Online Edition
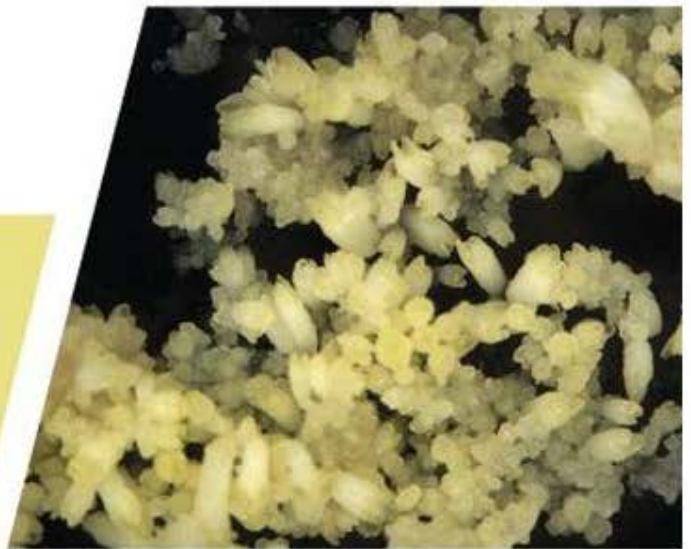
Vegetative Propagation of Forest Trees

Edited by
Yill-Sung Park, Jan M. Bonga and Heung-Kyu Moon

NIFoS
NATIONAL INSTITUTE OF FOREST SCIENCE

Vegetative Propagation of Forest Trees

Edited by

Yill-Sung Park
Jan Bonga
Hyeung-Kyu Moon

National Institute of Forest Science
2016

Micropropagation and production of forest trees

Olivier Monteuuis

CIRAD-BIOS, UMR AGAP, TA A-108/03 - Avenue Agropolis, 34398 Montpellier Cedex 5 – France. olivier.monteuuis@cirad.fr

Abstract

Forest tree species have been micropropagated *in vitro* for nearly 50 years by axillary budding first, then with increasing interest by *de novo* organogenesis, i.e., adventitious budding and somatic embryogenesis. The particularities of these three main techniques and more generally of *in vitro* micropropagation are reviewed, analyzing their respective pros and cons as well as their effectiveness and limitations for mass producing improved quality planting stock by comparison with more conventional propagation methods.

Keywords: Adventitious budding; Axillary budding; Field applications; Meristems; Nursery; Planting stock improvement; Rejuvenation; Somatic embryogenesis; Tissue culture; Vegetative propagation

1. Introduction

Plant micropropagation has been reviewed in the literature with special mention of its applications to forest trees (Bonga and Durzan 1982; 1987; Haines 1994) that were successfully cultured *in vitro* as early as the 1950's (Bonga and von Aderkas 1992). The purpose of the current paper is to reconsider this vegetative propagation technique from a broader point of view, highlighting its specificities and its usefulness for addressing issues related to the improvement and the production of forest tree planting stock.

2. Definition and expectations

Micropropagation literally refers to the propagation on a tiny scale of more or less differentiated cells that can be structured into organs, in order to produce, ultimately, complete plants. Micropropagation is a purely vegetative propagation technique, based on mitotic divisions that permit to replicate, theoretically unlimitedly, the original genotype while preserving all of its characteristics. The reality of cell totipotency as the conceptual basis of micropropagation (Durzan1984; Bonga and von Aderkas 1992) is strikingly demonstrated by single cell-derived

somatic embryogenesis (Yeung 1995). The very small size of the vegetative organs or tissues that are being micropropagated requires highly controlled environmental conditions for manipulating these structures as well as for ensuring their further development (Bonga et al. 2010). Axenic *in vitro* culture conditions have been proven to be the most suitable to meet these requirements (Bonga and Durzan 1982; Bonga and von Aderkas 1992; George 1993).

The use of the term micropropagation should, therefore, be restricted to vegetative propagation under *in vitro* conditions. Irrespective of the environment, primary meristems remain the basic structures as the origin of shoots and roots, and as such of micropropagation. *De novo* micropropagation should, however, be distinguished from micropropagation by axillary budding, although for certain species like *Eucalyptus spp.* the two ways may coexist (Le Roux & van Staden 1991).

3. *De novo* **micropropagation**

3.1 Somatic embryogenesis

Somatic embryogenesis (SE) consists in producing embryos by mitotic divisions from somatic cells while preserving their original genetic make-up. It is, therefore, a cloning technique, as opposed to zygotic embryogenesis in which germinal cells give rise to seedlings that are all genetically different from each other. Apart from a very few cases of direct embryogenesis, for example genotype-dependent cleavage polyembryogenesis (Durzan and Gupta 1987; Sharma and Thorpe 1995; Durzan 2008), SE is mainly indirect. The somatic embryos are formed *de novo*, usually after callus formation artificially induced by the application of strong growth regulators that are assumed to be partly the cause of somaclonal variation (Jones 2002; Menzies and Aimers-Halliday 2004; Bairu et al. 2011). In the most favorable situations, some undifferentiated cells of these calli can gradually evolve into somatic embryos characterized, similarly to zygotic embryos, by a shoot–root bipolar structure (Yeung 1995). This basically distinguishes somatic embryos from adventitious and axillary budding-derived microcuttings that consist of a shoot from which adventitious roots must develop subsequently. By virtue of this analogy with zygotic embryos, SE remains the only way of achieving complete ontogenetic rejuvenation. It resets the ontogenetic program to zero through the formation of embryonic structures that characterize the very first stages of the ontogeny. The older the mother plant the greater the magnitude of this ontogenetic rejuvenation. In this respect, *Hevea brasiliensis* (Carron and Enjalric 1985), *Quercus robur* (Toribio et al. 2004; San–José et al. 2010; Ballester and Vieitez 2012), and more recently *Quercus ilex* (Barra-Jiménez et al. 2014) deserve special consideration as, contrary to most woody species,

somatic embryogenesis can be obtained from sporophytic tissues of mature genotypes. Notwithstanding genotypic and culture medium interference (von Aderkas and Bonga 2000; Bonga et al. 2010; Monteuuis et al. 2011), the physiological rejuvenation associated with this SE ontogenetical rejuvenation has been helpful for subsequent mass clonal propagation by rooted cuttings of mature selected genotypes of rubber trees (Masson et al. 2013), and of other tree species (Lelu-Walter et al. 2013).

3.2 Adventitious budding

Similarly to SE, micropropagation by adventitious budding or organogenesis (Bonga and von Aderkas 1992) depends on the *de novo* formation of new meristems or meristemoids from specialized cells. These need first to dedifferentiate with the possible formation of a transitory callus before reinitiating shoot development from a newly formed shoot apical meristem (SAM). This process is generally induced by the addition of high concentrations of growth regulators into the initiation culture medium. Apparently, this dedifferentiation capacity can be found mostly in superficial cells of vegetative structures like cotyledons or hypocotyls that characterize the early stage of the ontogeny. Contrary to SE, roots are developed also *de novo* subsequently and not concomitantly to shoot formation. Shoot elongation followed by root formation requires transfer to suitable media. Usually, a substantial proportion of the adventitious shoots fail to develop true-to-type when transferred to the field, which may be due to the growth regulators added to the initiation medium (Bonga 1991; Timmis et al. 1992). This and a too high production cost may account for a much more limited operational use of adventitious budding than initially expected (Timmis et al. 1987; Menzies and Aimers-Halliday 2004).

