CURRENT STATUS OF BIOTECHNOLOGY DEVELOPMENT AND APPLICATION IN FORESTRY

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1. Introduction

Forests and other wooded areas provide wood and timber, as well as a vast array of economic, environmental and social products and services, including employment, shelter, energy, nutritious foods and a wide range of other goods and ecosystem services. Forests also play a very significant role in carbon sequestration, with a net removal of an estimated 2 million gigagrams of carbon dioxide equivalents per annum. The world's total forest area is just over 4 billion hectares, *i.e.* 31 percent of the total land area (2012). Approximately 13.2 million people are employed in the formal forest sector, 41 million in the informal sector, 840 million people collect woodfuel or charcoal, 1.3 billion people use forest products as main materials for shelter, 2.4 billion people use fuelwood to cook their food or to boil water (FAO, 2014a).

Forests host much of the world's biodiversity, but this diversity is threatened by a high rate of deforestation due to the expansion in global agricultural and industrial needs. Other challenges include forest diseases, pests and weeds, many of which are introduced from other regions, so that 50 percent of the forest tree species are threatened by, or subject to genetic erosion (FAO, 2014b).

Forest trees have certain characteristics that differentiate them from species used in other agricultural sectors, such as crops and livestock - for example, their highly heterozygous nature, long generation intervals, vulnerability to inbreeding depression, narrow regional adaptation and the fact that the majority of the species are undomesticated (FAO, 2011)[2], thus generating unique challenges and opportunities for biotechnology applications. As elaborated in this paper, biotechnological approaches have advanced considerably in the last decade and have contributed to creating more efficient and effective characterization, conservation and utilization strategies for forest genetic resources, as well as advanced technologies for mass propagation, genetic improvement and forest biomass utilization. Of the over 700 tree species reported by countries to FAO in the process of preparation of The State of the World's Forest Genetic Resources, as subject to tree improvement programmes, 241 species were mentioned as involved in biotechnology research (FAO, 2015).

This paper has the purpose of briefly reviewing the development of biotechnology and discussing its current and potential application in the forestry sector, with special attention to smallholders and to tropical areas. Based on the definition of 'biotechnology' in Article 2 of the Convention on Biological Diversity (*i.e.* "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use") the term 'biotechnology' is defined here in a broad way and encompasses a large number of technologies applied in the forest sector (IUFRO and FAO, 2010). Further description and background of the biotechnologies described in this paper can be found in Chapter 6 of FAO (2011).

[2] "The concept of plant domestication can be interpreted in a restrictive sense as a process of changing of the biological characteristics of a species, or in a more extended sense as a process of change in plant exploitation practices, which brings with it changes in the plant's morphology and genetics, as well as in its growing environment" (Wiersum, 1996).

2. Study of forest biological diversity and conservation of forest genetic resources

A. Molecular markers

Molecular markers are used to study the level, structure and origin of genetic variation within and among species in both naturally regenerated and planted forests (Lidder and Sonnino, 2012; Porth and El-Kassaby, 2014a). Molecular marker studies, thus far, have suggested that natural populations of most tropical tree species contain higher levels of variation than most other plants (Kindt *et al*., 2009). However, the extent of genetic diversity in tropical forest species is still largely unknown. Molecular markers have helped to differentiate species in forest inventories but a thorough cataloguing will require good linkages with field botanists (Dick and Kress, 2009). For tropical trees, a practical protocol guide on molecular marker methods (Muchugi *et al*., 2008a) as well as an accompanying guide on the effective handling and analysis of the datasets generated (Kindt *et al*., 2009) have been produced by the World Agroforestry Centre.

Genetic variation information provides important insights for decision-making in forest genetic resources conservation. For instance, molecular markers along with climate modelling allowed to forecast the long-term decline of the late-successional Australian rainforest conifer, *Podocarpus elatus*, due to habitat fragmentation, and to urge for adoption of adequate conservation strategies (Mellick *et al*., 2013). Molecular markers were used to identify those teak populations which should be given the highest priority for *in situ* conservation and to establish seed zones to safeguard the current genetic resources of teak in Myanmar (Thwe-Thwe-Win *et al*., 2015).

Accurate taxonomic identification together with the ability to discriminate hybrids from pure species and to estimate the degree of introgression is essential to develop effective conservation strategies (Wang and Szmidt, 2001). Molecular markers are used to help resolve phylogenetic relationships of tree species that are difficult to distinguish on the basis of morphological characteristics alone, for example between species of *Warburgia* (Muchugi *et al*., 2008b), *Populus* (Cervera *et al*., 2005) and *Quercus* (Zeng *et al*., 2010). A standard two-locus DNA barcode has been proposed for species identification of terrestrial plants, including forest trees (CBOL Plant Working Group, 2009).

Molecular markers have facilitated the identification of natural hybrids across species thus leading to a better understanding of introgression, for instance between *Fraxinus excelsior* and *F. angustifolia* (Fernández-Manjarrés *et al*., 2006), *Populus alba* and *P. tremula* (Lexer *et al*., 2005), *Pinus echinata* and *P. taeda* (Xu *et al*., 2008), and *Quercus suber* and *Q. ilex* (Burgarella *et al*., 2009). Furthermore, natural hybrid zones, besides being important sources of genetic diversity (Hamilton *et al*., 2013; De la Torre *et al*., 2014), are valuable for investigating evolutionary processes of speciation (Tovar-Sánchez and Oyama, 2004) as well as for identifying Quantitative Trait Loci (QTLs) for adaptive genetic variation (Lexer *et al*., 2004).

Molecular markers have offered insights into the domestication of forest trees, such as the origin of the cultivated olive tree, which originated from domestication of the *Olea oleaster* (Besnard *et al*., 2001; Breton *et al*., 2006). Additionally, based on nuclear and cytoplasmic markers, nine domestication events have been proposed for olive cultivars (Breton *et al*., 2009), while Amplified fragment length polymorphism (AFLP) data have suggested two distinct geographic origins of cultivated *Spondias purpurea* trees in Mesoamerica (Miller and Schaal, 2006). Molecular marker studies have also shed light on the evolutionary history of other tree species, for instance the postglacial routes of colonization of *Populus nigra* from two main refugia in Italy and/or the Balkans and Spain (Cottrell *et al*., 2005); the evolutionary pattern divergence bewteen two North American balsam poplars (*Populus trichocarpa* and *P.*

balsamifera), and one Eurasian aspen (*P. tremula*) (Ismail *et al*., 2012); and the loss of genetic diversity after domestication in *Jatropha curcas* (Sanou *et al*., 2015).

Since most forest trees are outcrossing, they do not generally show evidence of strong genetic differentiation among populations and the highest genetic diversity is found within populations. Molecular markers have been used to measure genetic variation in tropical trees, for example within and between populations of *Calycophyllum spruceanum* in the Peruvian Amazon Basin (Russell *et al*., 1999), *Sesbania sesban* in sub-Saharan Africa (Jamnadass *et al*., 2005), *Tectona grandis* in India (Narayan *et al*., 2007) as well as diverse geographical regions in Indonesia and Thailand (Shrestha *et al*., 2005), and *Guaiacum sanctum* in Costa Rica (Fuchs and Hamrick, 2010). Similar analyses on molecular genetic diversity have been carried out in temperate tree species (Krutovski, 2006), for example within and among populations of *Fagus grandifolia* in Mexico (Rowden *et al*., 2004), *Robinia pseudoacacia* in China (Huo *et al*., 2009) and *Sorbus torminalis* in Europe (Rasmussen and Kollmann, 2008).

