiary periods (~ 135-65 mya) (Ahuja, 2005, 2009a). *Sequoia* is an endemic restricted in its natural habitat to the fog belt of California and southern Oregon, USA. It grows from sea level to 900 m altitude, but prefers altitudes lower than 750 m,

in a frost-free period ranging from 6-11 months in a year (Olson et al. 1990). Because of its polyploid nature, meiosis is irregular and seed set is low in *Sequoia*, varying from 1-10 %, and only 5-10 % of the seeds are viable (Olson et al., 1990; Ahuja and Neale, 2002; Ahuja, 2005). Although older *Sequoia* trees (genotype dependent) are relatively less susceptible to frost, seedlings in the Pacific Northwest region, where

Sequoia is an endemic, are vulnerable to infection by a fungus *Botrytis* and frost damage (Olson et al., 1990). For this reason, clonal forestry offers prospects for propagating selected genotypes by tissue culture.

Sequoia has been grown out of its endemic region with varying degrees of success in different parts of the world, including France, Germany, Spain, United Kingdom, Turkey, Chile, South Africa, and New Zealand (Kuser et al., 1995; Meason et al., 2016; Klápště et al., 2020).

Micropropagation has been achieved in *Sequoia* by using meristems, nodal stem explants, and needles of juvenile explants, as well tissues from stump shoots of mature trees that were 90 years old (Boulay, 1978; Ball et al., 1978; Arnaud et al., 1993; Bon et al., 1994; Sul and Korban, 2005; Korban and Sul, 2007). Somatic embryogenesis and organogenesis was also reported by tissue culture of needles of *Sequoia* (Liu et al., 2006). In general, stump shoots from the base of mature *Sequoia* trees are more responsive to *in vitro* regeneration compared to shoots from the crown of the same mature trees.

During the winter of 1985-1986, a *Sequoia* plantation near Cologne, Germany was badly hit by the frost and extremely low temperature around -15°C. A number of trees survived the winter. The owner of the plantation sent branches from three (two 25 years old, and one aged 8) frost-tolerant *Sequoia* trees, to the Thuenen-Institute of Forest Genetics, Grosshansdorf, for clonal propagation and to test their ability to tolerate frost in Germany. We cultured bud meristems from the three trees on a modified woody plant medium fortified with low concentrations of BAP (0.2 to 0.5 mg/l) and NAA (0.02 mg/l) (Fig. 3 A), and rooted microshoots (Fig. 3 B) in a hormone-free medium, and *ex vitro* in a peat-perlite potting mixture, and later the young plants were grown in the greenhouse (Fig. 3 C) (Ahuja, 1996, 2011a). More than 1000 plants were produced from the three trees by tissue culture, and several hundred clones were tested under field conditions for testing their overwintering capacity. Field trials from the clones derived from three frost-tolerant trees were conducted for a number of years at Grosshansdorf and Trenthost, in northern Germany. Trees in Grosshansdorf arboretum seem to be frost-tolerant and have survived more than 25 winters in Germany (Fig. 3 D). However, under field conditions at Trenthorst, only those clones survived that were sheltered by a tree canopy towards the edge of the field. Therefore, sheltering of *Sequoia* clones derived from frost-tolerant trees were necessary during several years of their early growth period for their later survival in harsh winters in temperate climate zones (Ahuja, 1996, 2011a, 2017).

Based on these regeneration studies on forest trees, it is suggested that an ideal method for micropropagation should have the following criteria (modified from Ahuja, 1987):

- It is relatively simple,
- Gives high frequency microshoot multiplication rate,
- Has a high degree of reproducibility,
- Applicable to juvenile and mature tissue,
- Applicable to different genotypes

- Induces direct differentiation of microshoots, or somatic embryos, with minimum callus formation,
- Gives high frequency of plantlet formation *in vitro*, or *ex vitro*,
- Gives high survivable of plantlets or somatic embryos after potting and transfer to greenhouse,
- Easily adaptable to greenhouse and nursery conditions for acclimatization,
- Ensures, to a large extent, genetic stability of propagules under *in vitro* environment, *ex vitro* greenhouse, nursery and field conditions, and in progeny tests,
- Most importantly, it is cost effective, that is, costs of micropropagated plantlets or somatic embryos are comparable to zygotic seedlings.

