Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction.

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Title

Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction.

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Concise and informative title:
Somatic embryogenesis in forestry

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Abstract
Vegetative propagation of forest trees offers advantages to both tree breeders and the forest industry. This review will describe benefits, type of vegetative propagation, and its integration into breeding programmes. Of all of the different methods for vegetative propagation only rooted cuttings and somatic embryogenesis (and the combined use of both) offer any practical methods for large-scale commercial use. However, it is very difficult to fully appreciate the overall level of activity of the research and application of somatic embryogenesis of forest trees. Publications and reports only highlight a small fraction of the ongoing work. To this end, a survey was conducted across Europe (under EU Research Infrastructure Concerted Action "Treebreedex") to document the species involved, the state-of-the-art of somatic embryogenesis, its stage of development and its application in tree improvement programmes and to commercial forestry. The results of this survey are presented and discussed. In addition, this review presents the challenges (biological, economic, public acceptance and regulatory) and their relationships to European forestry. Finally, a strategy to promote the use of this technology is proposed.

Key words (4-6)
Forest biotechnology, clonal forestry, vegetative propagation, somatic embryogenesis, tree breeding, deployment

Introduction

Current world wood consumption is estimated to be about 3.4 billion m^3/year with about half of this is used for fuel and the balance as "industrial wood". It is predicted that by 2040 that world consumption of industrial wood will increase from the current 1.5 billion m^3/year to about 2.5 billion m^3/year. It is very unlikely that this increased demand will be met by existing natural forests and it has been suggested that between 50 to 75% of the world's future supply of industrial wood will have to come from planted forests (Sedjo 2004). In Europe currently forest occupy over 1 billion hectares or about 44% of Europe total area and of this about 26% are natural forests with only about 4% as plantation forestry (MCPFE 2007). Of this billion hectares only about 48% produces timber, fibre or fuel. Clearly an increase in the use of intensively managed plantations, which among other things will require the use of genetically improved (produced by conventional breeding, not "genetically engineered") varieties of trees to maximise the productivity of these new plantations and improve their adaptability is needed. New ways to efficiently propagate and deploy large amounts of improved material will be required. The use of vegetative propagation (VP) in forestry is the fastest, the most flexible and effective way to produce enough genetically improved material to meet future wood demands.

Most plants, including forest tree species, are propagated by seed because it is easy to collect, most can be stored for long periods and they are easy to handle during sowing. However, seeds are the result of sexual recombination which results in each individual having a unique combination of genes. The use of VP in forestry is not a new idea. Rooted cuttings of Japanese cedar (*Cryptomeria japonica*) have been used to establish forest plantations in Japan for the last 400 years (Toda 1974). In China rooted cuttings of Chinese fir (*Cunninghamia lanceolata*) have been planted for the last 800 years (Minghe and Ritchie 1999). More recently large commercial plantations of a range of *Eucalyptus* species have been planted with clones selected for specific end uses (Zobel 1993). An unpublished survey of VP (using rooted cuttings and somatic embryogenesis, SE) of both conifer and angiosperm forest tree species carried out in 2009 was able to identify the production of at least 700 million vegetatively propagated forest trees per year (Table 1). Vegetatively propagated forests are not as artificial as they might seem. Naturally occurring clonal populations of forest trees do exist in *Populus tremuloides* (Cheliak and Dancik 1982), *Picea mariana* (Legere and Payette 1981), *Quercus pyrenaica* (Valbuena-Carabaña et al. 2008) and several other species, demonstrating that such populations are both natural and sustainable.

The objective of this review is to first discuss the uses of VP of forest trees, then to discuss the various VP methods available and to show that SE and hybrid SE/rooted cutting propagation systems offer the fastest and most efficient way to produce the large amounts of improved material that is required. Next we will discuss the current state-of-the-art of SE in Europe, will present the challenges (biological, economic, public acceptance and regulatory) and how this all relates to European forestry. Finally we will comment on what we think needs to be done to ensure that this technology is developed and put into commercial use in Europe.

What are the advantages of vegetative propagation to forestry?

Improved material from most forest tree breeding programmes around the world is typically produced in seed orchards where selected parents are allowed to interbreed and produce seed which combine the selected characteristics of both parents. For a number of reasons seed orchards very often fail to deliver the full potential of the genetic improvement that they should be capable of. These reasons include: **1)** not all parents consistently contribute to seed crops, **2)** some seed results from self-pollination, which results in poor performing progeny and **3)** pollen from trees outside the seed orchard reduce improvement levels. The result is that seed orchards typically provide only about half of the potential improvement that should be possible. In an effort to increase the levels of improvement possible from tree improvement programmes attention has turned to VP. VP has a number of advantages that make it a useful tool in breeding programmes which include:

- It provides a faster and more stable delivery of improved material because it is not affected by periodicities in flowering and seed production. For example, there is no delay in seed production until a seed orchard starts to flower and produce seed. As a consequence, it also provides a greater flexibility in Forest Reproductive Material deployment. The genetic material being vegetatively propagated can be changed very quickly compared to the material in a seed orchard that cannot be changed.
- It reproduces exactly a unique genetic layout (both in additive and non-additive terms), unlike sexual propagation where only additive traits are transmissible. Through VP non-additive sources of variation are readily accessible and can be exploited for further enhancing selection intensity and thus genetic gain compared to sexual propagation methods.
- It is well suited for early selection schemes, especially those based on the use of molecular markers alone at early stages of development, allowing further shortenings of the generation interval between two consecutive selection cycles.
- It provides a powerful testing method for genetic evaluation with a more straightforward estimation of micro-environmental and G x E (Genotype by Environment) interactions than in progeny testing. Isik at al. (2003) showed that clonal testing increased the efficiency of testing compared with conventional seedling progeny testing.
- For genetic studies and genetic evaluation in tree breeding, there is the possibility to use clones to better evaluate genotypes, to study phenotypic plasticity, or to shorten the breeding cycle (e.g. when clonal values can accurately estimate general combining ability, *Prunus avium*).
- It can produce clonal material that grows at a uniform rate, making management more predictable as well as producing a more uniform and higher quality product (timber or fibre) which can have an important effect on both

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harvesting and processing costs. Product uniformity could be more important than increased growth rates (Shelbourne 1997). It is also possible to select clones for both specific site conditions as well as specific end uses.

How can vegetative propagation be implemented into breeding programmes?

Tree breeding programmes have two main objectives, first to develop breeding material that contains desired traits (growth, form, wood properties, etc.) and second to produce commercial amounts of this selected material for afforestation and reforestation. Although not a breeding tool, VP is a propagation method that multiplies desired genotypes and allows the gain that is achieved during breeding be put into commercial use quickly. Conventional multiplication is often achieved through seed orchards, where selected genotypes mate freely (or less often by means of controlled crosses) to produce the seed that becomes the planting stock. This is a lengthy process, as seed orchards often need up to 10 to 15 years after selection has taken place to be come fully productive. VP techniques can intervene at two phases of the improvement scheme (Fig. 1). The first phase is before testing takes place, where VP has the ability of providing multiple clonal copies of selected candidates for testing. Multiple clonal copies can provide accurate assessments of the stability of the breeding material over a range of environments that mimic those of commercial deployment. Elite genotypes are often evaluated through progeny testing, which can potentially be subjected to delays due to problems in flowering and in seed production. These delays are absent with testing through VP. Therefore, at this first phase, VP brings the possibility of faster implementation of testing compared to the evaluation via the performances of progenies. However, clonal testing should be seen as a complement of progeny testing. While the former allows fast evaluation, the latter provides finer decomposition of the genetic components under evaluation.

The second phase is after selection of candidates, where VP provides the way of producing copies of valuable genotypes for use as field planting stock. This has three potential benefits over sexual multiplication which include: **1)** genetic improvement is directly translated into the commercial phase with high accuracy, because the same genotypes resulting from the genetic improvement phase are those released for reforestation and afforestation programs **2)** time lag between selection and deployment is shortened, because there is no need of setting up a lengthy mating program; and **3)** selection intensity can be substantially increased, as multiplication can be concentrated on a few genotypes with the desired assemblage of traits.