Molecular markers have been utilized to help evaluate the efficiency of agroforestry systems for conservation of forest genetic resources by comparing the genetic variation across natural forest and proximate planted farm stands. Random Amplification of Polymorphic DNA (RAPD) studies of the timber tree Meru oak (*Vitex keniensis*) in central Kenya showed little differentiation between unmanaged and managed stands (Lengkeek *et al*., 2006). Another study assessing the genetic diversity between planted and natural stands of *Inga edulis* from five sites in the Peruvian Amazon demonstrated lower allelic variation in planted stands, even though on-farm stands contained on average 80 percent of the allelic diversity of natural stands (Hollingsworth *et al*., 2005). An explanation for the difference between the two studies could be that while all on-farm *I. edulis* was of planted origin, the Meru oak in Kenya may have been planted or naturally regenerated. Classification into ethno-varieties of *Vitellaria paradoxa*, useful in conservation and breeding programmes, was reconsidered using microsatellite markers (Gwali *et al*., 2014).

The levels of genetic variation within clonally propagated domesticated stands of *Spondias purpurea* were significantly lower than in sexually reproducing wild populations (Miller and Schaal, 2006). Within the cultivated populations, trees in orchards harboured less genetic variability than trees in backyard gardens and living fences.

Knowledge of mating systems and gene flow is important for understanding genetic drift, natural selection and population divergence, and when designing conservation strategies that maximize connectivity of populations in fragmented forests while minimizing unwanted gene flow. For example, microsatellite data evaluating the mating system and pollen gene flow in oak (*Quercus semiserrata*) in northern Thailand showed high outcrossing rates, high levels of gene flow from outside populations and heterogeneity in the pollen composition received by individual trees, suggesting strategies to prevent genetic diversity losses in at the study site (Pakkad *et al*., 2008). Genetic diversity within and between populations in disturbed and undisturbed teak forests within the natural range of the species, the mating system and contemporary gene flow have also been studied using molecular markers (Volkaert *et al*., 2008).

Maternally and paternally inherited markers make it possible to distinguish the separate contributions from pollen and seed in gene flow studies (Jones *et al*., 2006; Sork and Smouse, 2006; Dick *et al*., 2008; Hamza, 2010). Molecular information has demonstrated that gene flow through pollen dispersal is significantly higher (20 to nearly 200 times) than gene flow through seeds, at least among wind-pollinated species (Savolainen *et al*., 2007) and tree species, such as *Quercus macrocarpa*, that produce large, seeds which are not widely disseminated (Dow and Ashley, 1998). Molecular markers have been used to study pollen-mediated gene flow in populations of both wind pollinated and animal pollinated forest trees (Burczyk *et al*., 2004), finding that long distance pollen dispersal (over 5 to 10 km) is not uncommon (Petit and Hampe,

2006); this could have significant implications for the conservation of trees in fragmented stands (White *et al*., 2002; Kamm *et al*., 2009).

Gene flow from planted to natural stands, *i.e*. anthropogenic hybridization, can have a profound effect on the diversity and adaptability of wild tree populations. Most poplar plantations in China, Europe and North America represent a very narrow genetic base (since they are clonally propagated) and could lower the effective population size and alter the evolutionary potential of nearby native poplar populations. Molecular markers have provided evidence for gene flow between cultivated poplars and native black poplar trees, with the frequency of hybridization depending on the size of the native population compared to the plantations (Broeck *et al*., 2004; Broeck *et al*., 2005). Extensive hybridization has also been observed between the native North American butternut, *Juglans cinerea*, and the introduced Japanese walnut, *Juglans ailantifolia* (Hoban *et al*., 2009).

B. Genomics and other "Omics"

The first forest trees whose genome was sequenced were *Populus trichocarpa* (Tuskan *et al*., 2006) and *Eucalyptus grandis* (Myburg *et al*., 2011; Myburg *et al*., 2014). Both species are characterized by relatively small genomes (450 and 500 Mb, respectively) and high economic value. Sequencing of the very large genomes (10 Gb and greater) of gymnosperm forest trees became tractable only more recently, after the introduction of next-generation sequencing (NGS) technologies. Currently, complete draft genome sequences have been published for several conifers, including *Picea abies* (Nystedt *et al*., 2013), *Picea glauca* (Birol *et al*., 2013), and *Pinus taeda* (Neale *et al*., 2013). Draft genome sequencing has been completed also for *Populus tremula*, *P. tremuloides*, and their hybrids, *P. grandidentata*, *P. nigra*, *Salix purpurea*, *Eucalyptus camaldulensis*, *Corymbia citriodora*, *Betula nana* and *Fraxinus excelsior* (Neale *et al*., 2013). For other forest tree species, including *Azadirachta indica*, *Castanea mollissima*, *Larix sibirica*, *Pinus lambertiana*, *Pinus pinaster*, *Pinus sylvestris*, *Pseudotsuga menziesii* and *Quercus robur*, entire genome sequencing is under way or nearly completed.

Association genetics[3], including genome-wide association studies (GWAS)[4], is used to identify several promising candidates genes responsible for adaptive traits of forest trees, such as growth, wood properties and adaptation to environmental stresses (Eckert *et al*., 2009a; Eckert *et al*., 2009b; Dillon *et al*., 2010; Neale and Kremer, 2011; Harfouche *et al*., 2012). In addition to genome sequencing, significant activity is dedicated to transcriptome sequencing and resequencing for polymorphism discovery (Neale *et al*., 2013). Transcriptome profiling is particularly challenging in forest tree species due to their large genome sizes and lack of reference sequences. In spite of this, it has been utilized to study growth (Park *et al*., 2008; Grönlund *et al*., 2009), adaptation to biotic (Heller *et al*., 2008; Azaiez *et al*., 2009) and abiotic stresses (Holliday *et al*., 2008; Kreuzwieser *et al*., 2009, Chen *et al*., 2014), and wood production and quality (Carvalho *et al*., 2013). Comparative transcriptomics is being employed to study the molecular basis of fungal resistance (Barakat *et al*., 2009) and drought tolerance (Cohen *et al*., 2010). Proteomics research in forest trees is still limited and restricted to a few genera (Abril *et al*., 2011).

[3] Association genetics or linkage disequilibrium mapping is a strategy for identification of individual loci that contribute to variation in a trait, based on the detection of allelic variants differentially represented in phenotypic categories (Harfouche *et al*., 2012).