3.3 Micropropagation by axillary budding

Every part of a tree shoot system and all the vegetatively produced offspring derived from it arise from the organogenic activity of the initial SAM formed at the apical pole of the embryo. SAMs through intensive cell divisions produce leaf initia and primordia, which are going to develop into full leaves of limited growth, as well as newly formed axillary meristems, which are potential SAMs at the axil of each leaf. The secondary meristem located underneath the SAM in the main stem that is responsible for cambium formation arises also from SAM activity. Micropropagation by axillary budding stimulates the organogenic capacity of these preexisting axillary meristems that may remain quiescent under apical dominance for long time periods to become proventitious buds liable to produce epicormic shoots. *In vitro* culture boosts the potential of these axillary

buds to produce new shoots. This is, therefore, a much more natural process than the *de novo* micropropagation that occurs after cell dedifferentiation and callus formation, with the associated risks of unexpected occurrence of variants. Micropropagation by axillary budding is considered to be less powerful in terms of potential multiplication rates than *de novo* shoot formation (Haines 1994; Menzies and Aimers-Halliday 2004; Lelu-Walter et al. 2013). It has been proven, nevertheless, for different tree species to be more reliable and sustainable in the long term with a higher guarantee of phenotypic true-to-typeness (Goh and Monteuuis 2001; Monteuuis et al. 2008; Mankessi et al. 2009; Monteuuis et al. 2013). The shoots derived from axillary meristems are trimmed into microcuttings during each subculture transfer and need ultimately, like for *de novo*-derived shoots, to form adventitious roots in order to become independent and autotrophic plants.

4. Chronological steps

Except for SE, which must be considered as a special case (Bonga and von Aderkas 1992; Timmis 1998; Thompson 2014), micropropagation by adventitious and axillary budding involves different chronological steps which are: culture initiation, the stabilization phase, shoot production, rooting and acclimation to *ex-vitro* conditions.

4.1 Culture initiation

Primary culture or culture initiation is a crucial step of micropropagation as it is the starting point of the process. It consists in introducing primary explants, which can be of different types and sizes, to *in vitro* conditions. These primary explants must have at least one SAM for micropropagation by axillary budding, whereas *de novo* techniques are by definition more flexible. One has to apply disinfection protocols strong enough to destroy surface contaminants, while maintaining explant tissues alive.

The organogenic responsiveness of a primary explant is liable to vary tremendously according to its physiological status within the donor plant (Durzan 1984; Monteuuis 1989; Bonga et al. 2010). The stress caused by the excision itself, the smaller the quantity of tissues removed the stronger the impact, the storage conditions, the disinfection procedure before inoculation and the delays in placing the tissue onto proper *in vitro* culture medium can also interfere (Bonga and Durzan 1982; Bonga and von Aderkas 1992). The physiological status of the explants depends on metabolic activities under the influence of environmental conditions and of endogenous factors encompassing genotypic effects, ageing, short and long distance physiological correlations (Durzan 1984; Bonga et al. 2010). External as well as endogenous rhythms, too often neglected, can also

interact (Lüttge and Hertel 2009). Young tissues collected from actively elongating stems are usually less exposed to external contamination than older ones, which are less succulent and as such more resistant to strong disinfection procedures. Also, the smaller the explant, the lesser the surface exposed to contaminants, hence the contaminations risks, but also the higher the cut surface to volume ratio, thus the higher the degree of damage (Bonga and Durzan 1982). Tiny explants like SAMs are far more sensitive towards the composition of the culture medium than bigger ones like microcuttings (Durzan 1984; Monteuuis 1988, George 1993). This sensitiveness to medium composition increases also with the age of the donor plant (Monteuuis 1987). Although presenting, theoretically, the advantage of an higher effectiveness for initiating contamination-free cultures concurrent with the possibility of getting rid of endogenous contaminants (George 1993; Bonga et al. 2010), meristem culture remains in practice little used for forest tree species (Durzan 1984). SAM micrografting can be viewed as an elegant and useful alternative to meristem culture (Monteuuis 2012). An *in vitro* germinated seedling used as rootstock constitutes a more natural and suitable culture support for SAMs than synthetic culture media. In addition to their benefits for initiating healthy cultures, using SAMs as primary explants has been more efficient than using bigger explants for achieving the physiological rejuvenation needed for clonally multiplying true-to-type mature selected genotypes of several tree species (Bon and Monteuuis 1991; Monteuuis 1991; Monteuuis and Goh 2015). In spite of these arguments, meristem culture and micrografting remain in practice impeded by SAM size, which varies noticeably according to the species, its physiological stage and even to the plastochron (Romberger 1963; Mankessi et al. 2010). Personal dexterity for excising rapidly and without damage the SAMs used as primary explants also has a determining impact. For these reasons, shoot apices have replaced SAMs as primary explants for certain species (Monteuuis 1996). In practice 1cm long shoot tips and nodal explants are more widely used, the tissues beneath the organogenic meristems buffering the composition of the culture medium that is never optimal and usually enriched with growth regulators – auxins, cytokinins – for stimulating growth activity. The initiation phase ends with the first morphogenetic response from the contamination-free explants, at which time fungal contamination will be visible thus allowing removal of contaminated cultures. The use of transparent gelling agents like gelrite and phytagel permits better assessment of bacterial contamination diffusing into the culture medium than translucent agar (George 1993). In order to prevent the spread of contamination from one explant to others, especially for precious material and only partially effective disinfection protocols, it is safer to introduce only one primary explant per culture vessel, generally a test tube.

4.2 Stabilization phase

The stabilization phase involves explants that look contamination-free at the end of primary culture, although the risk that these explants may contain endogenous bacteria cannot be ruled out (George 1993). For higher efficiency, several explants can be cultivated in one flask or jar. The "memory" of their initial location within the original donor plant (Durzan 1984, von Aderkas and Bonga 2000) from which they have been collected disappears progressively under the effect of medium-added growth regulators resulting in a higher overall uniformity of the tissue cultured crop.

4.3 Production phase

The production phase corresponds to the sustainable propagation and development of shoots that can be rooted *in vitro* or in more natural *ex-vitro* conditions (Driver and Suttle 1987; Monteuuis and Bon 1987; Bonga and von Aderkas 1992). At regular time intervals the explants are transferred onto fresh culture media of well-defined and suitable composition in order to ensure, over time, sufficiently high multiplication rates, mortality and contamination losses included. This is the main requirement to ensure a sustainable production of microcuttings that can be used first for developing efficient rooting protocols. The production prospects of micropropagation are often overestimated in scientific publications: the size of the buds obtained is sometimes not even indicated, the multiplication rates are established over a too short a culture period and from a too limited sample size to be realistic and applicable on an industrial scale. The aim of such experimental studies seems to get the greatest attention in publications. The reported micropropagation results have usually been achieved by adding to the *in vitro* medium supraoptimal concentrations of growth regulators prone to be the cause of a rapid decline of the cultures which need then to be reinitiated with new explants (George 1993). A more sustainable, natural and thus preferable approach consists in adding to the culture media exogenous growth regulators at concentrations compatible with shoot elongation. On such media, multiplication by axillary budding is promoted by the suppression of apical dominance when the elongated shoots are trimmed into nodal explants at each subculture transfer. The multiplication rates X are lower, but they increase exponentially according to the number of successive subcultures n, resulting in an amount of X^n explants at the end of the process. Several tree species in various laboratories have been subcultured for many years and even decades using such practices, combining shoot elongation and multiplication by axillary budding (Bon et al. 1994; Dumas and Monteuuis 1995; Goh and Monteuuis 2001; Monteuuis et al. 2008; Mankessi et al. 2009; Monteuuis et al. 2013). On media with low cytokinin concentrations, microshoots can root spontaneously. Morpho-organogenic activities have been observed to vary significantly in the course of time according to species, clones and