It is, however, in advanced breeding programs where the full benefits of VP can be to be fully achieved, particularly when the testing and improvement schemes are able to provide high selection accuracies, thus accurately identifying the best candidates. In this sense, the advent of new evaluation methods with very high accuracies, like genome-wide evaluation (Meuwissen et al. 2001) is expected to stimulate interest in the implementation of VP techniques. The combination of very early selection based solely on genome-wide marker profiles and VP of selected embryos has the potential of revolutionising breeding schemes, both in terms of time required and the gains achieved. The possibility of long-term cryopreservation of embryos adds also new possibilities, serving for instance as a secure genetic diversity backup while field testing is on-going.

How is vegetative propagation accomplished?

VP includes methods of both “macropropagation” (air layering, grafting, rooting of cuttings) and “micropropagation” using *in vitro* tissue culture techniques e.g. either by organogenesis (adventitious budding, axillary shoot tips) or SE. Among the macropropagation methods both air-layering and grafting are too slow and expensive for large-scale propagation. Grafting, however, is used in forestry to establish clonal material, including from physiologically old trees, for the establishment of seed orchards and clone banks. The rooting of cuttings can produce commercial volumes of material as has been demonstrated in a range of forest tree species (Lambeth et al. 1994). However, due to higher production costs (rooted cuttings typically costs two or more times a similar plant produced from seed) and ageing of stock plants, these methods are mostly practical with certain species. Nevertheless, rooted cuttings, in some situations can be an effective propagation method for forest species. In micropropagation plant regeneration by organogenesis involves a multi step process in which **1)** a shoot bud is initiated, **2)** it is then elongated, **3)** then it is induced to form a root and finally **4)** to produce a complete plant. Each of the steps typically involves a manual transfer to different growth regulator treatments and environmental conditions which results in high labour requirements and thus high per plant costs. Multiplication rates from axillary shoot formation typically range from a 2 to perhaps 10 shoots per cycle, depending on the species. For most forest tree species while organogenesis is possible, they are simply not economic, due to the fact that tree seedlings are so inexpensive For example micropropagated *Pinus radiata* plants were reported to cost more than 7 times open-pollinated seedlings (Menzies and Aimers-Halliday 1997). In the process of SE, a complete miniature plant consisting of a shoot bud, a root tip and a stem, which is similar to a zygotic embryo that normally occurs in the developing seed (Fig. 2), takes place in the laboratory (Carney and Pullman 2007). Somatic embryos are formed in one or two steps thus minimising the handling required. Once an embryogenic culture has been developed it can produce a theoretically unlimited number of exact copies of that genotype. It has been estimated that one gram of embryogenic culture of Norway spruce (*Picea abies*) contains between 1,000 to 2,000 potential somatic embryos showing the potential multiplication rate possible with SE (Pâques et al. 1995). Not all of these potential somatic embryos will develop into plants (emblings), but even if only a percentage do, SE is still a faster process with a higher multiplication rate than possible by any other macro or micro propagation method.

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Somatic embryogenesis and how it is accomplished.

Background

SE was first identified in non-woody angiosperms in the late 1950s. The first report of SE in woody angiosperms was *Santalum album* in the 1960s (Rao 1965). Currently a wide range of woody angiosperms species have been propagated by SE and several recent reviews have been published (Merkle and Cunningham 2011; Pijut et al. 2011; Vieitez et al. 2011). SE in the gymnosperms was first reported in 1985 for *Picea abies* (Chalupa 1985; Hakman et al. 1985) and since it has been obtained in a large number of conifer species such as *Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, *Sequoia*. The work on conifer SE has been reviewed by Attree et al. (1993), Stasolla et al. (2002) and Klimaszewska et al. (2007).

Somatic embryogenesis process

SE is a complex process involving many factors in several different steps (Fig. 3). Establishing embryogenic cultures ("initiation") requires placing plant material under controlled conditions in culture that promote cell differentiation and the formation of embryogenic cells. One of the most important factors is the selection of a tissue that will produce embryogenic cells. Initial work in both the woody angiosperms and gymnosperms was with seed tissues and as experience developed, this progressed to young seedlings and later on with more mature tissues. However, the initiation of embryogenic cells from mature tissues has been more successful in woody angiosperms (Merkle and Nairn 2005) such as oaks (Vieitez et al. 2011), than gymnosperms (Bonga et al. 2011). The existence of a gradient of maturity within the tree is well known (Olesen 1978). Tissues from root or stump sprouts, or from epicormic shoots (induced by partial girdling of basal part of trunk) have been used as initial explants to induce SE in *Quercus robur* (Chalupa 2000). Epicormic shoots forced to flush on crown branches have been found to be good sources of embryogenic cells. Based on this observation, embryogenic cells were initiated from leaves of these shoots in species such as *Quercus suber*, *Quercus robur* and *Ulmus minor* (Table 2). Leaf explants were effective in several other species such as *Quercus ilex*, *Populus ciliata*, *Thevetia peruviana*, and *Terminalia arjuna*.

The timing of the explant excision may be important for the initiation and this may be related to the onset of developmental changes. In many species the culture of immature zygotic embryos results in higher embryogenic initiation rates than if mature zygotic embryos are cultured. Expanding leaves of cork oak used as initial explants, lose their ability to respond when they exceed a certain threshold size (Hernández et al. 2003). Shoot tips have also been used as initial explants to initiate SE in several species. The somatic embryos arose not from the apical meristem but from the first leaf primordia, and had the same origin and developmental pattern as those induced in expanding leaves from epicormic shoots (Corredoira et al. 2006a). A very similar pattern to that described above for SE from shoot tips has been recently described in a study on the induction of SE from primordial shoot explants that were excised from shoot buds of mature somatic embryo-derived *Picea glauca* (Klimaszewska et al. 2011). In addition, explants from a range of tissues involved in sexual reproduction (filaments and anthers, inner ovule integuments, Table 2) have also been used successfully to initiate embryogenic cultures, although their limited availability in a short period during the year is a drawback. *In vitro* conditions allow the sustained maintenance and proliferation of the embryogenic cultures to a very immature developmental stage. Once initiated embryogenic cells, known as "proembryogenic masses" (PEMs) and "embryonal masses" (EMs) for respectively woody angiosperms and gymnosperms, enter a continuous cycle producing further PEMs or EMs. This ability results in an unlimited multiplication of the original plant material. In some woody angiosperm species new somatic embryos may form directly on previously formed somatic embryos in a process known as "recurrent" SE. Embryogenic cultures can also be stored in liquid nitrogen (-196°C), allowing genetic resource conservation, as well as providing a way to maintain the material in a juvenile state under secure conditions (Park et al. 1998). Changing the environmental conditions of the embryogenic cultures allows somatic embryo development (maturation Fig. 3). Current maturation protocols lead to the development of mature somatic embryos that morphologically and physiologically resemble normal zygotic embryos. The use of abscisic acid (ABA), a natural plant growth regulator found in maturing seeds, is of major importance for maturation of coniferous somatic embryos with ABA promoting accumulation of storage reserves such as lipids, proteins, carbohydrates (Attree et al. 1992; Gutmann et al. 1996; Lipavská and Konrádová 2004). However, the use of ABA to mature somatic embryos of woody angiosperms appears to be less effective than in the gymnosperms. "Desiccation" or the lowering of the moisture content of the SE has also been shown to improve the quality of the SE and synchronise germination. As soon as asexual plants are obtained, they develop in the same way as their counterparts which are zygotic embryos. First roots develop ("germination" also called "conversion") giving rise to young plantlets or emblings (somatic embryo plants) possessing the same characteristics as zygotic seedlings. Subsequently, emblings are "acclimatised" e.g. transferred to soil under *ex vitro* conditions to be "acclimatised" to grow under normal environmental conditions.