Although such an ideal method of micropropagation is not yet available for organogenesis or somatic embryogenesis for forest trees, at least some aspects of such a procedure have been accomplished in *Populus* and some other tree species. And major biotech efforts are progressing rapidly for making clonal forestry by micropropagation commercially feasible.

Genetic engineering in trees

Although genetic improvement of forest trees has mainly progressed by selection and breeding methods (Zobel and Talbert, 1884; White et al. 2007; Neale and Kremer, 2011; Eriksson et al., 2013), these approaches have limited applicability in long-lived forest trees, with extended vegetative phases ranging from one to many decades. Genetic engineering (GE), on the other hand, offers prospects of transfer of desirable genes into selected genotypes at a comparatively faster rate by bypassing the prolonged breeding process. Thus, transfer of genes with desirable traits by traditional approaches, involving breeding and recurrent selection would that take decades to centuries in forest trees, can be accomplished by genetic engineering in a single generation. Furthermore, GE overrides incompatibility barriers and allows gene transfer not only in unrelated tree species, but also between widely divergent taxa. At the same time GE also removes potentially undesirable effects of linked alleles to transferred genes which could be inadvertently introduced to the progeny by conventional breeding programs (Ahuja, 2009b, 2011b).

After establishing well defined regeneration systems, both by organogenesis and somatic embryogenesis, it became possible to transfer recombinant genes in forest trees by GE. Ever since the first report on the *Agrobacterium*-mediated transfer of herbicide tolerant gene to poplars (Fillatti et al., 1987), there has been considerable interest in the application of GE to forest trees by *Agrobacterium*-mediated or biolistic methods. During the last three decades, a number of forest trees have been genetically engineered with a variety of different recombinant genes, using tissues derived from organogenesis (leaf discs, microshoots, different tissues/organs) and somatic embryogenesis (embryonal cells/mass) for the production of transgenic trees (Charest and Michel, 1991; Fladung, Muhs, Ahuja, 1996; Ahuja, 2000; Trontin et al., 2002, 2007; Tang and Newton, 2003, Charity et al., 2005; Klimaszewska et al.,

2007; Lee et al., 2014; Uddenberg et al., 2016). The first wave of GE involved the transfer of “foreign” genes (mostly bacterial genes) used as reporter genes to monitor the integration and expression of transgenes in forest trees. This was followed by a second wave which included transfer of economically important genes for herbicide tolerance, pest resistance, lignin modification, yield traits, and reproduction manipulation (both bacterial and plant genes) to the forest tree genome (Ahuja, 2001, 2009b, 2011b, 2014a; Boerjan, 2005; Brunner et al., 2007; Hoenicka et al., 2012; van Acker et al., 2014; Chang et al., 2018; Klocko et al., 2018; Bruegmann et al., 2019a). However, there were a number of technical problems and limitations to in vitro regeneration of selected genotypes in forest trees. Some of these problems have been addressed and progress has been made and genetically modified forest trees are undergoing confined field testing of their growth performance and biosafety (Häggman et al., 2013; Porth and El-Kassaby, 2014; Ahuja, 2014a, 2014b; Vettori et al., 2016).

Transgene stability

Forest trees have long generational cycles with vegetative phases extending from one to several decades. Genetic and phenotypic stability of genetically modified trees are important considerations for subsequent large scale plantations of GE trees (Ahuja, 1988b, 1997, 2000; Hawkins et al., 2003; Hoenicka and Fladung, 2006a; Brunner et al., 2007; Porth and El-Kassaby, 2014). In addition, the potential risk concerning escape of transgenes from GE trees, horizontal gene transfer, vegetative propagation capacity of root suckers (for example, in aspen), effect of GM trees on target species, and effects of GM trees on non-target feral tree populations acquiring the transgens needs to be addressed before commercial deployment of GE trees can begin (Brunner et al., 2007; Farnum et al., 2007; Smouse et al., 2007; Ahuja, 2009b, 2011b, 2014a). Because integration of transgenes in the genome of host-plants occurs randomly and at non-predictable genomic sites, variable transgene expression could be one of the consequences. Other possible consequences are putative disruption leading to knock-out or even over-expression of host-genes (Fladung et al., 2005). One possible solution could be the targeted integration of foreign genes into so-called “safe-heavens” of the host genome by the application of site-specific recombination systems, which has been successfully tested in poplars (Fladung et al., 2010; Fladung and Becker, 2011).