[4] Genomic-Wide Association Studies (GWAS) involve scanning markers genomes of many individuals of a particular species to identify genetic variations associated with a particular trait. Both population and family-based designs are commonly used in GWAS. The population-based design is used to detect a potential association, whereas the family-based design is used for replication (Harfouche *et al*., 2012)

Comparative genomics is a promising approach to elucidate gene function in forest trees, considering that the application of other approaches is more complex in the study of forest trees than in other organisms. Comparative genomics tools have become available for *Populus* spp. (Neale and Ingvarsson, 2008; Douglas and DiFazio, 2010) and *Eucalyptus* spp. (Külheim *et al*., 2009; Paiva *et al*., 2011).

NGS and third-generation sequencing technologies are generating tens of millions of increasingly longer DNA sequence reads. Development in this field is so rapid and the costs are decreasing so fast that genotyping-by-sequencing (GBS[5]) is now becoming the germplasm characterization method of choice (FAO, 2015). As GBS becomes more routinely applicable, the corresponding routine applications of association mapping, allele mining, genomic selection (GS) will benefit greatly forest tree improvement approaches, including the introgression of novel alleles (Thudi *et al.*, 2012). The 1000 plants (Onekp or 1KP) initiative, an international consortium engaged in the sequencing of over 1000 plant species, is an example of previously unimaginable endeavours that are taking advantage of the exponential growth in capacities and the accompanying decrease in costs to generate massive amounts of publicly accessible data (OneKP Capstone Wiki[6]).

Besides its application in conservation and utilization of forest genetic resources, forest tree genomics is a tool which can also be used in the management of natural forests, for instance in identifying natural stands that could be used as seed sources for forest plantations (Neale and Kremer, 2011).

C. Bioinformatics[7]

The availability of enormous amounts of data generated by the high-throughput "omic" technologies has necessitated the development of methods for processing, analyzing, integrating, and interpreting the data.

The principal repository of forest tree genomic data is maintained by the Dendrome Project (Wegryzn *et al*., 2008). The associated TreeGenes database[8] currently provides information related to 1290 species belonging to 101 genera, full genome sequences for 13 species, transcriptome or expression resources for 263 species and 106 genetic maps for 35 species (Grau, 2015). The Dendrome Project includes also the DiversiTree interface[9], and the CartograTree tool[10] which associate phenotypic, genotypic and environmental data in a geo-referenced application.

Populus specific databases include the RIKEN *Populus* database[11], the Database of Poplar Transcription Factors (DPTF)[12], and the *Populus* Genome Integrative Explorer (PopGenIE)[13], with expression tools as

[5] A method whereby individual genotypes at a large number of Single Nucleotide Polymorphisms (SNPs) are directly detected by resequencing the entire the entire or a selected fraction of the genome of the individuals to be genotyped. Methods for obtaining the selected fraction can be based on restriction enzyme digestion or on hybridization and capture to oligonucleotide probes (Harfouche *et al*., 2012).

[6] https://pods.iplantcollaborative.org/wiki/display/iptol/OneKP+Capstone+Wiki https://pods.iplantcollaborative.org/wiki/display/iptol/OneKP+Capstone+Wiki

[7] Bioinformatics refers to the research, development and application of computational and statistical tools and information processing methods for the management of biological information (Lidder and Sonnino, 2012).

[8] http://dendrome.ucdavis.edu/treegenes/ http://dendrome.ucdavis.edu/treegenes/

[9] http://dendrome.ucdavis.edu/DiversiTree/ http://dendrome.ucdavis.edu/DiversiTree/

[10] http://dendrome.ucdavis.edu/cartogratree/ http://dendrome.ucdavis.edu/cartogratree/

[11] http://rpop.psc.riken.jp/index.pl http://rpop.psc.riken.jp/index.pl

[12] http://dptf.cbi.pku.edu.cn/ http://dptf.cbi.pku.edu.cn/

[13] http://popgenie.org/ http://popgenie.org/

well as browser tools for synteny[14] and QTLs (Sjödin *et al*., 2009a). *Eucalyptus* specific databases include the EUCANEXT database[15] (Costa Nascimento *et al*., 2011).

D. Spatial analysis of genetic diversity for conservation

The study of genetic variation among trees or populations in combination with the study of their spatial, geographical and ecological distribution, or landscape genomics, allows fine correlative analysis between phenotypes and genotypes. This is used for instance to predict a species' ability to adapt to migrations or to environmental changes (Eckert *et al*., 2010; Sork *et al*., 2013; Lepais and Bacles, 2014). Molecular level study of the genetic variability of teak over the natural distribution area of the species revealed the existence of four centers of genetic variability (Fofana *et al*., 2009). Spatial genetic analysis has been conducted also for many other forest tree species, including *Araucaria angustifolia* (Medina-Macedo *et al*., 2015), *Fagus sylvatica* (Sjölund and Jump, 2015), *Pinus caribaea* (Sanchez *et al*., 2014) and *Populus trichocarpa* (McKown *et al*., 2014).

Altitudinal variation has also been observed within populations of forest tree species, indicating that both vertical and horizontal patterns of genetic diversity must be considered when designing conservation strategies (Ohsawa and Ide, 2008).

E. Studies of forest microbial communities

Biotechnology tools can provide important insights into the nature of entire tropical forest ecosystems, including the relationship between the forest trees and the microbial communities with which they interact (FAO, 2011). These plant-microorganism interactions hold particular relevance because they may confer drought and disease tolerance, which can be particularly beneficial under climate change conditions.

Molecular tools, including genome sequencing of the nitrogen-fixing actinomycete *Frankia*, and transcriptome analyses of inoculated roots, were used to study actinorhizal symbiosis in *Casuarina glauca.* (Zhong *et al*., 2013). These associations had remarkable impact on the adaptation to new sites and productivity of this species (Diagne *et al*., 2013).

Genomics-based research is further elucidating how the symbiotic associations between tree roots, bacteria (Bonfante and Anca, 2009) and fungi (Viera *et al*., 2009), including mycorrhizae, function. The emerging field known as community genomics uses DNA sequencing tools to clarify complex interactions between species within forest ecosystems (Whitham *et al*., 2006) and variations in their response to environmental changes (Ding *et al*., 2015).

Metagenomics[16] is a relatively new field of genetic research that enables studies of unculturable organisms as well as characterization of biodiversity at the ecosystem level (Lidder and Sonnino, 2012) and can provide insights into tropical forest ecosystems, which can influence the strategies employed for managing tropical forests.

F. Cryopreservation

The majority of recalcitrant seeds have been identified in trees and shrubs, with approximately 47 percent of the species from evergreen rain forests having seeds that are desiccation sensitive (Tweddle *et al*., 2003). Thus, cryopreservation is especially important for the long-term conservation of forest germplasm

[14] Synteny is defined as: 'The occurrence of two or more loci on the same chromosome, without regard to their genetic linkage. Increasingly used to describe the conservation of gene order between related' (FAO, 2002)

[15] http://bioinfo03.ibi.unicamp.br/eucalyptusdb/ http://bioinfo03.ibi.unicamp.br/eucalyptusdb/

[16] Metagenomics is defined as 'The study of the genomes of samples taken directly from the environment, for instance, soil samples' (Marco, 2010).