Laboratory propagation options

The majority of SE production systems use embryogenic cultures grown on a solidified nutrient medium. It requires a significant amount of handling which affects the production costs. However, it has been suggested that the growth and development of somatic embryos in liquid medium is needed to produce large numbers of somatic embryos profitably. Two main techniques have been used to produce somatic embryos in liquid medium, namely suspension cultures and temporary immersion systems.

One of the main goals in the SE process is to be able to produce synchronous populations of embryogenic cells or differentiating embryos. The mass production of somatic embryos has been greatly improved using synchronized suspension cultures, and the simplest procedure to achieve that has been size fractionation. Using these methods, 0.5g

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of PEMs of *Liquidambar styraciflua* x *L. formosana* could potentially produce 4,000-6,600 germinable embryos in 6 weeks, and the same amount of PEMs of hybrid *Liriodendron tulipifera* x *L. chinense* could potentially produce 10,800-24,600 germinable embryos in 4 weeks, both in just one 125 ml Erlenmeyer flask (Dai et al. 2004). Suspension cultures may considerably improve the quality of produced embryos. Although suspension cultures of conifers are highly productive, the development and maturation of gymnosperm somatic embryos in liquid media has proven to be more problematic (Timmis 1998; Gupta and Timmis, 2005; Salaj et al. 2007)

Temporary Immersion Systems have been used mainly with species with recurrent SE in clusters of differentiated embryos and also to mature and pre-germinate somatic embryos. They follow the successful experience with several woody crops such as *Coffea arabica* (Etienne et al. 2011) and *Coffea canephora* (Ducos et al. 2011) that are able to produce millions of somatic seedlings per year on a commercial scale. In *Quercus robur* the potential of Temporary Immersion Systems for proliferation of somatic embryos has been estimated at between 36,000 and. 85,000 per square meter depending on genotype, while cultures on semi-solid medium rendered 10,000 to 12,000 SE per m^2 (Mallón et al. 2012).

Embling use options

There are two main approaches that have been used in the large-scale production of emblings. The first approach, and probably the more widely used, is to produce emblings that will be planted directly in the field (sometimes referred to as "varietals" rather than clones). This is the approach that is used by some of the large commercial SE production companies such as CellFor (Sutton 2002) and Arborgen. The reason for this is that the main pine species they produce, loblolly pine (*Pinus taeda*), does not currently lend itself to propagation by rooted cuttings. While the direct planting of emblings in the field appears to be the most effective use of this technology, the increased cost to produce emblings makes this option economically very difficult to justify. Emblings in the US have been reported to cost between 5 to 6 times the costs of improved seedlings of the same species (Sorensson 2006). To establish an entire plantation with this material would be uneconomic for most forest tree species grown for timber production, however, both CellFor and Arborgen have developed some unique marketing approaches to encourage the use of this material. Arborgen has promoted "FlexStand" in which rows of improved "varietal" material destined for saw log are planted with alternate rows of material grown for biomass. This is a variation of MOCAS (planting of a **M**ixtures **O**f **C**lones **a**nd **S**eedlings) as suggested by Park (2002) as a way to reduce the per hectare plant costs. CellFor have promoted a "value based pricing" in which the emblings cost is based on a proportion of the future value of the product resulting from the use of this technology.

The second approach which is used on a large-scale by several organisations including the company Forest Genetics Ltd. in New Zealand and Coillte Teoranta in Ireland is to use emblings and grow them as juvenile stock plants to provide cuttings for rooting. This method works well with species such as *Pinus radiata*, *Picea sitchensis* and other species where methods for the rooting of cuttings are well developed and are effective. In this approach the high production costs of the emblings can be spread over the large number of cuttings produced by the stock plant throughout its life. This will be discussed in more details later.

Deployment Options

The same questions about how best to deploy rooted cuttings that have been discussed in the past are now being asked about the planting of SE produced material. Vegetatively propagated material can be either produced and planted as "bulk propagation" (where the clonal identity is not tracked during propagation and deployment) or as "clonal propagation" (where the clonal identity is tracked though the propagation process and to the plantation). This depends on both the level of genetic improvement to be captured and the regulatory environment.

Selected open pollinated families or full-sib crosses of selected parents can be propagated by "bulk or mass propagation" in which the clonal identity is not retained during propagation and deployment in the field. Provided enough genetically unrelated genotypes are used, the resulting plantation should be able to withstand potential losses from both abiotic and biotic risks during the rotation of the crop. Indeed the genetic diversity of a synthetic vegetatively propagated population could be greater than that of a seedling population originating from a naturally occurring seed stand.

Individuals phenotypically selected from outstanding open pollinated or full-sib families, can also be propagated as clones. This requires tracking the identity of every plant through the entire propagation process and even to the site where they will be planted. If clonal propagation is to be used it is necessary to test the clones. Clonal testing and selection, however, adds additional time and expense compared to the bulk propagation option discussed above. Besides the extra-costs for the maintenance of the clones, another drawbacks of this option might be the rapid physiological ageing of the stocks for some species, but this problem is mostly solved when SE is coupled with cryopreservation (Lelu-Walter and Pâques 2009). In any case, this option does provide the opportunity to select the best individuals originating from crosses of selected parents thus increasing the level of genetic improvement that is possible.

Embling field performance and genetic conformity

In several cases in which gymnosperm emblings have been established in the field, the initial growth is typically slower than control seedlings (Högberg et al. 2001, 2003; Niskanen et al. 2008). However, this is usually due to differences in initial plants size between emblings and seedling controls because the annual height growth in both

types of plants is usually the same later on. For interior spruce, Grossnickle (2011) summarised twenty years of experience showing similar dormancy, freezing tolerance and root growth patterns between emblings and seedlings Multisite growth performance of emblings have been reported after 4years for white spruce (Wahid et al. 2012), after 7 years for Douglas fir (Dean et al. 2009; Dean 2010).

The lack of pronounced phenotypic changes which are easily observed should not infer true-to-type genetic uniformity. Work is presently underway to develop methods for identifying genetic variation but none (outside still costly whole genome resequencing) are currently available to be powerful enough to detect significant morphological, molecular or cellular changes. In a few studies (Burg et al. 2007; Marum et al. 2009) only a few microsatellites covering a very small part of the genome were used, but these may not be sufficient to detect non random mutation particularly when conifers are involved.

It should be noted that somaclonal variation or mutation would not necessarily mean a dramatic morphological or behavioural abnormality and could result in increased fitness/performance as in ornamental plants and fruit trees. Burg et al. (2007) proposed that the family-dependent DNA instability seen in SE cultures from high altitude Norway spruce populations could be related to adaptation to a high UV environment.

Current state-of-the-art of somatic embryogenesis in forest tree species in Europe.

As part of the EU funded Research Infrastructure Concerted Action "Treebreedex" (http://treebreedex.eu, contract Number 026076), a survey was conducted on SE in Europe to document the species involved, the stage of development and the application of SE in tree improvement programmes. A questionnaire was sent to laboratories involved in VP. A total of 43 organisations belonging to 24 European countries (including Belarus) responded to the questionnaire. The majority of these organisations were university academic departments (43%), national forest research institutes (37%) or other state organisations (19%), public and private companies. In total, 45 forest tree species are being studied (27 gymnosperms and 18 angiosperms). The most studied gymnosperm species were some of the genus *Abies* (8 laboratories) *Picea* (18 laboratories) and *Pinus* (14 laboratories) while several of the genus *Quercus* (10 laboratories) were the most studied angiosperm species (Table 3). Most programmes considered that they were doing "basic research" (50%) while about 30% said they were doing both "basic and applied" research. Only about 20% of the programmes were part of a tree improvement programme (about 12% for angiosperm and 21% for gymnosperm species). Respondents of about 46% of the programmes said that although the SE process still requires further improvement, current SE technology was deemed to work well enough for industrial application for several species (*Abies*, *Larix*, *Picea*, *Pinus* and *Quercus*). The major technical bottleneck for most species was maturation (20%) and germination/conversion (20%). The majority (37%) of respondents said that the reason for using SE was to study the basic process of SE, although several programmes (Denmark, Ireland and France) are already producing commercial-scale numbers of emblings in species of *Abies*, *Picea* and *Pinus*. Other than propagation, the major applications of SE technology was for cryogenic storage of germplasm (29%), physiological and biochemical studies (23%) and comparison of zygotic and somatic embryos (22%). Improved production protocols (55%) and economic analysis of the process (28%) were seen as the major technical research needs.