Stability of transgenes in the vegetative propagules and across reproductive generations, and transgene containment are important considerations for long-lived forest trees that will ultimately determine their future in forestry (Ahuja, 2009b, 2011b; Ahuja and Fladung, 2014). Stability of transgene expression and functional utility of a transgene in time and space in the forest tree genome are also important considerations for transgenic research (Ahuja 2000). As trees grow, they increase in size and complexity with their increasing age, and changes in gene expression are likely to occur during seasonal cycles and in a changing environment. Therefore, promoter fidelity and genetic stability of transgene expression have to be viewed at several different levels in the long-lived forest trees. In

this context, the activity of some transgenes (for example, herbicide tolerance) may be required for a short duration during early growth, while others to remain active throughout the life (for example, pest resistance, lignin reduction, yield traits), and still others would be required at a specific time (for example, reproductive sterility) of the forest tree growth and development (Ahuja, 2009b, 2011b, 2014a).

Variation in transgene expression has been widely reported in transgenic herbaceous plants and forest trees. Variation in gene expression may be caused by somaclonal variation (Larkin and Scowcroft, 19981; Ahuja, 1987, 1998), integration patterns and copy number of a transgene, and inactivation/silencing of the transgenes in the host genome (Finnegan and McElroy, 1994; Ahuja and Fladung, 1996; Fladung et al. 1997; Ahuja, 1997; Kumar and Fladung, 2002; Butaye et al., 2005; Wagner et al. 2005; Ladics et al., 2015). Detailed studies have been carried out in *Populus* and several other forest tree species for determining the stability of transgenes on a short-term and long-term basis.

One study used the *rolC* gene from *Agrobacterium rhizogenes*, which is a dominant gene that affects the plant phenotype, particularly the leaf size and color when introduced in a plant. Since *rolC* gene expression can be monitored at the phenotypic level, it can be used as a selectable marker to assess its phenotypic and molecular expression, as well as genetic stability in the transgenic plants. The phenotypic effects of *rolC* gene have first been tested in the annual plant species tobacco (*Nicotiana tabacum*) (Spena et al. 1987; Schmuelling, Schell, and Spena, 1988) and potato (*Solanum tuberosum*) (Fladung, 1990; Fladung and Ballvora, 1992).

A morphological marker has a distinct advantage, when compared to the time-consuming analysis of biochemical markers, such as β -glucuronidase (GUS) gene, at specific intervals, because it can be easily observed at all times during the life cycle of a transgenic plant. Four selected clones from aspen (*P. tremula*) and hybrid aspen (*P. tremula* x *P. tremuloides*) were genetically transformed by using the leaf-disc *Agrobacterium* co-cultivation method. Two *rolC* reporter gene constructs with different promoters (35S::*rolC* and *rbcS*::*rolC*) were employed in this study, showing different leaf sizes (Fig. 4 A) (Ahuja and Fladung, 1996; Fladung et al., 1996, 1997). Transgenic aspens carried 1-2 copies of the *rolC* transgene (Fig. 4 B). Transgenic lines carrying the *rolC* transgene were grown in the greenhouse for more than 19 years (Fladung et al., 2013), and in a confined field trial carried out for 5 years on the grounds of the Thuenen Institute of Forest Genetics, Grosshansdorf, Germany (Fladung et al., 2004; Fladung and Hoenicka 2012; Pilate et al., 2016). Variation in phenotypic expression was observed in a number of transgenic plants both in the greenhouse (Fig. 4 C, D) and field conditions (Ahuja, 2000; Fladung et al., 2004). Results from this study have revealed that most of the transgenic plants carrying a single copy of the transgenes were relatively more stable than, those carrying more than one copy of the transgene (Ahuja, 2000; Fladung and Kumar, 2002; Kumar and Fladung, 2002; Fladung, Hoenicka, and Ahuja, 2013).