(Pritchard *et al*., 2014), even if this approach has generally been viewed as complementary to *in situ* conservation, as a short-term conservation strategy and a means of safety duplication (FAO, 2014b). The role of cryopreservation is further highlighted in situations where in situ conservation cannot be guaranteed or is difficult. A major disadvantage of cryopreservation is the overall difficulty associated with the regeneration of whole trees from cryopreserved tissues and organs.

As noted above, most forest trees are still undomesticated and cryopreservation protocols have thus been developed and/or optimized for only a limited number of species. Various tissues of softwood and hardwood forest tree species have been successfully cryopreserved, including embryos, embryogenic cultures, seeds, pollen and shoot tips (Panis and Lambardi, 2006). In softwood tree species, cryopreservation has been reported for more than 10 000 genotypes of over 23 conifer species and their hybrids (Tsai and Hubscher, 2004). In conifer clonal forestry, cryopreservation is used as a suitable and efficient means for the storage of embryogenic cultures of clones under selection awaiting field testing results. Clonal varieties can then be multiplied by thawing and propagating the cryopreserved embryogenic tissue clones that have been found to be superior in the field tests (Park, 2002; Sharma, 2005). Cryopreservation (predominantly of shoot tips) is being increasingly applied for hardwood trees such as *Populus*, *Robinia*, *Betula*, *Fraxinus*, *Morus* (Häggman *et al*., 2008), *Eucalyptus* (Kaya *et al*., 2013), and *Quercus* (Barra-Jimenez, 2015).

So far, there is little evidence of genetic alterations in forest trees caused by cryopreservation. Embryo recovery levels for cryopreserved oak (*Quercus* spp.) embryogenic lines ranged from 57 to 92 percent, with no genetic instability observed in the regenerated plants (Sanchez *et al*., 2008). Similarly, the genetic fidelity of silver birch (*Betula pendula*) meristems and mulberry (*Morus* spp.) axillary winter-dormant buds was maintained subsequent to cryopreservation (Ryynänen and Aronen, 2005; Atmakuri *et al*., 2009). The viability of cryopreserved material has also been determined. Dormant european ash (*Fraxinus excelsior*) seeds, cryopreserved for two years following desiccation to a safe water content, did not exhibit decreased germination after thawing after two years of storage at -3°C (Chmielarz, 2009). Likewise cryopreserved pecan (*Carya illinoinensis*) pollen, stored for 1-13 years, did not demonstrate reduced viabilty compared to fresh pollen (Sparks and Yates, 2002). Cryopreservation of dormant elm (*Ulmus* spp.) buds has been shown to be economically competitive to field clonal archives, with a two-fold cost saving in favour of the cryobank (Harvengt *et al*. 2004).

Implementation of cryopreservation in developing countries has been restricted by economic constraints. However, cryopreservation of forest trees has been initiated in some developing countries, for example for pollen of tree species at the National Bureau of Plant Genetic Resources (NBPGR), India, and for species of tree genera such as *Dipterocarpus*, *Bambusa* and *Dendrocalamus* in Indonesia (Jalonen *et al*., 2009). Cryogenic repositories for forest tree species include 420 accessions of mulberry (*Morus* spp.) at the National Institute of Agrobiological Resources, Japan and 440 elm (*Ulmus* spp.) accessions at the Association Forêt-Cellulose (AFOCEL), France (Engelmann, 2011). Since 2015 seeds of spruce (*Picea abies*) and pine (*Pinus sylvestris*) are stored under permafrost conditions in the Svalbard Global Seed Vault on the Spitsbergen Island[17].

G. *In vitro* slow growth storage

Slow growth *in vitro* culture is generally considered as a short- to medium-term conservation strategy because of problems in the management of *in vitro* collections, even if the intervals between subsequent sub-cultures are extended, and also because of concerns of possible genetic instability caused by

[17] http://www.euforgen.org/public-awareness/news/news-detail/first-seeds-of-forest-trees-deposited-at-svalbard/

somaclonal variation (FAO, 2014b). *In vitro* slow growth storage techniques require establishment of specific protocols depending on the type of explant and species under consideration. Another issue to be considered with tropical tree species is the presence of endophytes that can cause difficulties in the establishment of sterile cultures (Muralidharan and Kallarackal, 2005).

Successful protocols using this method have been developed for many forest tree species, including *Pinus radiata, Alnus glutinosa* and species of *Eucalyptus* and *Populus* (FAO, 1994; FAO, 2014b). *In vitro* cultures of *Melia azedarach* apical meristem tips can be maintained for one year without subculture or addition of fresh medium (Scocchi and Mroginski, 2004), up to 10 months for *Eucalyptus grandis* shoots (Watt *et al*., 2000), 11 months for *Garcinia indica* (Malik *et al*., 2005), 60 months for *Populus* species (Hausman *et al*., 1994) and 6 months for *Cedrus* species (Renau-Morata *et al*., 2006) and for *Cedrela montana* (Diaz-Quichimbo *et al*., 2013). An *in vitro* collection, with 32 selected European aspen (*Populus* spp.) clones, has been established in Spain (Martin *et al*., 2007).

3. Mass propagation

Clonal propagation of many economically important tree species is of importance in production forestry, and is used in both coniferous and hardwood species for the multiplication of clones of elite and rare genotypes. Additional advantages of vegetative propagation of forest trees, compared to trees produced from seedlings, include more uniformity in height and trunk girth and, if selected for, increased biomass production and reduced bark fissuring (Muralidharan and Kallarackal, 2005). *In vitro* tissue culture has become a preferred method of clonal propagation of many forest tree species. The development of large scale clonal plantations of some economically important species (*e.g. Eucalyptus* spp, *Tectona grandis*) using biotechnology has been reported by a number of countries including Brazil, Chile, the Republic of Congo, India, South Africa, etc. (FAO, 2015).

Micropropagation techniques have been applied to over 80 genera of forest trees, with five genera, *i.e. Pinus*, *Picea*, *Eucalyptus*, *Acacia* and *Quercus* (FAO, 2004), accounting for 34 percent of forest biotechnology activities reported in *The State of world's forest genetic resources* (FAO, 2014b). Several endogenous and exogenous factors influence *in vitro* growth and the eventual success of micropropagation. In addition to the seasonal effect, type and age of the explants, genotype is a crucial factor in determining the responsiveness of the material to micropropagate (Yasodha *et al*., 2004; Durkovic and Misalova, 2008; Mashkina *et al*., 2010).

In vitro multiplication of desired genotypes can be achieved via axillary budding, adventitious budding, or somatic embryogenesis. Axillary budding refers to the propagation of plants through shoot development from cultured axillary buds. It is the most successful clonal technique for angiosperms and produces the most true-to-type plantlets. Moreover, multiplication rates per subculture cycle can be higher using this technique than in adventitious budding. Protocols using this method have been developed for several species (Pijut *et al*., 2011; Ngezahayo and Liu, 2014), including *Populus tremula* (Peternel *et al*., 2009), *Tectona grandis* (Shirin *et al*., 2005), *Dalbergia sissoo* (Thirunavoukkarasu *et al*., 2010) as well as species of *Eucalyptus* (Glocke *et al*., 2006; Arya *et al*., 2009), *Acer* (Durkovic and Misalova, 2008), *Quercus* (Vieitez *et al*., 2009), and *Liquidambar* (Bayraktar et al., 2015).