Most of the material that is being produced as part of a tree improvement programme is used in clonal testing programmes (47%), in variety deployment (23%) and for General Combining Ability estimation (16%). For conifers, the majority of the material is used in mass propagation (55%), although some clonal propagation is used (*Abies* and *Larix*). Among the angiosperms propagation use was equally divided between mass and clonal. Most emblings produced are directly planted in the field (51%), whereas a few programmes (18%) use them as stock plants to produce rooted cuttings which then go to the field (*Picea sitchensis*). The main use of SE in *Abies* species is in the production of selected clones for Christmas tree production. Most programmes (81%) said that they were at the "experimental" stage while a few (27%) believed that they had advanced to the "pilot-stage" with 6% close to or at the commercial-scale. Most programmes reported that they produced less than 100 emblings per year, whereas several programmes working with *Picea*, *Abies* and *Pinus* ranged between 10,000 to 30,000 emblings produced per year. Many programmes were working with 10 to 20 clones, but the larger programmes worked with 10 to 200 clones (*Abies nordmanniana*, *Picea sitchensis*, *Pinus pinaster*). Most programmes deployed clones either in mosaics of monoclonal mixtures or in large monoclonal blocks. Multi-clonal mixtures were the least common deployment option. About 35% of the programmes had already material evaluated in field trials. The age of these trials ranged from 2 years for *Abies*, 4 years for *Pinus*, 10 years for *Quercus* and 15 years for *Picea*.

The main non-technical bottlenecks of the process were seen as the cost to produce emblings (60%) and a lack of appreciation by foresters of the value of the improved material (35%). Public acceptance and lack of customers for this material (both about 15%) were also seen as an important non-technical bottlenecks. Only about 9% of the respondents believed that their current national regulations presented a serious problem.

In conclusion, Europe appears to have the necessary scientific background (knowledge, skills and improved material) to develop and use SE on a larger scale. The fact that SE was first identified in gymnosperm species in Europe (Hakman et al. 1985) demonstrates that we have an adequate R&D capability in SE. However, the lack of a well developed market for this improved material seems to be a major constraint.

Commercial-Scale State-of-the-art of SE in the world other than Europe

World-wide several companies are currently producing commercial amounts of emblings of coniferous forest trees. Some of these companies are producing material for their own use, but there are also several commercial companies

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that successfully produce and sell gymnosperm planting stock produced by SE on the open market. These include CellFor, based in Canada, Arborgen in the US and Forest Genetics Ltd in New Zealand, which have been mentioned earlier (Douglas-fir, interior, white and Sitka spruces, radiata, loblolly and slash pines). Unfortunately very little information is available on how many emblings are produced and how much they cost. Nevertheless, the fact that these companies exist demonstrates that there is a market for improved material produced by SE. In 2008 it was reported that CellFor produced 10 million "seedlings" (SE "emblings") and Arborgen was expected to produce between 0.5 and 1.0 million "seedlings" of southern yellow pine (*Pinus taeda*) (Grossnickle and Pait 2008). Among the woody angiosperms the commercial process is less advanced. Currently the embryogenic systems with hybrid yellow-poplar, hybrid sweet gum and in a lesser extent American chestnut are considered the most advanced among those in woody angiosperms. US universities and private companies such as The University of Georgia, International Paper Company and ArborGen are carrying out breeding programs with the support of these cloning techniques. Field trials with thousands of plants derived from somatic embryos of hybrid sweet gum and yellow-poplar have been established by these companies to identify superior performing clones for biomass production. Although cloning by SE of these species is still at a research rather than at an operational level, it is considered that these protocols are readily scalable and that only investments to develop the production engineering systems required for scaling-up to the operational production are needed (Merkle and Cunningham 2011). Some of the most advanced SE protocols in woody angiosperms have been developed in Europe mostly by French groups for woody crops the planting of which takes place in other countries. This is the case of coffee previously mentioned, and oil palm, *Elaeis guineensis*, one of the crops in which cloning by SE plays a main role with 2.53 million clones produced in 2009 in Malaysia (Kushairi et al. 2010).

One of the largest field trials with emblings of woody angiosperms has been implemented with the rubber tree *Hevea brasiliensis*. French researchers at CIRAD (Carron et al. 2009) have done the laboratory work on this species. Up to 65 ha of field tests have been established in Ivory Coast, Nigeria and Thailand in collaboration with the companies Michelin (France) and RRIT (Thailand).

Applications of somatic embryogenesis of forest species in Europe

Woody angiosperms

The implication of woody angiosperms SE in genetic improvement programmes of forest tree species in Europe is quite limited at present. Although much work has been achieved in academic and public research institutions to develop protocols of SE for several hardwoods (Table 3), only a small number of plants have been produced. In several cases such as in *Quercus robur* established plants are used for physiological studies (Wilhelm, per. com). All of the emblings of *Castanea sativa* (about one hundred) that were transferred to the field survived. Many of them displayed an early sexual maturity, forming male catkins after 3 years and nuts the following year (Corredoira et al. 2006b). A small field trial of *Quercus suber* was established with plants from both zygotic and somatic embryos from a range of sources from one hundred-year-old trees. As regards the cork oak, the Spanish public company TRAGSA has established in Extremadura (Spain) large clonal tests with emblings obtained from half-sib zygotic embryos of selected trees, and with emblings obtained from those selected trees. More than forty selected hundred-year-old trees were cloned (Hernández et al. 2011) and hundreds of emblings have been established since 2005 in three experimental plots.

Gymnosperms

Among the coniferous species, the application of this technology is much further advanced.

In Sweden in 2004 several forestry companies became interested in SE and the role it could play in increasing the genetic quality of Norway spruce (*Picea abies*) planted in their forests. They set up a project with Skogforsk, the Forest Research Institute of Sweden to see if clonal testing of material from the Skogforsk Norway spruce (*Picea abies*) breeding programme could accelerate the development of new improved material. In 2006, the companies initiated a project, executed by SweTree Technologies to explore the bulk propagation of improved material from the Skogforsk breeding programme using SE technology. Although the original objective of the project was to work with tested clones, there was also interest in using untested clones derived from crosses between tested parents (referred to as "family forestry"). In this process 20 to 30 clones derived from 5 to 10 families can be expected to provide the same level of improvement, or higher, than would be expected from a seed crop collected in a seed orchard established with trees from the same generation as the parents (Lindgren 2009). The use of SE technology was partly to overcome the periodicity of good seed crops in Norway spruce.

In France, maritime pine (*Pinus pinaster*) material -mostly from full-sib families obtained from very high genetic value parents-, is being propagated using SE to produce material for field trials as well as to collect production cost data. Currently about 15,000 emblings per year are produced from a cryo-stored collection of more than 2,000 clones (Harvengt personal comm.). Commercial production is seen as a future perspective (perhaps in 10 years) due to a lack of willingness on the part of forest owners to plant SE material and overall a high reluctance to pay for genetic gain. Indeed, commercial nurseries are selling plant material at the same cost whatever their genetic value, but could not sustain high cost material such as SE. Their current strategy is to establish demonstration plots to convince the most competitive forest owners to plant the first small commercial plots while the technical issues are continuously improved. In order to increase the financial return of SE material, Interprovenance ('Interracial') crosses are included in the programme in order to maximise interest in the vegetatively propagated material (use of tested Corsican and Moroccan parents crossed with French Atlantic coast provenances).