steady culture conditions (Monteuuis 1988; Favre and Juncker 1989; Monteuuis 2004a). This is very likely due to the influence of endogeneous rhythms (Champagnat et al. 1986; Lüttge and Hertel 2009). Beside growth regulators, mineral components are also important: unsuitable salt compositions are liable to induce noticeable changes in the morphological and organogenic capacity of the explants, leading ultimately to culture failure (Monteuuis 1988).

4.4 Rooting and acclimation to *ex-vitro* conditions

The microshoots produced *in vitro de novo* or by axillary budding must ultimately be rooted to become a fully autonomous plant. There are several ways of producing adventitious roots from an *in vitro*-derived shoot (Monteuuis and Bon 1987). Basically, the process involves 3 successive phases: root induction, root initiation and root expression (Gaspar et al. 1994). Briefly, root induction corresponds to the biochemical/physiological signals sent to the target cells by the application of exogenous rooting substances or "auxins" at the base of the microcuttings as instant dips or during longer periods on an auxin-enriched *in vitro* rooting medium (George 1993). Consequently these target cells undergo concrete anatomical changes during the initiation phase to give rise to root primordia that elongate and become visible during the expression phase. For many tree species and conifers more specifically, root primordia require to be placed onto a specific auxin-free expression medium to elongate (Monteuuis and Bon 1986, Bon et al. 1994, Dumas and Monteuuis 1995). The whole process can be achieved entirely *in vitro*, or induced and initiated *in vitro* and then exposed to *ex-vitro* conditions for root elongation on more natural horticultural substrates (Driver and Suttle 1987; Monteuuis and Bon 1987; Bonga and von Aderkas 1992). The *in vitro* environment provides a better control of external parameters but is more costly, especially when specific media are required for root induction/initiation and expression. Moreover, microshoots *in vitro* are heterotrophic with limited capacity for photosynthesis which makes the transfer to *ex-vitro* conditions critical. Risk of hydric stress, especially for unrooted microshoots, must be prevented. Also, most of the time, the roots formed in gelled media differed anatomically and morphologically from roots adapted to a more natural environment (Monteuuis and Bon 1986; McClelland et al. 1990). According to species, these *in vitro* formed roots are often totally or partially replaced by more functional ones once transferred to *in vivo* conditions (Bonal and Monteuuis 1997). Most of the time, the new *ex-vitro* roots arise from the root structures developed *in vitro* which may justify, at least for certain species or for not fully rejuvenated material, to carry out the rooting process partially or completely *in vitro* (Hackett 1988; McCown 1988). However for cost, manipulation, time saving and greater efficiency reasons, it is usually preferable to

root directly the *in vitro* derived shoot in *ex vitro* conditions (McCown 1988; Bonal and Monteuuis 1997; Goh and Monteuuis 2001).

5. Usefulness

The advantages of using micropropagation to improve forest tree species planting stock have been discussed for several decades already. From a practical standpoint and with the benefits of hindsight, its main advantages seem to be:

5.1 Propagation efficiency

Providing suitable protocols can be developed, micropropagation permits to mass produce, theoretically, unlimited numbers of selected plants from a small group of cells that are more or less organized and that could not survive in *in vivo* conditions. This is particularly true for organs which, once removed from the donor plants, cannot be rooted *ex vitro* or grafted. Such rootless explants can be maintained and serially subcultured on proper culture medium during the time needed to ensure their mass multiplication or to restore their ability for adventitious rooting resulting from a sufficient degree of physiological rejuvenation (Bonga and Durzan 1982; Durzan 1984; Hackett 1988). Another main advantage of micropropagation is the possibility to mass produce in a restricted space, year around, regardless of the local outdoor conditions, enough material to make it more cost efficient than propagation under nursery conditions, especially when simple *in vitro* protocols are used (Monteuuis 2000).

5.2 Alternative to outdoor stock plants

Adapted micropropagation procedures permit to mass multiply sustainably by serial subcultures selected plant material without resorting to stock plants that need to be intensively managed to ensure the production of rooted cuttings in properly equipped facilities. The greater the production targets, the larger the required stock plant areas and rooting beds and the higher also the number of qualified staff that is needed to run all this efficiently. The overall cost of producing plants by rooted cuttings in nurseries together with the constraints this imposes increases dramatically with the quantity of planting stock needed. This should not be underestimated (Monteuuis 2000).

5.3 Establishment of contamination-free *ex-situ* gene banks

Tissue culture is by definition contamination-free, although endogenous contaminants like bacteria may exist surreptitiously for years to invade unpredictably the culture medium and thus affect all the explants of the same origin

after several subculture cycles. Shoot apical meristem culture has proven its efficiency for getting rid of such problems (George 1993). Cultures can be stored *in vitro* a long time at a temperature low enough to limit explant growth, reducing thereby the frequency of the subcultures. The most effective storage method is cryopreservation which requires special pre and post conditioning treatments (Bonga and von Aderkas 1992; George 1993; Jones 2002). Such an *ex situ* gene pool stored *in vitro* can be helpful for various species irrespective of the local natural conditions and can be used for different purposes, including DNA characterization in the absence of exogenous microbial contaminations.

5.4 International exchanges of vegetative material

Thanks to being contamination-free, tissue-culture remains to date the only way to introduce vegetative plant material to countries with very strict phytosanitation rules. Micropropagation is, therefore, essential for the international exchange and acquisition of germplasm for research as well as for operational and commercial purposes.

5.5 Requisite for GMO evaluation

Micropropagation of *in vitro* genetically transformed cells or group of cells to produce complete plants is also crucial for assessing the expected benefits resulting from genetic engineering experiments. Such assessment should be done *in vitro* first, and then ultimately outdoors (Bonga and von Aderkas 1992; Haines 1994; Timmis 1998).

5.6 Physiological rejuvenation

The possibility offered by tissue culture, in comparison with nursery techniques, to cultivate miniaturized organs, in particular SAMs that can be micrografted *in vitro,* is a real asset with regard to physiological rejuvenation prospects (Durzan 1984; Monteuuis 1989; Bonga and von Aderkas 1992). This is essential for successful true-to-type cloning of mature selected trees (Bonga 1991). In some cases, e.g., for clonal seed orchard establishment, it can be advantageous to rejuvenate the mature genotypes only to the degree needed to get rooted shoots, while avoiding too much vegetative vigor, delayed flowering and seed production that can result from a more advanced physiological rejuvenation.