Adventitious budding refers to the induction of adventitious buds on non-meristematic tissue and is the preferred method for micropropagation of conifers. Induction rates can be quite high but there is more risk for somaclonal variation. Species of *Pinus* (Alonso *et al*., 2006; Alvarez *et al*., 2009), *Prunus*, *Ulmus* and *Fraxinus* (Durkovic and Misalova, 2008), among others, can be propagated by this technique.

Somatic embryogenesis (SE) is the process of differentiation of somatic embryos from vegetative cells through the application of exogenous growth regulators to juvenile tissue. SE systems have been developed for both conifer species (Nehra *et al*., 2005) and temperate hardwood species (Pijut *et al*., 2007; 2011), with conifer species usually being more intractable (Bonga *et al*., 2010). It has the largest potential multiplication rate and is amenable to handling in automated bioreactors (FAO, 2011). While theoretically and technically feasible for a number of species, the high costs associated with somatic embryogenesis have as yet prevented its use in the commercial propagation of forest trees (Thompson, 2014). It is however likely that production costs will soon be abated through refinements of current protocols.

In Malaysia, teak (*Tectona grandis*) clones with superior wood qualities, have been micropropagated and exported to Australia, Brazil and the United Republic of Tanzania, where they outperformed clones from other sources, displaying a 30 percent increase in yield (Goh *et al*., 2010). *Eucalyptus* hybrid clones constitute a considerable portion of existing commercial plantations, particularly in South America. Clonal forestry of selected *E. grandis* hybrid clones has been shown to reduce wood specific consumption (the amount of wood in cubic meters necessary to produce one ton of pulp) by 20 percent, while second generation clones derived from hybridization with *E. globulus* have led to a further reduction of 20 percent (Grattapaglia and Kirst, 2008).

One criticism raised regarding clonal forestry is that due to a reduction of genetic diversity it may render clonal plantations vulnerable to unexpected outbreaks of diseases and pests. Deploying more clonal lines in mixtures or monoclonal blocks could lower the risk, but also reduce genetic gain. Park (2014) proposes a combination of somatic embryogenesis and Multi-Varietal Forestry to ensure an appropriate balance between genetic gain and genetic variation in the material planted.

Molecular markers can be used to manage clonally propagated domesticated forest tree stands by aiding the selection and identification of clones (Hiraoka *et al*., 2009; Toral Ibañez *et al*., 2009; Aravanopoulos, 2010) and verifying the expected genetic stability of propagated material (Gangopadhyay *et al*., 2003; Lopes *et al*., 2006; Goh *et al*., 2007; Chandrika and Rai, 2009; Huang *et al*., 2009b). Molecular markers are also routinely used in the identification of clones in commercial *Eucalyptus* breeding and production forestry in Australia, Brazil, Chile, Portugal, Spain and South Africa (Grattapaglia, 2008).

Determination of the origin of forest reproductive material used in the establishment of planted forests may be crucial. Molecular markers have been applied to improve traceability through reliable identification of the origin of such material (Finkeldey *et al*., 2010).

National certification schemes have been developed in many countries to ensure production of virus-tested material of forest trees. Sensitive, reliable, and rapid detection techniques to check the health status of the nursery materials are important tools that can be used to reduce the risk of spread of new and emerging plant diseases, as well as to implement effective sanitation procedures for the elimination of viruses from infected materials prior to their multiplication. Biotechnological tools, including NGS, are expected to further enhance efficacy and efficiency of certification schemes and production of virus-tested material (Varveri *et al*., 2015). Regional plant protection organizations have made considerable efforts to harmonize procedures, methodologies, and techniques of disease detection in order to assure the safe movement around the world of propagation materials.

Genomics tools allow for real-time detection and mapping of known and novel pathogens. These tools are utilized to monitor and protect natural forests from invasive pathogens (Porth *et al*., 2015), including nematodes (Kikuki *et a*l., 2009).

4. Genetic Improvement

A. Ploidy level manipulation

Doubled haploid (DH) plants, produced using in vitro anther culture (or alternatively by ovary/ovule culture) and chromosome doubling, are valuable in breeding programs since they are completely homozygous (that is, recessive genes are readily apparent), and the need for numerous cycles of inbreeding is thus considerably reduced by shortening the time needed to select desired lines (Dunwell, 2010). Due to their long regeneration time and tendency to express strong inbreeding depression, forest tree species especially stand to benefit from the production of DH plants. In addition to shortening the breeding period, production of DH trees can be beneficial for the isolation of recessive traits at sporophytic level. Haploids can be useful also for DNA extraction and decoding of large genomes such as those in forest trees (Neale *et al*., 2014; Arrillaga *et al.*, 2014).

Induced haploid production through anther culture has been reported for 32 woody species, including *Populus* spp. and *Quercus* spp., but the success rate has been low and efficient anther culture systems are still limited (Andersen, 2005; Mao *et al.*, 2014). Many forest tree species are intractable in anther cultures and a major impediment is the conversion of calli and embryos into plantlets (Srivastava and Chaturvedi, 2008). An alternative method for obtaining haploid cell lines is the culture of megagametophyte explants, which has been used in *Pinus* spp. (Arrillaga *et al.*, 2014), and *Larix sibirica* (Krutovski *et al*., 2014).

Tetraploids are of potential economic value in forest trees since they have better wood quality than the corresponding diploids, while sterility of triploids can prevent the spread of potentially weedy, invasive species and hybrids, such as *Acacia* spp. (Griffin *et al*., 2015). Triploid breeding and intensive silviculture of triploid hybrid *Populus tomentosa* have increased forest plantation productivity significantly in northern China (Zhang *et al*., 2015). Triploids are normally obtained through hybridization between diploids and naturally occurring or colchicine-induced tetraploids (Cai and Kang, 2011; Lam *et al*., 2014).

B. Molecular marker-assisted selection (MAS) and genomic selection (GS)

Most tree breeding programmes rely on recurrent selection, *i.e*. implementing cycles of selection with inter-mating, generation after generation. The goal is to improve the overall performance of the population while maintaining genetic diversity, rather than to develop outstanding varieties for immediate use. Thus, advantages of using MAS [18] in tree breeding include reduction of generation time, correspondingly decreased field-testing costs and increased efficiency of selection for low-heritability traits (Neale and Kremer, 2011).

However, the potential of MAS in forest tree breeding is limited by a number of factors, including the high heterogeneity of breeding populations, the lack of simply inherited traits that could be easily targeted, the weak linkage disequilibrium (LD) among families, the narrow allelic range captured in QTL studies, and the limited number of scientists working in this area (Guimaraes *et al*., 2007; Grattapaglia and Kirst, 2008; Harfouche *et al*., 2012).