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In Denmark, Nordman fir (*Abies nordmanniana*) is an important Christmas tree species. Christmas trees represent a unique forest product that commands high prices for trees with desired characteristics. Therefore it is an area where if superior individuals could be reproduced clonally, they could command a high price which would help to offset the high VP costs. Propagation of most true fir species by rooted cuttings is not possible. Currently 250 embryogenic cultures (clones) from 27 selected elite trees have been established and cryopreserved. Emblings from each clone will be produced for field testing and new sources of material will be added each year. The use of robotics is being explored as a way to help reduce production costs (Find and Krogstrup 2008).

In Ireland, Sitka spruce (*Picea sitchensis*) is the major commercial timber species. Seed orchards to produce improved material are yet to come into production and are unlikely to supply enough improved seed on a regular basis to meet current needs. Therefore VP of material from the breeding programme is seen as a possible solution. All material is bulk propagated from families or clones. The cost of a finished 15 cm embling (which requires an additional year of growth in the nursery before planting out) is estimated to cost between 2 and 3 Euros per plant. It is more than 10 times the cost of a 3 year-old bare-root unimproved seedling ready for planting in the field (Thompson personal comm.). Therefore it would be economically impractical to plant them directly in the field. So, emblings are grown on as stock plants in an outdoor hedge orchard to produce cuttings for rooting. A stock plant will remain in the hedge for 5 years. During this time the stock plant will produce more than 250 cuttings, of which about 80 % will root. Thus the cost for the embling stock plant itself disappears when spread large numbers of rooted cuttings (Thompson personal comm.).

Challenges

The main challenges for the use of VP in forestry (including SE technology) can be divided into 4 main areas which include: **1)** biological, **2)** economic, **3)** public acceptance and **4)** regulatory challenges.

Biological Challenges

In the past “maturation” particularly in coniferous species or the loss of the ability to vegetatively propagate an individual as it ages, was the major biological challenge facing VP by cutting. However, with the use of juvenile tissue and the cryogenic storage of embryogenic tissue while regenerated plants are tested in the field, the importance of maturation has decreased.

Some genotypes will simply not respond to VP and as a result they are considered as lost. It would be possible to develop “customised” protocols for certain genotypes, but this would not be practical on a large-scale. Recalcitrant clones can reduce the genetic diversity and may require the initiation of a larger number of genotypes in order to maintain the genetic diversity of the VP population.

Maintaining a wide genetic diversity in VP material is essential to avoiding potential problems due to both abiotic and biotic threats to the VP population. This point will be discussed further in the later section on Public Acceptance Challenges.

Clonal testing increases the accuracy of the selection process, but it takes time and costs money. If short-term testing or early evaluation through the assessment of physiological parameters or the use of genome-wide marker profiles could be developed, this could make clonal testing and thus the use of clonal propagation and deployment more attractive. Clones may show greater interaction with the environment as compared to seedling progenies. It may result in the need to develop specific clones for specific environments.

In spite of these potential biological limitations, none of them are insurmountable and thus there are currently no major biological impediments to prevent the widespread utilisation of SE technology.

Economic Challenges

The main economic concern about SE and VP in general is the higher cost of SE planting stock. A detailed economic analysis of the SE process (Cervelli and Senaratna 1995) showed that the most labour intensive steps in the SE process were where emblings had to be individually handled, which is typically at the end of the process (maturation, desiccation, germination/conversion). The use of computer selection of quality somatic embryos (Zhang et al. 1999) and robotic handling of emblings (Find and Krogstrup 2008) have been proposed to help further reduce these costs. A “SE Fluidics System” has been developed for Norway spruce in which the entire SE process is carried out in liquid medium allowing easy transfer without handling of individual somatic embryos (Aidun and Egertsdotter 2012). Similarly the encapsulation of the somatic embryo to form an “artificial seed” (Lulsdorf et al. 1993) has also been proposed as another way to minimize handling and thus reduce production costs. All of these methods are yet to be demonstrated to be practical on a commercial scale as well as effective in reducing production costs.

As mentioned earlier, rooted cuttings currently cost at least twice the price of comparable seedling produced material and emblings of loblolly pine in 2008 cost between 5 to 6 times that of comparable seedlings (Sorensson 2006) while European experience with spruce and pine suggests this may in fact be currently closer to 8 to 10 times. All these costs need to be balanced against the advantages which include a shorter time to get improved material into commercial use, increased flexibility of genetic composition, possible reduced rotation lengths, possible reduced establishment costs (quicker occupancy of the site), increased productivity and increased uniformity. However these positive arguments in favour of VP might still not be enough to break the strong psychological barriers of forest owners. Selling the idea of these benefits to foresters and landowners is the challenge. Meanwhile, there are several

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ways to reduce the per hectare planting costs, by planting mixtures of SE and unimproved plants or by using a lower cost plants produced by a combined process of SE and rooted cutting technology.

Connected to this production cost challenge, is also the question on how many SE lines could be economically handled and released at the same time so that genetic diversity in plantation could be maintained. Technical options such as to release different sets of a few clones over years exist but should require regulation adaptation.

Public Acceptance Challenges

The main public concern about the use of vegetatively propagated forest trees stems from a basic concern of man's "manipulation of nature", specifically the concern that vegetatively propagated material could potentially result in a loss of genetic diversity which could lead to a catastrophic failure of intensively managed forest plantations. Examples of catastrophic failures such as Dutch elm disease and chestnut blight are cited as examples of devastation yet to come from plantations with reduced genetic diversity, yet the wide genetic diversity of the natural population of both these species failed to provide any protection against the pathogen. Similarly monocultures are usually condemned without a proper understanding of the term or the actual levels of genetic diversity in both natural forests and plantations. Only monocultures with restricted genetic variation are indeed at risk. It is entirely possible to produce a vegetatively propagated plantation that has a greater genetic diversity than is available even in natural forests by including material from a wide range of populations.

A process for addressing environmental concerns about clonal forestry has been proposed by Stelzer and Goldfarb (1997) which should help allay many of the fears and concerns about clonal forestry. It basically depends on involvement of stakeholders from the beginning in the planning and testing of clonal forestry to demonstrate that public concerns are being addressed. Continued communication is essential in this process. Failure to address these concerns could lead to increased regulation.

Regulatory Challenges

Public concerns over the seemingly unrestricted use of vegetatively propagated forest material could result in regulations governing the use of this material. These regulations could originate from several different sources including forest certification organisations, national governments and the European Union. Regulation could be more rigorous for vegetatively propagated native species than with non-native species.

In the early 1980s the possible widespread use of vegetatively propagated forest trees led several countries establishing strict regulations governing the number of clones and the area that could be planted with this material. In particular, Sweden and Germany set some of the strictest regulations, however, in the years since then these original regulations have been relaxed. There is also some confusion of the terms "genetically improved" and "genetically modified" in the minds of some of those concerned about the use of this technology.

All member states in the European Union (EU) have agreed to Council Directive 1999/105/EC on the marketing of forest reproductive material. In this directive are definitions of how clones are defined (clones or clonal mixtures). However, it is up to the individual member states to each regulate (if they deem it necessary) the number of clones and their proportion as well as how long clones are to remain in production. In some cases member states have done this, but in most, no specific regulations dealing with clonally propagated material currently exist. However, this could change due to public pressure. The following is a summary of the current regulations specifically covering the use of vegetatively propagated material in Europe:

Germany- (2002)- only tested material can be vegetatively propagated and it must be planted in clonal mixtures.

France- the situation is similar to Germany.