5.7 Economics

Due to certain particularities developed previously, micropropagation can be economically more profitable than conventional propagation by rooted cuttings

from stock plants in the nursery. This mostly depends on the production scale and also on the capacity of the plants to be micropropagated using simple protocols. The coexistence of the two systems developed for teak within the same company in Sabah, East Malaysia established that if more than 100 000 teak plants are produced per annum, micropropagation was more cost effective than nursery techniques. This was mainly due to the savings made because the intensive and time consuming management of stock plants is not needed when propagating by tissue culture (Monteuuis 2000). *In vitro* culture cost can also be significantly reduced by micropropagating plants in countries where the financial investment needed for setting up and then running proper tissue culture facilities is lower, mainly because manpower in developing countries is far less than paid in developed ones.

6. Current limitations

It appears from the literature that micropropagation protocols have been successfully established for various forest tree species. This might be true at an experimental scale but not operationally where micropropagation development remains impeded by serious limitations.

6.1 Availability of responsive primary explants

Culture initiation success depends greatly on the type and the physiological condition of the explants inoculated (Durzan 1984; Bonga et al. 2010). Easy access to nearby donor plants to provide primary explants will definitely be beneficial. Also, resort to efficient nursery methods for preconditioning these explants prior to their introduction to tissue culture may greatly help, according to species and circumstances. These methods include grafting and optional use of BA sprays on successfully grafted scions, as well as keeping portions of branches or sticks under humid conditions in order to stimulate the production of young sprouting shoots to be utilized as responsive primary explants (Monteuuis et al. 2011).

6.2 Genotype responsiveness

The capacity for micropropagation often varies tremendously according to the genotype. For instance, at the genus level and notwithstanding a strong between and within species genotypic influence (Park et al. 1998), *Picea spp* demonstrate overall a higher capacity for somatic embryogenesis than pines or firs and douglas fir. For this latter species cleavage polyembryogenesis is strongly influenced by provenance (Durzan and Gupta 1987). Likewise, poplar (McCown et al. 1988) and

radiata pine (Aitken-Christie et al. 1988) have a higher predisposition for adventitious budding or meristematic nodule formation than other species. Also, marked differences of *in vitro* rooting capacity were observed between closely related *E. urophylla X grandis* hybrid clones derived from the same mother tree – half-sib genotypes (Nourissier and Monteuuis 2008, Mankessi et al. 2009).

6.3 Physiological ageing

The capacity for micropropagation decreases more or less rapidly according to species as genotypes physiologically age (Bonga and Durzan 1982, Durzan 1984, Hackett 1988). This is especially true for adventitious budding and SE which, except for a few exceptions like *Hevea brasiliensis* (Carron and Enjalric 1985), *Quercus spp* (San-José et al. 2010, Barra-Jiménez et al. 2014), remain restricted to very young individuals, mostly immature or mature embryos, too young for reliable selection (Bonga et al. 2010). When SE is successful part of the resulting emblings will be used for establishing clonal tests, while the others will be cryopreserved for as long as it takes to get results from the clonal tests, which allows a sounder selection (Park et al. 1998; Sutton 2002; Lelu-Walter 2013). Notwithstanding variations in the course of time, axillary budding multiplication rates are generally higher for physiologically juvenile explants than for more mature ones. These latter usually require higher concentrations of cytokinins in the nutrient medium, at least during the initiation and stabilization phases (Monteuuis 1988; 2004a). The negative influence of natural ageing on adventitious rooting ability and phenotypic true-to-typeness of the clonal offspring is well known (Bonga and Durzan 1982; Bonga 1991). More insidious is the *in vitro*-induced physiological ageing liable to affect prematurely soft and permeable cells exposed to non-optimal SE or adventitious budding culture media (McKeand 1985; Frampton and Isik 1987; von Aderkas and Bonga 2000).

6.4 Composition of the culture medium

In vitro culture media are usually synthetic, gelled or liquid, and consist of a combination of a restricted list of salts, vitamins, sucrose and growth regulators (Bonga and von Aderkas 1992; George 1993). The characteristics of these components as well as their interactions are likely to change uncontrollably during the autoclaving process, as well as during each subculture cycle due to nutrient uptake by the explants, evaporation, pH variation, and influence of temperature and light (George 1993). These unexpected changes are totally independent of the metabolic requirements associated to explant development in the course of time. Stress caused, for example by inappropriate medium components, unsuitable matrix strength, excessive concentrations of growth regulators and macro-salts,

ammonium especially, can affect the physiology and responsiveness of the explant (von Aderkas and Bonga 2000). *In vitro* micrografting can be an alternative solution to such limitations especially for tiny explants that are more sensitive (Monteuuis 2012).

6.5 Laboratory requirements

Micropropagation activities require proper facilities, equipment and human resources. These include a permanent supply of electricity and good quality water, as well as judiciously partitioned and equipped building facilities (George 1993). Location wise, the vicinity of a big city offers a lot of advantages like airport facilities, external services for easier maintenance and good delivery as well as more daily life convenience. Conversely, easy access to donor plants and suitable nursery facilities can help for initiating the *in vitro* cultures and for testing the post *in vitro* behavior of the tissue-cultured plants and adapting the protocols accordingly, bearing in mind the benefits of *ex vitro* rooting (Monteuuis et Bon 1987; McCown 1988; McClelland et al. 1990).

6.6 Ecomomics

Whatever the technique used, economics have a determining influence on the operational utilization of micropropagation and on how it benefits forest plantations. The main issues to be addressed should be: *i)* Is micropropagation the most suitable way of propagating the selected species taking into account its specificities? *ii)* For what end-use? *iii)* And what ultimate return on investment?

7. *In vitro*-induced effects

Contrary to more conventional vegetative propagation techniques, micropropagation can modify certain characteristics of the *in vitro* cultured plant material.

7.1 Rejuvenation

From an ontogenic standpoint, SE-derived offspring must be duly considered as completely rejuvenated, the more developed the initial donor plant, the greater the rejuvenation achieved. The maturation symptoms that can be observed within such ontogenetically-rejuvenated embling populations are likely due to non-optimal culture media (von Aderkas and Bonga 2000; Monteuuis et al. 2011). Serial micropropagation of microcuttings by axillary budding can also induce a certain degree of mature to juvenile reversion affecting traits like leaf

morphology, particularly visible in heteroblastic species like *Pinus sp* or *Acacia sp,* or in species with a conspicuous dimorphism between juvenile and mature foliage (Mullins et al. 1979; Hammatt and Grant 1993; Monteuuis et al. 2011). In the case of *Acacia mangium*, unpredictable morphological reversions of the mature phyllode type to the juvenile compound leaves at the SAM level during shoot elongation have been noticed only *in vitro* so far (Hatt et al. 2012). Higher capacities for growth, for adventitious rooting as well as for multiplication by adventitious and axillary budding have been noticed as the numbers of subcultures increased for various tree species (Fouret et al. 1986; Walker 1986; Monteuuis 1988; Dumas and Monteuuis 1991; Monteuuis 2004a and b). These changes must objectively be interpreted as physiological rejuvenation indicators influenced by the macro-salt composition of the culture medium, the addition of activated charcoal or exogenous cytokinins (Walker 1986; Monteuuis 1988; Dumas and Monteuuis 1991; 1995). Hence, micropropagation can be useful for at least partially physiologically rejuvenating *in vitro* of mature genotypes, even if most of these rejuvenations revert to the mature phase after acclimatization to *ex vitro* conditions (Mullins et al 1979; Fouret et al. 1986; Pierik1990).