A

B

C

D

Fig. 4

Transgenic hybrid aspen clone Esch5 carrying two and one copies of the *35S::rolC* (left, middle, respectively), along with a wild-type Esch5 clone (right), showing different sizes of leaves conditioned by the *rolC* gene (A); Southern blot showing one and two copies of the transgenes in transgenic hybrid aspen Esch5 clone (B). Bottom row shows chimeras in *35S::rolC* transgenic hybrid aspen clone Esch5 (C), and Brauna 11 (D) carrying *rbcS::rolC* transgene"

Detailed confined field studies, ranging from 3 to 8 years have also been carried out in genetically modified poplars and white spruce. Transgenic poplars showed relatively stable expression of the herbicide tolerant gene up to 8 years under confined field trials (Li et al., 2008). Genetically modified dwarf white spruce (*Picea glauca*) carrying the *Bt* gene (derived from soil bacterium *Bacillus thuringiensis*). *Bt* gene produces an insecticidal protein) also showed a continued insecticidal activity in confined field trials for 5 years (Lachance et al., 2007). Generally, stable expression of transgenes was detected with one or a few copies of transgenes in these studies (Brunner et al., 2007; Li et al., 2008). However, there were exceptions in another study, where one or fewer copies of a transgene were not always associated with stable expression of the transgene, as was reported in the antifungal transgene (pinosylvin synthase) activity in poplars (Seppänen et al., 2004).

Because of the randomness of transgene integration and instability in the *Agrobacterium*-mediated and biolistically transformed plants, a site-specific, and a highly efficient and precise gene integration strategy, involving CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS (CRISPR)-associated protein9 (Cas9) genome editing system (CRISPR-Cas9) has been used for targeted mutagenesis in forest trees (Tsai and Xue, 2015; Fan et al., 2015; Zhou et al., 2015; Brueggemann et al., 2019b). CRISPR has become one of the most promising tool in genetic engineering, and offers the potential for substantial improvement over other gene editing technologies in its efficacy, speed and low cost. The power of the CRISPR-Cas9 platform lies not only in its ability to precisely engineer “foreign genes” (genes derived from other organisms) at specific locations in the host genome, but also native genes for the production of transgenic plants. Another utility of this gene targeting system lies in the potential for activating/editing native plant genes for drought tolerance, frost tolerance, disease resistance and other qualitative and possibly quantitative traits, rather than introducing exogenous genes (derived from outside the host organism) for these traits. Such innovations will pave the way for next generation biotech crops/trees 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 (Ahuja, 2014b). Although still in infancy, the CRISPR-Cas9 genome targeting system is evolving rapidly and seems to accomplish both gene insertion and gene mutation in plants/trees (Li et al., 2015; Ma et al., 2016; Tycko et al., 2016; Puchta, 2017; Sukegawa et al., 2021). The CRISPR-Cas9 gene targeting system also offers prospects for altering the genome in somatic embryogenesis or organogenesis cultures of forest tree species for genetic improvement and perhaps scaled-up, cost effective clonal forestry.

Transgene containment and regulation

Several approaches have been proposed to impede the escape and containment of transgenes (Brunner et al., 2007; Ahuja, 2011b; Hoenicka et al., 2012b). These include: (1) reproductive sterility and early flowering (Strauss et al., 1995; Hoenicka and Fladung, 2006b; Brunner et al., 2007; Traenkner et al., 2010;

Klocko et al., 2016, 2018; Briones et al., 2020), (2) site-specific excision of transgene mediated by recombinase systems, where the functional transgene is removed from the gametes before flowering (Luo et al., 2007; Gidoni et al., 2008; Fladung et al., 2010), and (3) introducing transgenes into the chloroplasts genome to reduced transgene escape via pollen (Bock 2006; Verma and Daneill, 2007). Transgene escape from transgenic populations is a virtual reality, and none of these approaches would likely achieve 100 percent transgene containment in the forest trees in a real world (Ahuja, 2009, 2011b; Ellstrand, 2018). Although, studies on containment of transgenes are being actively pursued, their successful application to forest trees, with extended vegetative phases, still remains a challenging problem. One alternative non-GE approach would be by isolating the transgenic populations to delimited areas, or using incompatible genotypes of wild tree populations neighboring the genetically modified trees (Ahuja, 2011b).