Finland- material in the "qualified" category of forest reproductive material can be vegetatively propagated up to 1 million copies (2 million for birch). For "qualified" material more than 11 clones must be used to plant clonal mixtures and with "tested" material a minimum of 4 clones are needed in mixtures. For "tested" material there are no restrictions.

Sweden- (2000)- up to 5% of the site (up to 20 ha.) can be planted with one or more clones.

Norway- using bulk propagation with "tested" material the clones must originate from a minimum of 10 half-sib or full-sib families with clones originating from a minimum of 100 seedlings per family. For "qualified" material it must originate from 10 families as above but with the clones originating from a minimum of 100 seedlings per family. For clonal propagation a minimum of 30 clones from 10 unrelated families are required with a maximum of 50 plants per clone per site.

Denmark- only the use of clones of *Populus* are regulated for planting in Denmark.

For all other European member states- no national regulations specifically concerning the use of clonally propagated material exists.

Challenges for the increased use of somatic embryogenesis technology in European forestry.

There is little doubt that SE can propagate large amounts of genetically improved planting stock faster than any other VP method as has been demonstrated in other parts of the world. It also allows for the capture of genetic gain not possible through sexual propagation. The question then becomes, "if VP and SE are such useful technologies, why have they not taken off in Europe unlike other parts of the world?" While there are several relatively small, localised and specialised European SE programmes, there is nothing to compare with what is happening elsewhere. The answer to this is not simple and probably is due to several factors.

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Most European forests are small and about 60% of total forest area are privately owned, unlike the large areas of forest owned by the government or private companies in other parts of the world, although this is changing. Large areas of forest land around the world are now owned by Real Estate Investment Trusts (REITs) and Timber Investment Management Organisations (TIMOs). Because these are mainly investment organisations, whether they will be interested in planting vegetatively propagated improved material on their land remains to be seen. However, in Canada where SE technology was practised on a large scale most of the land is owned by the state. In this case it is the need to reforest land within a specified time after harvesting that has encouraged the use of SE planting stock.

Forestry tends to be a very conservative business where profits are limited and costs must be carried for many years before a return is realised. Perhaps the main problem is that the advantages of planting improved material, including vegetatively propagated improved material have not been fully and clearly demonstrated and proven to foresters and landowners. Further efforts are needed here. Part of the problem in Europe may be that there is no large, well developed market for genetically improved planting stock, especially when price becomes the major factor in deciding what type of planting stock to use. The added cost of improved material may be seen as just another added cost. Many economic studies have shown that improvements in the growth and productivity of forest trees resulting from tree improvement programmes more than cover the costs of planting improved material. Nevertheless about 35% of the respondents to the SE survey cited the "Lack of interest in improved material" as a bottleneck for the increased use of SE. Even if the additional cost of this material will be recovered from the resulting crop, there still is the problem of having to have to wait for the full rotation (40 to 100 years or more) to benefit fully from an investment made today. Even for organisations used to long-term investment in forestry, the additional up front costs may discourage the use of high cost planting stock. Perhaps the fact that forestry has become more of a business with short-term targets rather than a long-term investment has resulted in the loss of the long-term view.

In the time since the first draft of this manuscript was written the Canadian forest biotechnology company CellFor has filed for bankruptcy. While the company cites several reasons for the failure, part of the problem was due to the world-wide economic recession and its effect on the demand for timber. Clearly the high price of SE produced plants played a role in the failure of the company.

Unless SE production costs can be dramatically lowered to somewhere between the cost of a seedling and a rooted cutting (generally 2 to 3 times the cost of a seedling), or unless there is a large demand for uniform clonal material, such as Christmas trees, SE technology in forestry will remain a tool for researchers. Hybrid production systems where SE is the first of a two step propagation process also hold promise for large-scale use in some species where rooted cuttings are successful. The use of robots and artificial seed technologies may also help reduce SE production costs in the future, but this has not yet been demonstrated.

Finally there are the perceived concerns about clonal forestry such as the biological/technical, economic/marketing and environmental concerns, including regulatory issues that have been discussed earlier which may tend to inhibit landowners from considering planting vegetatively propagated material. Public acceptance and the possible regulatory issues that may develop from them could become a limiting factor if not handled properly. This will require the development of confidence by the public in the idea that the use of vegetatively propagated planting stock, if done correctly, can enhance, rather than reduce genetic diversity. The programme suggested by Stelzer and Goldfarb (1997) provides a scheme for how this can be accomplished. If at least a majority of the public can accept the use of this technology then the potential for government regulations can be greatly reduced.

Strategy for the implementation of somatic embryogenesis technology in European Forestry

For VP and SE to be practical and useful it must meet a set of criteria which might include some of the following:**1)**There needs to be a strong reason to use VP and SE, **2)**There needs to be a source of high value improved material, **3)** There needs to be a demonstrated efficient propagation method, **4)** The reasons to use VP would include: *i*) late and irregular flowering species such as for the spruces, larches, *ii*) valuable material that is not easy to propagate by other means such as interspecific (larch, aspen) or inter-populations (pines) hybrids, *iii*) species where uniformity and consistency are important characteristics such as for Christmas trees, *iv*) high value timber species such as wild cherry, walnut, and many fruit trees.

Of the species mentioned above hybrid larch (*Larix* x *eurolepis*) perhaps offers the greatest potential. There is a demand for true hybrid material which cannot be consistently provided by seed orchards. Seed source trials have shown that the best hybrids perform well across most of Europe thus providing a large potential market area. Finally there is a well developed SE technique available for the species (Lelu-Walter and Pâques 2009). Other good candidate species include Norway spruce (*Picea abies*) for Scandinavia (Högberg et al. 1998) and Sitka spruce for the United Kingdom and Ireland. Both species have seed periodicity problems and have well developed SE protocols. Although work has been done on maritime pine (*Pinus pinaster*, Lelu-Walter et al. 2006) and Scots pine (*Pinus sylvestris*, Lelu-Walter et al. 2008) further work would be required for these species. Among the broadleaf species there would need to be improved material from high value species that cannot be easily or cheaply propagated by alternative sexual or vegetative methods. However, some species of the genus *Quercus* currently offer high potential because SE can be induced in tissues from adult trees allowing the clonal testing of selected phenotypes.

What is needed is an information campaign designed first to inform foresters about the potential benefits of using SE and perhaps to re-enforce an understanding of the value of improved material. In addition, it would also be helpful to have the following: **1)** Field demonstrations plots with vegetatively propagated material, **2)** A completed economic analysis, **3)** A detailed plan for further development and scaling-up of mass-production at the European level, **4)** A

customer for the product(s), **5)** An individual willing to promote the benefits of SE and VP, **6)** No major public opposition or adverse regulations.

Concluding Remarks

Currently, in some parts of the world, VP of forest trees, in some cases using SE technology is well developed and used commercially. In Europe, in spite of the significant level of research and development in this field, as evidenced by the results of the survey carried out for this review, VP and the application of SE in forestry are not well developed. The basic understanding of the process of SE, while not complete, is sufficient for it to be used commercially. The main limiting factors appear to be **1)** the cost, and **2)** public acceptance. Public acceptance is currently not a significant factor, but it could become one if the use of VP in long-lived species such as trees is not adequately addressed. Concerns about the narrowing of genetic diversity in VP crops need greater attention. Stelzer and Goldfarb (1997) have provided a model to help develop public confidence in the use of VP forestry which will help to avoid future regulation of its use in Europe. The greater immediate problem is the cost of VP material which results in an unwillingness to plant this material. As discussed earlier, VP material can range from 2 to perhaps 10 times the cost of seed propagated material, mainly due to the increased labour required to produce this material. While some of these additional costs can be recovered through the increased wood volume, quality and uniformity in the VOP material, the problem is one of cash flow. The additional cost today will not be fully recovered until the forest crop is harvested which may be many years in the future. More effort needs to be directed toward reducing the cost to produce VP material especially SE plants if they are to become commercially acceptable. Methods to reducing the handling of individual somatic embryos, such as the use liquid culture including automated SE selection and handling, seem to be promising. The use of hybrid systems that combine SE and conventional VP methods have been shown to be successful. The VP of species such as Christmas trees that can command a high price for high quality trees is also an option. Examples and demonstration of how VP and SE technology can be applied are needed.