The rare although demonstrative rejuvenation cases obtained from SAM cultures either directly on synthetic media or by micrografting may be due to the removal of potentially juvenile SAMs from ageing-induced inhibiting correlative systems to which they are exposed within the mature donor tree (Durzan 1984; Monteuuis 1989; Bonga et al. 2010; Monteuuis et al. 2011). It can be assumed that their inoculation on a suitable *in vitro* culture medium free of inhibitory ageing factors, while possibly benefitting from rejuvenating substances from the juvenile rootstock in the case of micrografts (Monteuuis 2012), will allow the expression of their juvenile characteristics. Explant miniaturization as well as the timing of SAM excision seems to have a determining influence on *in vitro* physiological rejuvenation, the juvenile "window" becoming more and more time and space restricted as the ortet develops (Monteuuis 1989; Bonga et al. 2010; Monteuuis et al. 2011), which is consistent with the cyclophysis concept (Schaffalitzky de Muckadell 1959; Olesen 1978). Conversely, the incomplete or transitory rejuvenation that is observed when bigger primary explants are used might be due to the persisting negative ageing influence by the mature tissues that are removed together with the meristems from the donor plant.

7.2 SAM characteristics

Cytohistological investigations of *Acacia mangium* have shown that SAMs of juvenile and mature origins displayed morphological and infrastructural similarities with SAMs of outdoor juvenile plants when micropropagated *in vitro*, even at the nucleus level (Hatt et al. 2012). *In vitro* culture of SAMs of *Eucalyptus*

urophylla x Eucalyptus grandis also resulted in a noticeable reduction of SAM size and cell numbers, depending on the plastochron (Mankessi et al. 2010; 2011a). This strengthens the assumption of a possible rejuvenating effect of tissue culture at the SAM level for different tree species (Fouret et al. 1986; Pierik 1990; Hammatt and Grant 1993). In contrast, cells of SAMs *in vitro* that had been excised from juvenile and mature ortets are characterized by a large vacuome which is more representative of the mature state. This reinforces the opinion that *in vitro* culture could also have a maturing effect on plant tissues (George 1993), which could account for the incomplete or transitory *in vitro* rejuvenation that has been reported (Pierik 1990; von Aderkas and Bonga 2000, Monteuuis et al. 2011).

7.3 DNA methylation

According to several reports, repeated *in vitro* subcultures of tree species could induce an overall increase of DNA global methylation as well as DNA methylation profiles that are different from those of outdoor growing plants (Li et al. 2002; Valledor et al. 2007; Monteuuis et al. 2008; 2009). A progressive re-methylation due to prolonged *in vitro* culture has been hypothesized (Lambé et al. 1997). However, reports are not consistent (Mankessi et al. 2011b) and are prone to vary according to species, *in vitro* culture conditions and duration (Lambé et al. 1997; Hasbun et al. 2005; Valledor et al. 2007).

8. Practical considerations

A lot of papers have been published on successful micropropagation of various forest trees, illustrating an intensive activity at the experimental level during the past 50 years. However, reliable reports on operational applications of these research activities are few (Lelu-Walter et al. 2013; Thompson 2014) and progress so far has been below expectations. This might be due to several reasons.

8.1 An increasing gap between research and short term applications

Research quality, especially in the public sector, is more and more evaluated with regard to the number of papers published in high ranking scientific journals. Consequently, research topics are getting more and more basic in nature, with far reaching and ambitious targets that are more and more disconnected from short term applications, and most of the time conducted by researchers who have not been exposed to the constraints associated with operational activities. As a matter of fact, the number of publications on micropropagation of forest trees has dramatically declined during the past decades, in spite of urgent needs to meet with the shortest delays a constantly increasing wood demand for various end-uses.

8.2 What species for what end-uses?

Forest tree species are highly diverse. Some have been selected, domesticated, genetically improved and planted for specific end-uses that encompass pulp and paper, particle boards, multipurpose lumber, veneer and slicing for the most precious timber ones. It can logically be assumed that the value of the final end product will have an impact on the selling price of the planting material: planters will be more eager to buy costly planting stock if the return on investment or the added value is higher. *Picea abies* is in this respect demonstrative: the mass production of selected clones by rooted cuttings initiated during the 1970-1980s in Germany (Kleinschmit 1974; Kleinschmit and Schmidt 1977) has progressively declined as this costly planting material failed to be economically profitable for chip or even lumber end-uses. Contrarily, producing *Abies nordmanniana* clones to be sold as Christmas trees (Nielsen et al. 2008), with a much higher added value and a better return on short term investment seems more justified (Lelu-Walter et al. 2013).

8.3 The best propagation strategy to meet the objectives: seed vs vegetatively produced plants.

In contrast with propagation by seeds, in which every seedling is genetically different from one another, asexual or vegetative propagation consists in duplicating, theoretically endlessly, genotypes while preserving through mitotic divisions the integrity of their original genetic make-up – and thus, consequently, all their individual characteristics. This is essential to ensure the transfer of phenotypic traits that are under non-additive control, especially for those that have a strong economic impact.

The choice of the propagation method remains highly dependent on species characteristics and more particularly on the range of variation of economically important traits among seedlings, especially for genetically related ones like half-siblings issued from the same mother tree. The greater this variability, the more justified the vegetative propagation option, at least theoretically (Bonga and Durzan 1982). Practically, how well plant material can be efficiently mass propagated vegetatively has a determining impact. Vegetatively produced plants are usually more expensive than seedlings. This is why the respective pros and cons of the sexual vs asexual propagation systems in relation to end-use targets and added value must be wisely pondered. For particle board and chip production, seedlings from good provenances are generally preferred for various reasons, cost especially, over vegetatively produced planting stock, as argued for *Acacia mangium* (Monteuuis et al. 2003). This basic question seems particularly relevant for *Picea spp* and *Pinus spp* considering the huge investment put into SE research

activities with these species during the past decades, unfortunately with concrete returns that are still far below expectations (Thompson 2014). Will all this pay off one day? The answer is obvious for teak due to the very cost efficient mass clonal micropropagation techniques that have been developed lately for producing, in short rotations, a high yield of premium quality timber of great market value (Goh and Monteuuis 2015 in this book).

8.4 Bulk vs clonal propagation

Bulk propagation consists in the vegetative propagation of a group of mixed genotypes without maintaining any individual identification. This can be useful for increasing the number of plants of presumably high genetic value but available in insufficient quantities, like for example those obtained by controlled pollinations. In clonal propagation, by contrast, genotypic identity is rigorously and individually preserved through successive propagation cycles, which may last several centuries in certain cases.