Also association genetics indicated as a promising tool to identify genetic polymorphisms of use for MAS (Neale and Kremer, 2011), has limitations in weak LD (Eckert *et al.*, 2009b) and resulted in identification of a few markers with limited effect on phenotypic variance (Grattapaglia and Resende, 2011). LD mapping uses natural tree populations, and relies on past recombination events within the population. LD mapping is therefore potentially more feasible for forest trees than MAS as it does not require controlled

[18] MAS is defined as the use of DNA markers to improve response to selection in a population. The markers will be closely linked to one or more target loci, which may often be quantitative trait loci (FAO, 2002).

crosses and analysis of second-generation progenies. The advent of NGS technologies has enabled the undertaking of GWAS in forest trees (Khan and Korban, 2012). LD mapping has been used in *Eucalyptus nitens* to identify alleles associated with a wood quality trait (Thumma *et al*., 2005).

The innovative strategy "breeding without breeding" (BWB) allows the capture of 75 to 85 percent of the genetic response to selection achieved through conventional tree breeding programmes, but without performing any controlled crosses or experimental field testing, which are costly and time-consuming (El-Kassaby and Lstibůrek, 2009; El-Kassaby *et al*., 2011; El Kassaby *et al*., 2012). BWB combines phenotypic pre-selection of superior individuals with molecular markers for parentage analysis and pedigree reconstruction to identify elite genotypes retrospectively for establishing seed orchards. It is thus an effective and economic approach that seems to be a viable option for developing countries (Wang *et al*., 2010). This strategy always presupposes that also non-selected genetic material is conserved for possible future use.

GS[19] has also been proposed for tree breeding. Initial results have been promising but are contingent upon requirements of effective population size and genotyping density being met (Denis and Bouvet, 2011; Grattapaglia and Resende, 2011). The impact of GS approaches on breeding of forest tree species is predicted to be much larger than in any other crop species because of long cycles of tree breeding (Isik, 2014).

Early applications of GS have been undertaken in several forest tree species, including *Eucalyptus* spp. (Resende *et al*., 2012a; Denis and Bouvet, 2013), *Pinus taeda* (Resende *et al*., 2012b and 2012c), and *Picea glauca* (Beaulieu *et al*., 2014), but routine application of GS to most forest trees has yet to be realized.

C. Mutagenesis

Mutation breeding is complicated in forest tree species because recessive mutations are masked in heterozygous plants and it is difficult to obtain homozygous lines. As such, efforts have focused on the creation of dominant mutations, primarily in *Populus* species and hybrids, by activation tagging[20], and enhancer and gene traps[21] (Busov *et al*., 2005). Activation tagging involves insertion of strong enhancers via *Agrobacterium*-mediated transformation, followed by screening of the resulting phenotypes in primary transformants and identification of candidate gene(s) (Busov *et al*., 2011).

Alternative techniques to produce dominant phenotypes include gene and enhancer trapping. Gene trap vectors contain a reporter gene without a functional promoter, while enhancer trap vectors carry a reporter gene preceded by a minimal promoter. The reporter gene is expressed in a fashion that reflects the normal expression pattern of the tagged gene (Groover *et al*., 2004).

[19] Genomic selection (GS) or genome-wide selection: genetic improvement based on prediction models developed by estimating the combined effects of all existing markers simultaneously on a phenotype. Models are developed by phenotyping and high-density genotyping a training population, so that all loci that regulate a phenotype are in linkage disequilibrium with at least one marker. Therefore, contrary to MAS, the identification of specific markers that regulate a trait is unnecessary (Harfouche *et al*., 2012).

[20] Activation tagging: a technique that utilizes heterologous enhancers elements to boost the expression of a gene adjacent to its site of insertion. Over-expression of the affected gene may lead to an identifiable phenotype. Subsequent cloning of the gene responsible for the mutant phenotypes is relatively straightforward (Harfouche *et al*., 2012).

[21] Gene trapping is a high-throughput approach that is used to introduce insertional mutations across the genome. The mutation is generated by inserting a gene trap vector construct into an intronic or coding region of genomic DNA. The gene trap vector constructs contain selectable reporter tags used to identify mutants where the vector has successfully interrupted a gene.

TILLING (Targeting Induced Local Lesions In Genomes) has been developed for identification of mutants based on DNA sequence deviations, including recessive mutations, in heterozygous mutants. Several induced mutations of hybrid poplar have been isolated by this method (Mattsson *et al*., 2007). EcoTILLING, that detects mutations by using NGS platforms, has been used to detect DNA variation in natural populations of *Populus trichocarpa* (Gilchrist *et al*., 2006) and to identify rare mutations in *P. nigra* (Marroni *et al*., 2011; Vanholme *et al*., 2013).

D. Wide crossing

Interspecific hybridization/wide crossing involves crossing plants belonging to two different species that are not normally sexually compatible. It is used to transfer useful characteristics from wild relatives to cultivated species (Santos *et al*., 2014; Zarpelon *et al*., 2015) or to combine favourable traits of two different species, but significant amounts of time and scientific expertise need to be invested. Biotechnology approaches such as *in vitro* embryo rescue are crucial to overcome sexual incompatibility and to speed up the process (Lidder and Sonnino, 2012). *In vitro* embryo rescue utilizes tissue culture techniques to enable a fertilized immature embryo, resulting from an interspecific or intergeneric cross, to avoid abortion caused by unbalanced endosperms and to continue growth and development until it can be regenerated into an adult plant. It has been utilized for crosses between *Salix* and *Populus* and between *Populus* species from different sections of the genus (Bagniewska-Zadworna *et al*., 2011; Payamnour *et al*., 2013).

E. Genetic engineering (GE)[22]

In recent years, GE has been used at the research level to successfully transfer an increasingly broad spectrum of traits in forest trees. These include biochemical and biopolymer production (Costa *et al*., 2013; Dalton *et al*., 2012); wood quality (Skyba *et al*., 2013; Wagner and Donaldson, 2014); pulping efficiency (Wilkerson *et al*., 2014); biofuel production (Nieminen *et al*., 20012; Peter, 2012); growth (Dubouzet *et al*., 2013; Han *et al*., 2011); stress (Hinchee *et al*., 2011), pathogen (Newhouse *et al*., 2014), and herbicide tolerance (Walter *et al*., 2010); and floral control (Zhang *et al*., 2012). These traits are amongst those that have been tested in over 700 field trials with GE trees (Zhang *et al*., 2012).

More complex GE strategies, including gene stacking[23] (Gartland and Gartland, 2014) and metabolic engineering[24] (Wagner and Donaldson, 2014), have also been developed. Gene stacking facilitates the combination of different target traits *(e.g.* pulping efficiency, enhanced growth and freezing tolerance), while metabolic engineering enables the establishment and optimisation of novel pathways. Cisgenics, which allows the generation of new plant varieties without insertion of foreign DNA into the host genome, has also gained some momentum including demonstration in poplar, *Populus* spp. (Han *et al*., 2011). Transplastomics[25], despite its significant potential (including high protein expression, transgene containment in non-conifer species, and lack of gene suppression) and an early demonstration in poplar (Okumura *et al*., 2006), has not yet realised its potential in forest trees. While agricultural crops are being developed with genes modified using synthetic biology technology (Scott *et al*., 2015), no forest tree species resulting from this technology appears to be obtained neither for experimental purposes nor for commercial release.