Unless the economic aspects of VP and SE are addressed, this technology will remain a research tool when it could be so much more.

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Figure captions

Figure 1. Place of Vegetative Propagation (VP) such as somatic embryogenesis (SE) in breeding programme and mass propagation. **A:** way only available for angiosperm species ()

Figure 2. Differences () between zygotic and somatic embryogenesis: example with maritime pine.

Figure 3. Somatic embryogenesis steps in forest trees (broadleaves and conifers).

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Table 1. Estimated usage of vegetatively propagated forest tree species in 2007 (Thompson unpublished).

Species	Number (million)
Eucalyptus	433
Pines	164
Cunninghamia lanceolata	65
Crytomeria japonica	17
Spruces	13
TOTAL	692

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Table 2. Explant used to induce somatic embryogenesis in angiosperm and gymnosperm species.

	Explant type	Species	Review
Angiosperms			Merkle and Cunningham 2011 Pijut et al. 2011 Vieitez et al. 2011
	Zygotic embryo	all species	
	Mature tree		
	Root	*Quercus* sp.	
	Epicormic shoot	*Quercus* sp., *Ulmus*	
	Leaf	*Quercus* sp., *Populus*, *Terminalia*	
	Shoot tip	*Quercus* sp.	
	Filament, anther	*Aesculus*, *Quercus* sp.	
	Inflorescence	*Quercus* sp., *Liquidambar*	
Gymnosperms			Attree and Fowke 1993 Stasolla et al. 2002 Klimaszewska et al. 2007
	Zygotic embryo	all species	
	Seedling parts	*Picea* sp.	
	Needle	*Picea glauca*	
	(emblings 10 ys old)	(Klimaszewska et al. 2011)	

Table 3. Countries in Europe involved in somatic embryogenesis of forest trees (angiosperm and gymnosperm species).

Country	**Species**										
	Angiosperm										
	Acer	***Aesculus***	***Castanea***	***Eucalyptus***	***Fagus***	***Fraxinus***	***Juglans***	***Populus***	***Prunus***	***Quercus***	***Sorbus***
Austria										spp.	
Belarus										*robur*	
Bulgaria										*robur*	
Czech Rep										*petraea*	
Denmark					*sylvatica*					*robur, rubra*	
France							*nigra*		*avium*		
Germany	*pseudoplatanus*				*sylvatica*						
Hungary		*hippocastanum*						x *canescens*			
Italy						*excelsior*					
Lithuania								*tremula* x *tremuloides*			
Portugal				*globulus*						*suber*	
Romania						*excelsior*			*avium*	*petraea, robur*	*domestica*
Serbia		*carnea, flava*									
Spain			*sativa, s.* x *crenata*							*ilex, robur suber*	
	Gymnosperm										
	Abies		***Larix***			***Picea***		***Pinus***			***Pseudostuga***
Belgium	*nordmanniana*										
Croatia						*omorika*					
Czech Rep	*alba, cephalonica*					*abies*					
Denmark	*nordmanniana, procera*										
Finland								*sylvestris*			
France			*decidua, x eurolepis,* x *marschlinsi*			*abies*		*pinaster, sylvestris, taeda*			*menziesii*
Germany	*nordmanniana*		*decidua,* x *eurolepis*			*abies, schrenkiana*					*menziesii*
Ireland						*sitchensis*					
Norway	*lasiocarpa*					*abies*					
Poland	*alba*		*decidua*			*abies, breweriana, omorika, pungens*					
Portugal								*pinaster*			
Romania						*abies, pungens*		*cecembra*			
Russia			*sibirica*					*sibirica*			
Serbia						*omorika*		*heldreichii, peuce*			
Slovakia	*alba, alba* x *numidica,*							*nigra*			