The main advantage of bulk propagation lies in the unnecessity to label and keep separated each genotype. This means that less handling and management is required than is needed for the clonal option, especially when large numbers of clones are concerned. Vegetatively propagating a mixture of unidentified genotypes will maintain a certain degree of genetic variability, depending on the number of genotypes involved, at least at the beginning. This may no longer be the case as the number of propagation cycles increases in the course of time as the genotypes with the higher multiplication and rooting rates are likely to supplant progressively the others. Clonal propagation while keeping each genotype separated, prevents such risks, in addition to a number of other advantages, including the possibility to mass produce superior planting material for establishing high yielding and uniform large-scale plantations of premium quality (Libby and Rauter 1984). Another issue to consider is that each seed-issued genotype is unique and there will always be a a "risk" that the time, energy, land and cost investments required by advanced tree breeding programs may not deliver, ultimately, genotypes that are as good as a particular outstanding one selected from the wild. This is partly due to biological processes like the DNA recombinations associated with chromosome crossing overs over which breeders have no control. Being able to mass clonally propagate true-to-type any selected individual regardless of its age is therefore of paramount importance. Practically, the success is highly dependent on the efficiency of the vegetative propagation methods used. In other words, special efforts must be devoted for adapting the cloning techniques to the particularities of the selected genotypes, rather than the other way around. As an illustration, clonal selection based on rooting capacity can be skewed by inefficient

rooting protocols and also by the lack of a strong positive correlation between rooting ability and other field traits of commercial importance.

8.5 Micropropagation vs more conventional vegetative propagation methods.

For micropropagation as for any other plant propagation technique, the simpler and therefore the cheaper to meet the ultimate objective the better. A wise approach of developing vegetative propagation protocols for new plant material should be to test first its responsiveness to conventional nursery techniques. Certain species like *Gmelina arborea*, *Populus spp* can be easily and advantageously mass clonally propagated from mature selected individuals by rooted cuttings in nursery facilities or even directly out planted. In case of nursery technique limitations and if the species is economically really worthwhile, then resort to tissue culture can be considered. In *Sequoiadendron giganteum* (Monteuuis 1988), the effectiveness of various conventional techniques like propagation by rooted cuttings, air layering, grafting for cloning mature selected individuals was first assessed and the information thus obtained warranted to work with smaller ramets (Monteuuis 1985). This was done by using *in vitro* techniques to miniaturize more and more the explants, starting with microcuttings (Monteuuis and Bon 1986), then shoot tips and finally SAM culture and micrografting (Monteuuis 1986, 1987).

Resorting to tissue culture must be warranted. There are too many examples of programs embarking on sophisticated *in vitro* programs without assessing first the capacities of simpler procedures to meet the actual needs. For *Eucalptus urophylla X E. grandis* hybrid clones for instance, the minicutting technique associated with intensively managed container-grown stock plants (Saya et al 2008) was found more efficient than micropropagation by axillary budding (Nourissier and Monteuuis 2008, Mankessi et al 2009).

9. Conclusion

Micropropagation is a remarkable tool for improving the quality of forest tree planting stock. Its usefulness has, however, to be seriously pondered according to the ultimate objectives and the particularities of the plant material to meet the desired goals. The advantages and limitations of vegetatively multiplying selected trees by tissue culture rather than in more natural and cheaper nursery facilities deserve special consideration. Practically, producing with the shortest delays and at the cheapest cost the needed quantity of improved quality planting stock to meet plantation requirements must remain the priority.

10. References

Aitken-Christie J, Singh AP, Davies H (1988) Multiplication of meristematic tissue: A new tissue culture system for radiata pine. In: Hanover JW, Keathley DE (eds) Genetic Manipulation of Woody Plants, Plenum Press, New York, pp 413-432

Bairu MW, Aremu AO, van Staden J (2011) Somaclonal variation in plants: causes and detection methods. Plant Growth Regul 63:147-173

Ballester A, Vieitez AM (2012) Partial rejuvenation of mature hardwood trees through somatic embryogenesis: The example of pedunculate oak. In: Park, YS, Bonga JM (eds) Proceedings of the IUFRO Working Party 2.09.02 conference on "Integrating vegetative propagation,biotechnologies and genetic for tree production and sustainable forest management"June 25-28, 2012, Brno Czech Republic. Published online:http://www.iufro20902.org,: pp 47-55

Barra-Jiménez A, Blasco M, Ruiz-Galea M, Celestino C, Alegre J, Arrillaga I, Toribio M (2014) Cloning mature holm oak trees by somatic embryogenesis. Trees 28:657-667

Bon MC, Monteuuis O (1991) Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through *in vitro* meristem culture. II. Biochemical arguments. Physiol Plant 81: 116-120

Bon MC, Riccardi F, Monteuuis O (1994) Influence of phase change within a 90-year-old *Sequoia sempervirens* on its in vitro organogenic capacity and protein patterns. Trees 8:283-287

Bonal D, Monteuuis O (1997) *Ex vitro* survival, rooting and initial development of *in vitro* rooted vs unrooted microshoots from juvenile and mature *Tectona grandis* genotypes. Silvae Genetica, 46(5):301-306

Bonga JM (1991) In Vitro propagation of conifers: fidelity of the clonal offspring. In: Ahuja MR (ed) Plant Biotechnology. Plenum Press, New York, pp 13–21

Bonga JM, Durzan DJ (1982) Tissue Culture in Forestry. Martinus Nijhoff/W. Junk, Boston, Massachusetts, 420p.

Bonga JM, von Aderkas P (1992) In Vitro Culture of Trees. Kluwer Academic Publishers, Dordrecht, 236 p

Bonga JM, Durzan DJ (1987) Cell and Tissue Culture in Forestry. Volume 1. General Principles and Biotechnology. 422 pp Volume 2. Specific Principles and Methods. 447 pp. Volume 3. Case histories. 416 pp. Martinus Nijhoff Publishers, Dordrecht, Netherlands.

Bonga JM, Klimaszewska KK, von Aderkas P (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tissue Organ Cult 100: 241-254

Carron MP, Enjalric F (1985) Embryogenèse somatique à partir du tégument interne de la graine d'*Hevea brasiliensis* (Kunth., Müll., Arg.). CR Acad Sci III-Vie 300 (17):653–658

Champagnat P, Barnola P, Lavarenne S (1986) Quelques modalités de la croissance rythmique. Naturalia Monspeliensia. Colloque International sur l'Arbre, Montpellier. pp 279-302.

Driver JA, Suttle GRL (1987) Nursery handling of propagules In Bonga, JM, Durzan, DJ (eds). Tissue Culture in Forestry, Vol. 2, Scientific Principles and Methods. Martinus Jijhoff/Dr. W. Junk, Publishers, Dordrecht, Netherlands; pp 320-335

Dumas E, Monteuuis O (1991) Régénération in vitro de pins maritimes âgés par bourgeonnement adventif sur euphylles. Annales AFOCEL 1989-1990: pp 43-58

Dumas E, Monteuuis O (1995) *In vitro* rooting of micropropagated shoots from juvenile and mature *Pinus pinas*ter explants: influence of activated charcoal. Plant Cell Tissue Organ Cult 40:231-235

Durzan DJ (1984) Special problems: Adult vs. juvenile explants, Chap. 17. In: Sharp WR, Evans DA, Ammirato PV, Yamada Y (eds) Handbook of Plant Cell Culture, Crop Species. MacMillan Publishing Company, New York, pp 471-503

Durzan DJ (2008) Monozygotic cleavage polyembryogenesis and conifer tree improvement. Cytol Genet 42(3):27-44

Durzan DJ, Gupta PK (1987) Somatic embryogenesis and polyembryogenesis in Douglasfir cell cultures. Plant Sci 52: 229–235

Favre JM, Juncker B (1989) Variations in expression of episodic growth by in vitro-culture shoots of oak *Quercus robur* L.). Annales des Sciences Forestières 46:206-210

Fouret Y, Arnaud Y, Larrieu C, Miginiac E (1986) *Sequoia sempervirens* as an *in vitro* rejuvenation model. NZ J For Sci 16:319-327

Frampton LJ Jr, Isik K (1987) Comparison of field growth among loblolly pine seedlings and three plant types produced *in vitro*. TAPPI J 7:114-120

Gaspar T, Kevers C, Hausman JF, Ripetti V (1994) Peroxidase activity and endogenous free auxin during adventitious root formation. In: Lumdsen PJ, Nicholas JR, Davies WJ (eds), Physiology, Growth and Development of Plants in Culture. Kluwer Acad. Pub, Dordrecht, pp 289– 298

George EF (1993) Plant Propagation by Tissue Culture. Exegetics Ltd, Edington. England.