The commercialization status of genetically modified forest trees is presently at the crossroads. The future of genetically modified forest trees still remains controversial and uncertain, as a number of biological and regulatory issues relevant to transgenic trees need to be addressed and resolved before their commercial deployment (Ahuja, 2009b, 2011b, 2014a, Bradford et al., 2005; Finstad et al., 2007; Custers et al., 2016). Forest scientists view that the regulatory obstacles have hindered progress of transgenic forest biotechnology (Strauss et al 2009a). Until now the US regulatory requirements had focused on the insertion and integration of the foreign genes and possible genetic changes caused by the transgene (process), and not in the trait (product) itself in the host plant (Sederoff 2007; Strauss et al., 2009a). The consensus among forest scientists has been on the contrary; that the regulatory scrutiny should be on the product and not on the process (McHughen and Smyth, 2008; Strauss et al., 2009b). More recently, USDA (2019, 2020) has revised its regulation status and has relaxed its rules on the genetically modified crops. The regulatory oversight will now be based on the product (trait) and not entirely on the genetic engineering process. Hopefully, this change in the policy would open new avenues for deregulation of forest trees in USA. Although, pest resistant poplars have been commercialized in China since 1990's (Ewald et al., 2006), only recently frost-tolerant transgenic eucalypts hybrid (*Eucalyptus grandis* x *E. urophylla* S) (Wear et al., 2014) and genetically modified loblolly pine (*Pinus taeda*) engineered for increased wood density (Miller et al., 2019) are undergoing the deregulation process in the United States of America, and are expected to be released in the United States of America's market in the near future.

Climate change impacts on forest tree

Global climate has been changing in the past and will continue to change in the future. What is new is that according to current estimates by meteorologists global warming has been occurring at an accelerated rate. The Intergovernmental Panel on Climate Change (IPCC) has made projections on increase of global mean temperatures by the end of the current century. The Fourth Assessment Report (AR4) by IPCC (2007) predicted

a global temperature increase between 2.4°C to 6.4°C, accompanied by significant changes in precipitation (Allan and Sodan, 2008; O’Gorman and Schneider, 2009; Trenberth, 2011), while the AR5 report by IPCC (2014) predicted a temperature rise between 2.6°C and 4.8°C, depending on the locations with low or high emission of carbon dioxide (CO₂). However, a recent IPCC (2018) assessment report suggests that global warming is likely to reach 1.5°C between 2030 and 2052, if it continues at the current rate. These changes in global warming are likely caused by anthropogenic greenhouse gases, mainly CO₂, resulting from the industrial combustion of fossil fuel in the atmosphere, and have contributed to about 78 % of the total greenhouse gas emissions increase from 1970 to 2010 (IPCC 2014).

Rapid climate change may impact plant/tree species in a number of different ways, including: (1) range shifts, migration, and geographical redistribution (Aitken et al., 2008; Iverson et al., 2005, 2008; Mckennedy et al., 2007; Kelly and Goulton 2008; Thuiller et al., 2008; Iverson and McKenzie, 2013; Monleon and Lintz 2015; Takolander et al., 2019); (2) shifts in phenology (e.g. flowering time) (Franks et al., 2007; Craufurd and Wheeler, 2009; Anderson et al., 2012; Gaira et al., 2014; Prevy et al., 2019); (3) threat to biodiversity (Botkin et al., 2007; Bellard et al., 2012; Javeline et al., 2013); (4) ecosystem disturbance and wildfires (Kirilenko and Sedjo, 2008; Littell et al., 2010; Stuetzel et al., 2013; Bellard et al., 2014; Mahi et al. 2019); (5) forest growth, drought and mortality (Battles et al., 2008; Allen et al., 2010, 2015; Quirk et al., 2013); (6) changes in genetic and evolutionary response (Davis et al., 2005; Hoffmann and Willi, 2008; Hoffmann and Sgro, 2010; Rehfeldt et al., 2014); and (7) extinction risks (Thomas et al., 2004; Pearson et al., 2014; Urban, 2015). In addition to the above mentioned impacts on plants, climate change is also presumable causing changes in the environment, including increased drought conditions, changes in precipitation, and increase in sea levels. Clonal forestry and transgenic forestry will have to cope with rapidly changing climate and global warming.