alba x *cephalonica*
Spain
Sweden
UK

abies
sitchensis

pinaster, *pinea*, *radiata*
sylvestris

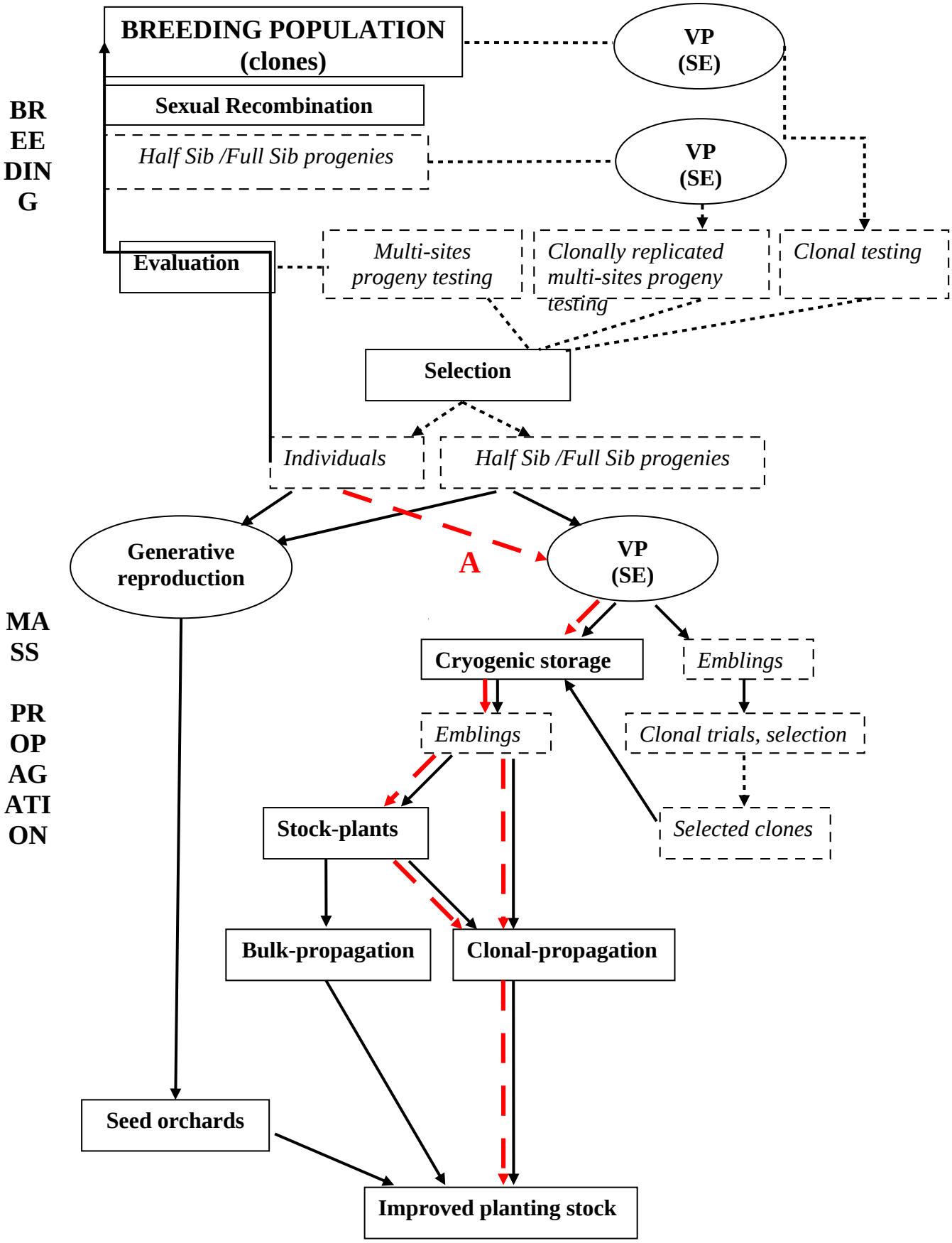

Figure 1

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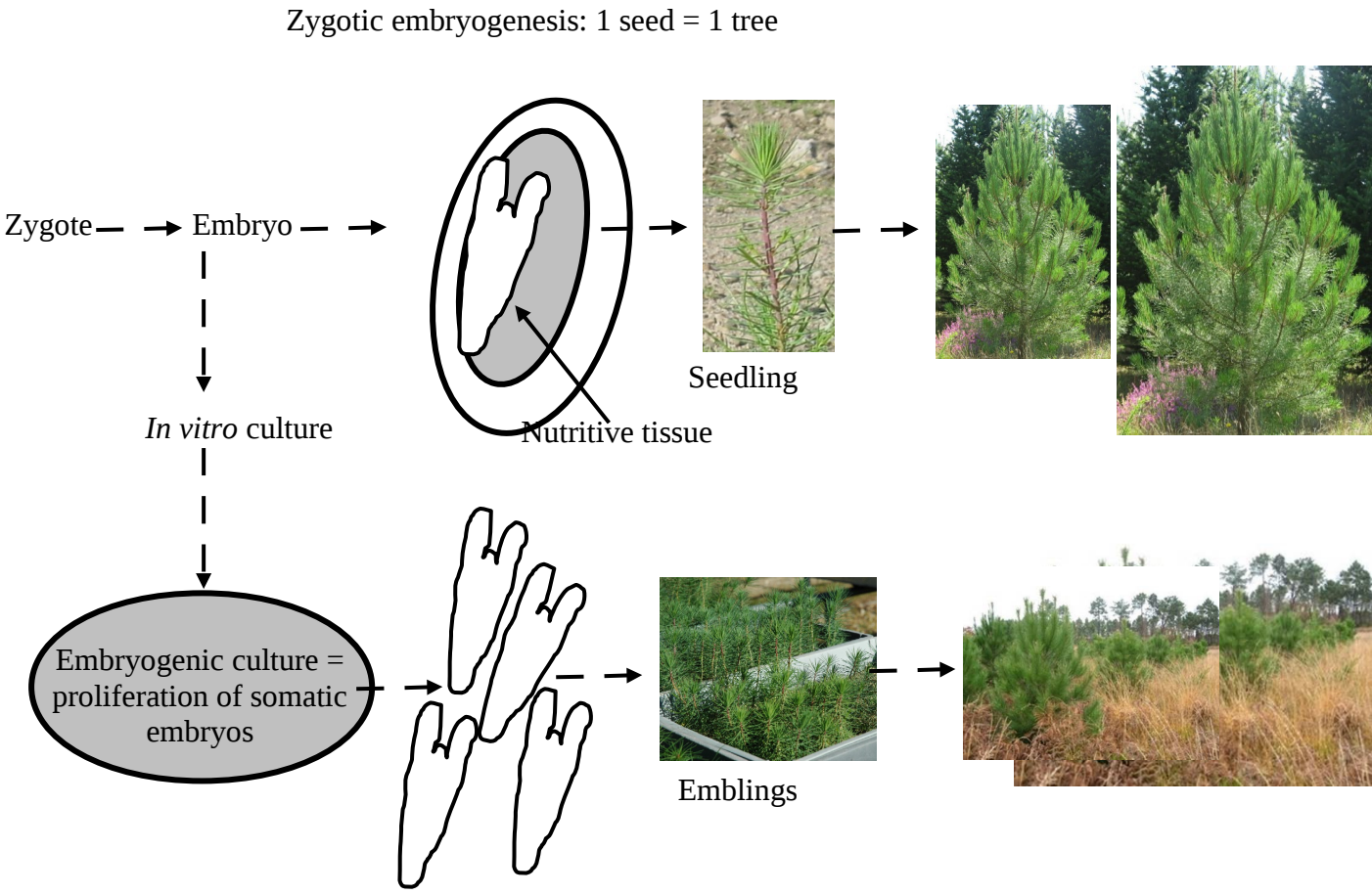

Figure 2

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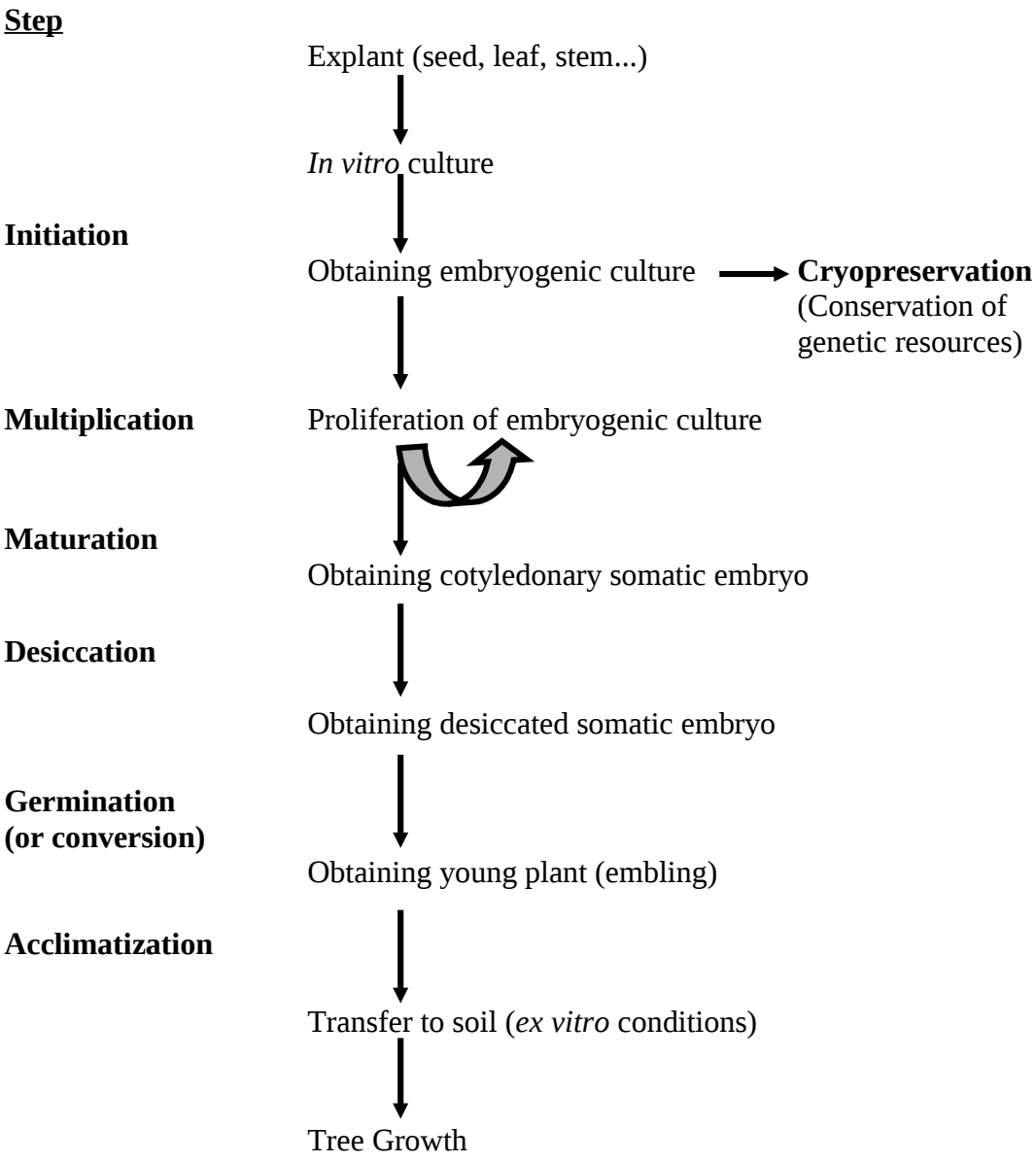

Figure 3