Goh D, Monteuuis O (2001) Production of tissue-cultured teak: the Plant Biotechnology Laboratory experience. In: Proc. of the Third Regional Seminar on Teak: "Potential and opportunities in marketing and trade of

plantation Teak: Challenge for the New Millenium". July 31 – Aug. 4, 2000, Yogyakarta, Indonesia, pp 237-247

Goh D, Monteuuis O (2015) Teak. In this book.

Hackett WP (1988) Donor plant maturation and adventitious root formation. In: Davis TD, Haissig BE, Sankhla N (eds). Adventitious Root Formation in Cuttings. Dioscorides press. Oregon. US pp 11-28

Haines RJ (1994) Biotechnology in forest tree improvement, with special reference to developing   countries. FAO Forestry Paper No. 118, 226 pp

Hammatt N, Grant NJ (1993) Apparent Rejuvenation of Mature Wild Cherry (*Prunus avium* L.) during micropropagation. J Plant Physiol 141:341-346

Hasbun R, Valledor L, Santamaria E, Cañal MJ, Rodriguez R, Rios D, Sanchez M (2005) *In vitro* proliferation and genome DNA methylation in adult chestnuts Acta Hortic 693:333–340

Hatt C, Mankessi F, Durand JB, Boudon F, Montes F, Lartaud M, Verdeil JL, Monteuuis O (2012) Characteristics of Acacia mangium shoot apical meristems in natural and in vitro conditions in relation to heteroblasty. Trees 26:1031-1044

Jones N (2002) Somatic embryogenesis as a tool to capture genetic gain from tree breeding strategies: Risks and benefits. Southern Afric For J 19 (1):93-101

Kleinschmit J (1974) A program for large-scale cutting propagation of Norway spruce. N Z J Forest Sci 4:359-366

Kleinschmit J, Schmidt J (1977) Experience with *Picea abies* cutting propagations in Germany and problems connected with large scale applications Silv Genet 26(5-6):197-203

Lambé P., Mutambel HSN, Fouché JG, Deltour R, Foidart JM and Gaspar T (1997): DNA methylation as a key process in regulation of organogenic totipotency and plant neoplastic progression? In Vitro Cell Dev Biol-Plant 33:155-162

Lelu-Walter M-A, Thompson D, Harvengt L, Sanchez L, Toribio M, Pâques LE (2013) Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction. Tree Genet Genomes 9:883–899 doi:10.1007/s11295-013-0620-1

Le Roux JJ, van Staden J (1991) Micropropagation and tissue culture of Eucalyptus - a review. Tree Physiol 9:435-477

Libby WJ, Rauter R (1984) Advantages of clonal forestry. For Chronicle, June 1984:145-149

Li X, Xu M, Korban SS (2002) DNA methylation profiles differ between field- and *in vitro*-grown leaves of apple. J Plant Physiol 159:1229-1234

Lüttge U, Hertel B (2009) Diurnal and annual rhythms in trees. Trees 23:683-700

Mankessi F, Saya A, Baptiste C, Nourissier S, Monteuuis O (2009) *In vitro* rooting of genetically related *Eucalyptus urophylla X Eucalyptus grandis* clones in relation to the time spent in culture. Trees 23:931-940

Mankessi F, Saya AR, Boudon F, Guedon Y, Montes F, Lartaud M, Verdeil JL, Monteuuis O (2010) Phase change-related variations of dome shape in *Eucalyptus urophylla X Eucalyptus grandis* shoot apical meristems. Trees 24:743-752

Mankessi F, Saya AR, Montes F, Lartaud M, Verdeil JL, Monteuuis O (2011a) Histocytological characteristics of *Eucalyptus urophylla x Eucalyptus grandis* shoot apical meristems of different physiological ages. Trees 25: 415-424

Mankessi F, Saya AR, Favreau B, Doulbeau S, Conejero G, Lartaud M, Verdeil JL, Monteuuis O (2011b) Variations of DNA methylation in *Eucalyptus urophylla x Eucalyptus grandis* shoot tips and apical meristems of different physiological ages. Physiol Plant 143:178–187

Masson A, Julien JM, Boedt L (2013) Industrial propagation by rooted cuttings of mature selected clones of *Hevea brasiliensis*. Bois et Forêts des Tropiques, 317(3):51-58

McClelland MT, Smith MAL, Carothers ZB (1990) The effects of *in vitro* and *ex vitro* root initiation on subsequent microcutting root quality in three woody plants. Plant Cell Tissue Organ Cult 23:115-123

McCown BH (1988) Adventitious rooting of tissue cultured plants. In: Davis TD, Haissig BE, Sankhla N (eds) Adventitious Root Formation in Cuttings. Dioscorides press. Oregon, US: pp 289-302

McCown BH, Zeldin EL, Pinkalla HA, Dedolph RR (1988) Nodule culture: a developmental pathway with high potential for regeneration, automated micropropagation, and plant metabolite production from woody plants.In: Hanover JW, Keathley DE (eds) Genetic Manipulation of Woody Plants. Plenum Press, New York, pp 149-166

McKeand SE (1985) Expression of mature characteristics by tissue culture plantlets derived from embryos of loblolly pine. J Am Soc Hortic Sci 110:619–623

Menzies MI, Aimers-Halliday J (2004) Propagation options for clonal forestry with conifers. In: Water C, Carson M (eds). Plantation Forest Biotechnology for the 21st Century. Kerala (India): Research Signpost. pp 255-274

Monteuuis O (1985) La multiplication végétative du séquoia géant en vue du clonage. Annales AFOCEL 1984, pp 139-171

Monteuuis O (1986) Microgreffage de points végétatifs de *Sequoiadendron giganteum* Buchholz séculaires sur de jeunes semis cultivés in vitro. CR Acad Sci Paris, 302(III):223-225

Monteuuis O (1987) *In vitro* meristem culture of juvenile and mature *Sequoiadendron giganteum*. Tree Physiol 3:265-272

Monteuuis O (1988) Aspects du clonage de séquoias géants jeunes et âgés. Thèse de Doctorat d'Etablissement, Univ Blaise Pascal, Clermont-Ferrand, 190 p

Monteuuis O (1989) Maturation concept and possible rejuvenation of arborescent species. Limits and promises of shoot apical meristems to ensure successful cloning. In: "Breeding Tropical Trees: Population Structure and Genetic Improvement Strategies in Clonal and Seedling Forestry". Proc Conference IUFRO, Pattaya, Thailand, 28 Nov.-3Dec. 1988, 106-118