[22] Borrowed with modifications from MacRae *et al*. 2014.
[23] Gene stacking refers to the insertion of two or more genes into the genome of an organism (FAO, 2002).
[24] Application of recombinant DNA methods to restructure metabolic networks to improve production of metabolite and protein products by altering pathway distributions and rates (Bailey, 1991).
[25] Transfer of genes into chloroplasts.

The potential of GE for forestry is significant, and can be expected to grow as transformation technologies mature and genes for a larger number of target traits become available. Insect-resistant poplars planted in China are currently the only transgenic forestry trees approved for commercial use. Such plantations covered ca. 500 ha in 2011 (FAO, 2004; Zheng, 2010; FAO, 2011). Brazil has very recently approved the commercial release of a GE *Eucalyptus* with accelerated growth and improved wood production[26]. Worldwide, a number of GE trees are either in advanced trials or close to deregulation for commercial application (Porth and El-Kassaby, 2014b); these include fast-growing (Gartland and Gartland, 2014) and freeze-tolerant (Hinchee *et al*., 2011) *Eucalyptus*. Much of the science required for the implementation and testing of traits affecting productivity (*e.g.* herbicide and insect resistance traits) is in place. Likewise, there is a considerable body of knowledge based on laboratory and glasshouse studies indicating the genes likely to be beneficial to traits associated with sustainability, health, maintenance of biodiversity, and adaptation to climate change.

Many developing countries currently have biosafety regulations for agricultural crops, including fruit-trees, although many others lack such frameworks and the capacity to implement them. According to available information, there are no regulations specific to the use of GM forest trees and therefore regulations adopted for agricultural crops are usually applied also to forest trees, in spite of the uniqueness of their features. Decision-making is complicated by the fact that while agriculture is primarily viewed as a system for the production of goods, forests are generally viewed as a natural system, important not only for production of a range of goods, but also for provision of services, including the conservation of biodiversity and for social and cultural values. The use of GM forest trees is therefore more a socio-political and environmental issue than as a technical or trade issue (FAO, 2011).

Major forest product certification schemes approach the issue of GM forest trees differently. The Forest Stewardship Council (FSC) explicitly prohibits the use of GM trees[27], whether trial or otherwise, while the Programme for the Endorsement of Forest Certification (PEFC) specifies that areas in which GM trees are planted will not be certified if they do not comply with local, national, or international legislation[28]. PEFC is scheduled to review their biotech position at the end of 2015. The prohibitive stance of the FSC standards has often been translated into national and regional forest management schemes, further compounding their inhibitory affect. Apart from these, there is currently no specific statements on transgenic trees in various other wood product certification schemes, including chain-of-custody and illegal logging and bio-based or sustainability initiatives, such as, those being developed by the Global Consumer Goods Forum (Coventry, 2001). However, a recent Australian illegal logging standard does not tolerate transgenic trees (MacRae *et al*. 2014).

[26] http://www.ctnbio.gov.br/upd_blob/0002/2055.pdf
[27] https://ic.fsc.org/preview.fsc-pol-30-602-2000-fsc-interpretation-on-gmos-genetically-modified-organisms.a-143.pdf
[28] http://www.pefc.org/standards/chain-of-custody

F. New breeding technologies

The recent development of a number of new breeding technologies show considerable promise in forest biotechnology, not least because some of these seem likely to be defined as non-GE and therefore not regulated as transgenic trees. Techniques, such as, transcription activator-like effector nucleases (TALEN)[29], zinc finger nuclease (ZFN)[30], and clustered regularly interspaced short palindromic repeats (CRISPRs/Cas)[31] allow precise targeted gene editing (TGE), targeted gene integration and other genetic manipulations useful to establish trait variation (Gaj *et al*., 2013; Vojtas, 2013; Zhang *et al*., 2013). TGE has already been widely used in crop and model plants for scientific discovery and improvement of economically relevant traits, such as, growth, yield, product quality, pathogen resistance, and herbicide tolerance (Russell, 2013), but, to our knowledge, this is all still at the research stage and no crop varieties resulting from this technique have been released yet. Work is underway, using ZFN, to modify flowering in poplar (Strauss, 2010). The reader is referred to recent reviews for more detail (Dubouzet *et al*., 2013; Häggman et al., 2013; Suzuki and Suzuki, 2014; Tang and Tang, 2014; Vanholme *et al*., 2012).

If it is assumed that transgenic trees (and their products) are defined as those that have foreign DNA inserted into the genome, then the new biotech approaches would not be certified against by the previously mentioned certification schemes. Notably, the new biotech methods are undetectable in the products by an external agency, such as a certifying organization.

5. Use of forest products

A. Tracking the origin of forest products to reduce illegal logging

The analysis of DNA isolated from timber and wood products can be used to track their origin and thus to help reduce illegal logging and associated environmental problems (Lowe and Cross, 2011). Molecular tools are applied to test the declared origin of internationally traded timber and wood products. For instance, mechanisms to improve the control of the trade in wood of the important Southeast Asian family *Dipterocarpaceae* have been developed in Germany (Finkeldey *et al*., 2010). Deguilloux e*t al*. (2003) proposed to use chloroplast DNA markers for the purpose to trace oak wood used in the barrel industry. A DNA-based method to control the geographical origin of timber from *Entandrophragma cylindricum* populations located in three forest concessions in Eastern Cameroon has been proposed by Jolivet and Degen (2012). Other examples are discussed in Ekué and Loo (2012). The identification of spatially isolated populations is however restricted by their differentiation, which is often weak.

B. Conversion of lignocellosic (LC) biomass to liquid biofuels

The interest in moving from the current first-generation of liquid biofuels, obtained from the conversion of agricultural feedstock to second-generation biofuels is based on the fact that LC biomass, including forest-based products, is a not edible, very abundant biological material, and therefore it does not compete

[29] Transcription activator like effectors (TALEs) are a structurally and functionally distinct class of proteins from the plant pathogenic bacteria *Xanthomonas* that are injected via the type III secretion pathway into plant cells. Once in the plant cell, they enter the nucleus, bind to TALE-specific DNA sequences, and turn on the downstream genes (Bogdanove *et al*., 2010).

[30] Zinc fingers are DNA-binding protein motifs, characterized by two closely spaced cysteine and two histidine residues that serve as ligands for a single Zn2+ ion. When bound, the structure take on a conformation in which amino acid side chains protrude in a way that allows interaction with the major DNA groove (FAO, 2002). ZFN technology is used to selectively delete or edit endogenous genes in plants by generating sequence-specific breaks in double-stranded DNA (Harfouche et al., 2012).

[31] A class of precision genome engineering tools based on the RNA-guided Cas9 nuclease from the type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system (Le Cong *et al*., 2013).

directly with food production. Biotechnology has extensive applications for the conversion of LC biomass to biofuels by biochemical processing (Ruane *et al*., 2010), mostly for enzymatic hydrolysis of cellulose and hemicellulose and the fermentation of resulting sugars.