Climate change mitigation and forest tree adaptation

A range of technologies has to be employed for mitigating climate change and global warming. There are at several geo-engineering mitigation and adaptation strategies that are currently employed to cope with CO₂ and global warming (Zhang et al., 2015; Fawzy et al., 2020). One is land-based for CO₂ sequestration by large scale reforestation programs (Nave et al., 2019; Domke et al., 2020). A recent study (Bastin et al., 2019) suggests that about 0.9 billion hectares of land world-wide would be suitable for reforestation, which could ultimately capture two thirds of human-made carbon emissions. Such a reforestation program would serve as a powerful tool for carbon sequestration from the atmosphere. In order to meet the 1.5°C target, it would be required to remove at least some of the carbon dioxide that was previously emitted. The future goal of global warming mitigation would be to achieve **Net-Zero** carbon emissions by 2050 (Matemilola and Salami, 2020). Net-Zero emissions by 2050 would require global alliances

between nations for rapid and large scale reduction of greenhouse gases and other toxic elements in the atmosphere, and carbon sequestration (Fuss et al., 2020; Rogelj et al., 2021)). Second approach is *atmosphere-based* and involves injecting aerosols (e.g. sulfur dioxide) into the stratosphere by aircrafts for solar radiation management. The sulfur dioxide is oxidized in the atmosphere to sulfuric acid, which has a low enough vapor pressure to form cloud droplets to block solar radiation (Robock, 2014). The third approach is *ocean-based*, and involved ocean fertilization by adding iron (iron sulfate), urea (source of nitrogen), and phosphorus to stimulate phytoplankton growth and population. Phytoplanktons absorb CO₂ through photosynthesis and form a food chain in the ocean. When phytoplanktons die, they sink taking the photosynthate with them to ocean floor, thus sequestering atmospheric CO₂ (Williamson et al., 2012). The fourth approach is *albedo-based* that involves reflection of incoming short-wave radiation from sunlight by the leaf surface by plants and tree canopy (Davies-Barnard, 2014). The *albedo* affect could be overcome by growing plants/trees with that have broad and waxy leaves/needles to deflect solar energy.

There are at least four broad options for adaptation of forest trees to rapid climate change: (1) strategies for mitigation of climate change, (2) translocation of clonal material by managed relocation (one type of assisted migration) to new locations to pursue biological diversity, ecosystem functioning as an adaptation strategy for coping with climate change, in the event that the current habitat is to become unsuitable because of climate change (Richardson et al. 2009; Schwartz et al. 2012; Locatelli et al., 2015), (3) selection of genotypes from the germplasm in the forest tree species of interest that are tolerant to environmental stresses, including drought, frost, and pests, and are amenable for *in vitro* clonal propagation, and (4) employment of biotechnological approaches to produce novel genotypes that are resilient and tolerant to climate change effects. I shall discuss climate change mitigation, and biotechnological approaches to cope with climate change in the following sections.

Biotechnological strategies for coping with climate change

Global climate change is impacting species distributions and functioning and their terrestrial ecosystem (Parmesan, 2006; Thuiller et al., 2008). There is substantial evidence to suggest that species ranges are shifting (Iverson and McKenzie, 2013; Monleon and Lintz 2015; Takolander et al., 2019) and some species are facing extinction risks (Thomas et al., 2004; Schwartz et al., 2006; Cahill et al., 2013; Romn-Palacios and Wiens, 2020). Different models have been used to estimate plant migration patterns to potentially suitable habitats under the future climate change scenarios, and it appears that many North American species will likely shift their ranges at a rate of 10 to 100 km/100 years to keep pace with the predicted climate change in this century (Davis et al., 2005; Iverson and Prasad, 2005; Iverson et al., 2019). Iverson et al., (2008) have examined the potential response of 134 tree species in the USA under different climate change scenarios in this century. Depending on

the climate change model, they predicted that more than a one-third of the tree species could shift their ranges more than 400 km northwards, and in the hottest climate change scenario, most of the species would advance up to 800 km northwards (Iverson et al., 2008).