Monteuuis O (1991) Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through *in vitro* meristem culture. I. Organogenic and morphological arguments. Physiol Plant 81:111-115

Monteuuis O (1996) *In vitro* shoot apex micrografting of mature *Acacia mangium*. Agroforestry Systems, 34(2):213-217

Monteuuis O (2000) Propagating teak by cuttings and microcuttings. In: Proc. of the international seminar "Site, technology and productivity of teak plantations" FORSPA Publication N°24/2000, Teaknet Publication N°3, 209-222

Monteuuis O (2004a) *In vitro* micropropagation and rooting of *Acacia mangium* microshoots from juvenile and mature origins In Vitro Cell Dev Biol-Plant 40:102-107

Monteuuis O (2004b) *In vitro* rooting of juvenile and mature *Acacia mangium* microcuttings with reference to leaf morphology as a phase change marker. Trees 18(1):77-82

Monteuuis O (2012) *In vitro* grafting of woody species.   Propagation of Ornamental Plants 12(1):11-24

Monteuuis O, Bon MC (1986) Microbouturage du séquoia géant. Annales AFOCEL 1985, 49-87

Monteuuis O, Bon MC (1987) Enracinement et acclimatation de vitro-plants forestiers. C.R. Symposium "Plant micropropagation in horticultural industries", Florizel 87:160-169

Monteuuis O, Alloysius D, Garcia C, Goh D, Bacilieri R (2003) Field behavior of an in vitro-issued *Acacia mangium* mature selected clone compared to its seed-derived progeny. Aust For 66(2):87-89

Monteuuis O, Doulbeau S, Verdeil JL (2008) DNA methylation in different origin clonal offspring from a mature *Sequoiadendron giganteum* genotype. Trees 22:779-784

Monteuuis O, Baurens FC, Goh DKS, Quimado M, Doulbeau S, Verdeil JL (2009) DNA methylation in *Acacia mangium in vitro* and *ex-vitro* buds, in relation to their within-shoot position, age and leaf morphology   of   the   shoots. Silvae Genet 58(5-6):287-292

Monteuuis O, Lardet L, Montoro P, Berthouly M, Verdeil JL (2011) Somatic embryogenesis and phase change in trees. In: Park YS, Bonga JM, Park

SY, Moon HK (eds), Proc. of the IUFRO Working Party 2.09.02 on "Advances in Somatic embryogenesis of Trees and Its Application for the Future Forests and Plantations" Aug 19-21, 2010, pp 21-29

Monteuuis O, Galiana A, Goh DKS (2013) Chapter 15: *In vitro* micropropagation and rooting of *Acacia mangium* and *A. mangium x A. auriculiformis*. In: Lambardi M, Ozudogru EA, Jain SM (eds) "Protocols for Micropropagation of Selected Economically-Important Horticultural Plants" Springer Science-Business Media, New York. pp 199-211

Monteuuis O, Goh DKS (2015) Field growth performances of different age teak genotypes clonally produced by rooted cuttings, *in vitro* microcuttings and meristem culture. Can J For Res 45:9-14

Mullins MG, Nair Y, Sampet P (1979) Rejuvenation *in vitro*: induction of juvenile characters in an adult clone of *Vitis vinifera* L. Ann Bot 44:623-627

Nielsen UB, Rasmussen HN, Jensen M (2008) Rooting Nordmann fir cuttings for Christman trees? In: Vegetative propagation of conifers for enhancing landscaping and tree breeding, September 10-11, 2008, Punkaharu, Finland, Working papers of the Finnish Forest Research Institute 114, pp 48-52

Nourissier S, Monteuuis O (2008) *In vitro* rooting of two *Eucalyptus urophylla* X *Eucalyptus grandis* mature clones. In vitro Cell Dev Biol-Plant 44:263-272

Olesen PO (1978) On cyclophysis and topophysis. Silvae Genet 27: 173-178

Park YS, Barrett JD, Bonga JM (1998) Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones, In Vitro Cell Dev Biol-Plant 34:231-239

Pierik RLM (1990) Rejuvenation and micropropagation. In Nijkamp HJJ, Van Der Plas LHW, Van Aartrijk J (eds) "Progress in Plant Cellular and Molecular Biology". Amsterdam, Netherlands, pp 91-101

Romberger JA (1963) Meristems, Growth and Development in Woody Plants. USDA For Serv Tech Bull No 1293, 214 p.

San-José MC, Corredoira E, Martínez MT, Vidal N, Valladares S, Mallón R, Vieitez AM (2010) Shoot apex explants for induction of somatic embryogenesis in mature *Quercus robur* L. trees. Plant Cell Rep 9:661-671

Saya RA, Mankessi F, Toto M, Marien JN, Monteuuis O (2008) Advances in mass clonal propagation of *ucalyptus urophylla X E. grandis* in Congo. Bois et Forêts des Tropiques 297:15-25

Schaffalitzky de Muckadell, M. 1959. Investigations on aging of apical meristems in woody plants and its importance in silviculture. Kandrup and Wunsch's Bogtrykkeri, København, pp 313-346

Sharma KK, Thorpe TA (1995) Asexual embryogenesis in vascular plants in nature. In: Thorpe TA (ed) *In Vitro* Embryogenesis in Plants. Kluwer Academic Publishers, pp 17–72

Sutton B (2002) Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis. Annals of Forest Science, 59(5-6):657-661

Thompson D (2014) Challenges for the large-scale propagation of forest trees by somatic embryogenesis - a review In: Park YS, Bonga JM (eds.). Proceedings of the 3rd international conference of the IUFRO unit 2.09.02 on "Woody plant production integrating genetic and vegetative propagation technologies."September 8-12, 2014. Vitoria-Gasteiz, Spain. Published online: http://www.iufro20902.org.: pp 81-90

Timmis R (1998) Bioprocessing for tree production in the forest industry: conifer somatic embryogenesis. Biotech Progr 14:156-166

Timmis R, Abo El-Nil M, Stonecypher RW (1987) Potential genetic gains through tissue culture. In: Bonga JM, Durzan DJ (eds.) Cell and Tissue Culture in Forestry. Vol. 1. General Principles and Biotechnology. MartinusNijhoff, Dordrecht, pp 198-215

Timmis R, Ritchie GA, Pullman GS (1992) Age-and position-of-origin and rootstock effects in Douglas-fir plantlet growth and plagiotropism. Plant Cell Tissue Organ Cult 29:179-186

Toribio M, Fernández C, Celestino C, Martínez MT, San-José MC, Vieitez AM (2004) Somatic embryogenesis in mature *Quercus robur* trees. Plant Cell Tissue Organ Cult 76:283-287

Valledor L, Hasbun R, Meijon M, Rodriguez JL, Santamaria E, Viejo M, Berdasco M, Feito I, Fraga MF, Canal MJ, Rodriguez R (2007) Involvement of DNA methylation in tree development and micropropagation. Plant Cell Tissue Organ Cult 91:75-86

von Aderkas P, Bonga JM (2000) Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. Tree Physiol 20:921-928

Walker N (1986) *Sequoia sempervirens*: réjuvénilisation et culture de méristèmes en cascade. Annales AFOCEL 1985:25-47

Yeung EC (1995) Structural and developmental patterns in somatic embryogenesis. In: Thorpe TA (ed) *In Vitro* Embryogenesis in Plants. Kluwer Academic Publishers, pp 203-247