Recent research aimed at improving enzymatic hydrolysis focuses on the search for high cellulase-producing organisms; the production of hypercellulolytic mutants of organisms suitable for cellulase production; genetic modification to develop high cellulase-producing organisms with high specific activity; and theoretical studies on the mechanism of action of a multi-enzyme system on a complex polymer (Bon and Ferrara, 2007). Engineering of enzymes using advanced biotechnologies is ongoing to develop enzymes with improved characteristics such as higher efficiencies, increased stability at elevated temperatures and at certain pH levels and higher tolerance to end-product inhibition (Bon and Ferrara, 2007). As stated by Arshadi *et al*. (2014), "production efficiency of cellulases has been increased more than 10-fold nowadays resulting in a serious decrease in their price, rendering enzymatic saccharification more economical than physicochemical methods".

Unlike production of bioethanol from first-generation sugar crops or starchy materials, fermentation of the sugars resulting from the breakdown of cellulose and hemicellulose to bioethanol is more complicated as it involves a mixed-sugar fermentation (involving pentose and hexose sugars) and it takes place in the presence of inhibiting compounds released and formed during pre-treatment and hydrolysis. Because of their larger sizes, thicker cell walls, better growth at low pH, less stringent nutritional requirements and greater resistance to contamination, yeasts are preferred to bacteria for commercial fermentations (Jeffries, 2006). However, LC biomass, in particular of hardwoods, can contain 5–20% (or more) of the pentose sugars xylose and arabinose, which are not fermented to ethanol by the yeast *Saccharomyces cerevisiae*, the most commonly used industrial fermentation micro-organism.

To overcome these problems, several different approaches are being explored. One is to develop efficient xylose-fermenting strains of *Saccharomyces cerevisiae* using a range of biotechnologies, including GE (Gonçalves *et al*., 2014). Another approach is to focus on yeast species that naturally ferment xylose, including *Pichia stipitis*, whose genome has been sequenced (Jeffries *et al*., 2007). The productivity of *P. stipitis* has been recently enhanced through genome shuffling[32] (Shi *et al*., 2014). Another approach is to focus on bacteria, including *Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis* instead of yeast. GE strains have been produced for each of them for use in bioethanol production (Balat *et al*., 2008).

Enzymatic hydrolysis and fermentation of forest-based LC biomass can also be carried out together, in a process called simultaneous saccharification and fermentation (SSF) (Romanì *et al*., 2012). This has a number of advantages, as they take place in the same reactor, thus reducing costs and increase the hydrolysis rate, since the sugars resulting from hydrolysis are fermented and thus do not inhibit cellulase activity. On the negative side, the ideal pH or temperature conditions for the saccharification step may differ from those for the fermentation step (Balat *et al*., 2008).

Through the steps described above, two of the three main components of LC biomass, *i.e.* cellulose and hemicellulose, are converted to bioethanol. The third component, lignin, as well as its by-products, need to be removed before fermentation takes place as they are often toxic to micro-organisms and inhibit the activity of the enzymes used for hydrolysis, which can reduce the conversion efficiency. GE poplars with reduced lignin content can lower the cost of bioethanol production (Littlewood *et al*., 2014). Harfouche *et al*. (2014) describe several strategies pursued to develop lignin-degrading microorganisms.

[32] Whole-genome shuffling is a process that combines the advantage of multi-parental crossing allowed by DNA shuffling with the recombination of entire genomes normally associated with conventional breeding (Zhang *et al.*, 2002).

In conclusion, even if several improvements are yet to be done, significant progress has been made (Arshadi *et al*., 2014) and this indicates that commercial production of second-generation biofuels from forest-based feedstock can become soon feasible.

C. Bio-based polymers and biomass-based plastics from forest biomass

Bio-based polymers (Babu and Seeram, 2013) and biomass-based plastics (Doug, 2014) can both replace conventional, petroleum-based polymers and thus contribute to alleviating related environmental problems (Saygin *et al*., 2014), and provide new polymers with improved performance. Bio-based polymers of first generation were strongly based on the use of agricultural feedstocks, but the competition with food production is likely to promote a shift towards forest biomass as raw material (Arshadi *et al*., 2014). For example, polyhydroxyalkanoates (PHAs; biological polyesters) are produced through bacterial fermentation, using forest biomass as feedstock (Keenan *et al*., 2006). Applications for bio-based polymers are found in a broad array of industries and day-to-day applications, including electronics (*e.g.* polylactic acid, PLA), packaging (*e.g.* starch, cellulose), textile industries *(e.g.* cellulose), medicines, pharmaceutics and cosmetics (*e.g.* starch, chitin, chitosan), agriculture (bio-based polyethylene) and food industries (*e.g.* pullulan). Advanced production of different bio-based polymers occurs throughout the world (Arshadi *et al*., 2014).

6. Conclusions and outlook

Forest biotechnology and crop biotechnology are often considered to be comparable, but the benefits, goals, risks and deliverables are distinctly different. Forest biotechnology applications are developing along a separate path from those in crop biotechnology. As illustrated in this paper, biotechnology and its applications in forest genetic resources characterization and conservation, mass propagation, tree breeding, and forest biomass utilization, are expanding very rapidly; cover an increasing number of taxa; and are no longer restricted to tree species used in plantation forestry and tree planting. However, in spite of their importance, forest trees and other forest plant species have received less attention than crop plants and domestic animals. The development and application of biotechnology are progressing at a much lower pace in developing countries and in the tropics in general than in industrialized countries and in temperate areas (FAO, 2015). Biotechnology applied to tropical forest species suffers therefore from a double gap: forest trees in general receive less attention than crops and domestic animals, and tropical trees less attention than temperate forest trees. This situation persists in spite of the strategic importance of tropical forests from a productive, environmental, economic and social point of view. This gap leads to an important knowledge deficit, pointed out at the FAO International Technical Conference on Agricultural Biotechnologies in Developing Countries in 2010 (FAO, 2011), that needs to be urgently addressed.

The biotechnology tools used in naturally regenerated forests and in planted forests differ to some degree. For naturally regenerated forests, molecular markers and genomics provide important information on genetic variation within and between populations (FAO, 2015). Today, findings are available to guide operational forest management plans, including in developing countries, but only for a still limited, even if growing, number of the hundreds of tree species that are managed in naturally regenerated tropical forests. Biotechnology provides important insights into the nature of entire tropical forest ecosystems, including the relationship between forest trees and the soil microbial communities with which they interact, which can influence the strategies employed for managing tropical forests (FAO, 2011).

As far as planted forests are concerned, a variety of different biotechnological tools can be used, depending on the level of management intensity and the genetic material used. For less intensively managed planted forests a range of vegetative propagation methods (including tissue culture-based micropropagation), the use of biofertilizers (not covered in this paper) and molecular markers-based clonal identification and

genetic stability verification technologies, are widely utilized. Biotechnologies applied to intensively managed planted forests that provide industrial raw materials on a large scale include mass propagation through somatic embryogenesis, selection based on molecular markers and quantitative trait locus (QTL) analyses, whole genome sequencing and functional genomics. The most sophisticated group of biotechnologies, which includes backward and reverse genomics approaches, whole-genome sequencing, low-cost large-scale vegetative propagation, genetic modification of forest trees, and conversion of LC biomass to liquid biofuels, is under rapid development and is likely to be applied on commercial scale in a near future.

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