These studies suggest that it would be necessary to devise strategies for the future survival and evolution of forest trees facing unpredictable climate change. In this direction, biotechnological approaches involving gene editing offer several options for producing transgenic trees that can adapt to climate change. These include: (1) engineering trees for improved growth rates, canopy morphology, and enhanced photosynthetic activity for improved carbon sequestration; (2) genetically engineer to produce transgenic trees that have broad and waxy leaf surface facing sunlight to reflect heat back into space, and (3) produce transgenic trees that are stress tolerance. Here I shall focus on two stress-related traits that will be important for the adaptation of forest trees to climate change. These include tolerance to drought and frost (cold) stress. Drought tolerance would be critical for forest trees for their continued adaptation and survival in their original locations affected by global warming. Frost tolerance, on the other hand, would be important for those tree species that could not adapt to global warming and had to be moved by assisted migration to colder climates for their survival.

One approach involves use of *somaclonal variation* in tissues of selected clones *in vitro* to induce epigenetic or genetic changes for stress tolerant trait. In several studies, polyethylene glycol (PEG) has been used in plant tissue cultures to induce stress tolerance in plants (Joshi et al., 2011; Montalbán et al. 2014; Mishra et al., 2021). Towards this goal, I suggest, induction of callus formation in both organogenesis and somatic embryogenesis cultures of forest tree species by using appropriate levels of PEG and/or 2,4-dichlorophenoxyacetic acid (2,4-D) (Liu et al., 2013), at temperature ranges (25–30°C), and select for plantlets or somatic seedlings tolerant to drought and/or frost.

The second strategy calls for transfer of stress tolerant genes (drought tolerant or frost tolerant) into selected clones of a forest tree species by *genetic engineering*. Although both drought tolerance and frost tolerance may be controlled by a number of genes, their biosynthetic pathways are still unknown. Since drought and frost tolerance are polygenic traits, genetic transformation of a single gene appears to have limited potential on drought and frost stress tolerance. However, a number of transcription factor binding proteins related to drought and frost tolerance have been isolated and identified. These include *CDPK* (Calcium dependent protein kinase), *DREB* (dehydration responsive element binding protein), *CBF* (c-repeat binding factor) transcription factors for drought; and *CBF1* (DRE-binding protein) and *COR* (cold responsive gene) for frost tolerance isolated from plant/tree species (Winiewski et al., 2014; Polle et al. 2019; Chang et al., 2021). The transgenes encoding transcription factors binding proteins activate the drought tolerance (Kudo et al., 2017; Polle et al., 2019; Schulz et al., 2021) and cold tolerance (Chinnusami et al., 2007; Wisniewski et al., 2014; Wani et al., 2016; Gao et al., 2021) inducible

genes, which have been used for genetic transformation of a number of mostly annual plant species.

There are two options for stress tolerance gene transfer: (1) transfer of stress tolerant transcription factor binding protein coding gene (drought tolerance or frost tolerance), along with a pest tolerant (*Bt*) gene in a selected forest tree clone, and/or, (2) genetically engineer drought tolerance or frost tolerance transcription factor genes into a transgenic genotype that already carries a transgene for an economic trait (for example, pest resistance). As it turns out, both pest resistance and drought tolerance traits would be useful, as with increasing temperatures and humidity, insect pests population will also increase (Battisti and Larsson 2015; Sangle et al., 2015; Skendvžić et al., 2021). Test these genotypes in greenhouse with drier or colder atmospheres. Alternatively, move transgenic genotypes by managed relocation (a type of assisted migration) to pole-ward locations to test for frost-tolerance, or in drier southward locations for testing drought tolerance. It is suggested that tissues/organs from these transgenic genotypes should be stored in gene banks using cryopreservation techniques (Keller and Senula 2010; Gonzalez-Arnan et al., 2014) for future exploitation to cope with the rapid climate change.

Concluding statement

Clonal forestry by organogenesis and somatic embryogenesis has been making progress. However, there are still some limitations to the commercial deployment of clonal material. Among them are the technical problems to clonally propagate mature selected genotypes *in vitro*, and costs of propagule production. These problems may be addressed by innovative techniques in biotechnology for cost-effective production of forest tree clones by *in vitro*. Clonal forestry and transgenic forestry will have to cope with rapid climate change. CO₂ sequestration by large-scale reforestation programs (Boyd 2010), and geo-engineering (Zhang et al., 2015) offers prospects for combating climate change. Managed relocation offers opportunities to test the capacity of the forest clonal material to adapt to the new environment in the new locations in the face of climate change. Biotechnological approaches using somaclonal variation and genetic engineering would be necessary to produce clonal transgenic materials that are tolerant to climate change stresses.

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