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Somatic Embryogenesis and Phase Change in Trees

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Abstract

The advantages of clonal plantations are obvious for a lot of tree species. Somatic embryogenesis is a clonal propagation method with the greatest potential for achieving this goal, especially if combined to genetic engineering. However, more than for other vegetative propagation techniques, the practical use of somatic embryogenesis remains strongly impeded by the genetic identity and the physiological age of the mature selected trees to be cloned. So far, somatic embryogenesis has been successfully obtained from mature individuals only for a very limited number of broad-leaved or deciduous species using as primary explants leaves in a proper physiological condition and also sporophytic tissues from the reproductive organs. It is currently still limited to the embryonic phase of the ageing process for many evergreen coniferous species of high industrial impact. Shoot apical meristems owing to their key role in phase change warrant special consideration for attempting to clone mature trees by somatic embryogenesis. If direct induction from *in situ* collections is still hazardous in the absence of reliable indicators for the more responsive physiological stage, preconditioning *in vitro* procedures are worth considering when attempting to succeed in somatic embryogenesis from mature trees. These *in vitro* techniques include serial microcutting in subcultures as well as meristem culture and micrografting. With these techniques meaningful results have been obtained for different tree species in terms of rejuvenation. If some are limited to *in vitro* conditions, others are more unequivocal.

Abbreviations: BA: 6-Benzylaminopurine; SAM: Shoot apical meristem; SE: Somatic embryogenesis

Foreword

The meaning of the terms "*phase change*" and "*rejuvenation*" can be controversial and has often been debated (Wareing 1987, Pierik 1990, Jones 1999). They are considered in this paper in their most simple and literal form: *Phase change*: change from juvenile to mature characteristics and *vice-versa*: it includes therefore rejuvenation; various traits can be involved; *Rejuvenation*: recovery, even partially, of juvenile characteristics. The production of leaves exhibiting a juvenile-like morphology by shoot apical meristems (SAMs) from mature trees demonstrates the possibility for such SAMs to recover a certain degree of physiological juvenility. This reversion from mature to juvenile characteristic features must be considered in all objectivity as a demonstration of rejuvenation at the SAM level, even if this recovery is often fugacious. In this respect, the rationale for using heteroblastic species exhibiting a conspicuous foliar dimorphism between the juvenile and the mature phases for studying SAM phase change must be emphasized (Von Passecker 1947, Schaffalitzky de Muckadell 1959). Rejuvenation should be distinguished from reinvigoration (Wareing 1987, Pierik 1990). The latter means literally the regaining of vigor and is restricted to this definition in this paper. The most juvenile plants *i.e.* young germinants, with the highest capacity for adventitious rooting - which remains one of the most commonly acknowledged indicators of juvenility - are indeed not vigorous. Vigor corresponds to the attainment to a certain degree of maturation, or physiological ageing, generally associated to a decline of capacity for adventitious rooting (Borchert 1976): vigorous shoots generally do not root easily. This is why rejuvenation and reinvigoration must be differentiated.

Introduction

The advantages of clonal plantations are obvious for a lot of arborescent species including rubber (Carron *et al* 2009), fruit (Jain and Ishii 2003), ornamental and forest trees (Libby and Rauter 1984, Lindgren 2002). Except in some contexts, where grafted plants may be preferable, somatic embryogenesis (SE) is the clonal propagation method with the greatest potential for achieving this goal (Park *et al.* 1998, Jones 2002, Park 2002). In addition, SE can be combined with genetic engineering (Jones 2002, Malabadi and Nataraja 2007). However, more than for other vegetative propagation techniques used for producing plants with their own roots, the practical outcome of SE remains strongly impeded by the genetic identity and the physiological age of the selected trees to be cloned (Park *et al.* 1998, Bonga *et al.* 2010). So far, SE has been obtained from mature individuals only for a limited number of broad-leaved or deciduous species using as primary explants leaves in a proper physiological condition and also sporophytic tissues from the reproductive organs (Dunstan *et al.* 1995, Von Aderkas and Bonga 2000, Bonga *et al.* 2010). It is currently still limited to the first stages of embryo development for a few evergreen coniferous species of high industrial impact (Park 2010). SAMs, owing to their key role in phase

change and to the juvenile potential of their meristematic cells, warrant special consideration when attempting to clone mature trees by somatic embryogenesis (Monteuuis 1989). Induction could be done directly from SAMs collected *in situ*, or after *ex-vitro* and *in vitro* preconditioning.

Prospects of inducing SE directly from SAMs collected *in situ*

Through their organogenic capacities, SAMs ensure the aerial development of plants according to their ontogenetical program with associated morphological and anatomical characteristics (Sussex 1989, Weigel and Jürgens 2002). SAMs are prone to physiological ageing in relation to growth cycles and distance to the root system (Schaffalitzky de Muckadell 1959, Borchert 1976, Kerstetter and Poethig 1998). Such sequential phase changes at the SAM level are reflected, more or less saliently, according to species by various morphological indicators such as leaf shape or appearance of flowers (Schaffalitzky de Muckadell 1959, Robinson and Wareing 1969, Jones 1999). This has been called cyclophysis and it suggests that shoots can show "windows" of juvenility that are more tip and time restricted as ageing increases during the ontogenetical process (Schaffalitzky de Muckadell 1959, Olesen 1978). These windows of physiological juvenility will become ultimately confined to the SAMs, or even to within SAM zones or cells during the period of higher metabolic activity preceding shoot expansion or flush (Krenke 1940, Monteuuis 1988, 1989), as illustrated in Figures 1. These windows correspond to budbreak in temperate countries. A that time, SAMs from mature and juvenile genotypes demonstrate similarities in many respects, including *in vitro* culture success rates (Monteuuis 1987a).

Figures 1. *Examples of cyclophysis in Callistemon sp (left) and in Sequoia semperviens*

Recent findings have established that there are similarities between SAMs from mature and juvenile trees in relation to particular plastochronic phases (Mankessi *et al.* 2010, Mankessi *et al.* 2011). Another factor to consider for inducing SE from SAMs collected *in situ* is their size, liable to vary a lot from species to species, with the age of the donor tree and the within tree location of the SAMs (Parke 1959, Owston 1969). Too often, shoot apices are confused with true SAMs. These latter consist of the apical dome and emerging leaf initia or primordia (Romberger 1963). SAMs of about 100µm as overall size seem to be the smallest that can be introduced routinely in culture, bearing in mind that SAMs of a lot of species are much tinier than this (Romberger 1963, Mankessi *et al.* 2010). According to Nozeran (1978, 1984) and Nozeran *et al.* (1982), the more mature the tree, the smaller the portion of SAM tissue liable to contain cells that have remained juvenile and that would be responsive after excision and *in vitro* culture. The aim is to prevent the negative ageing influence of the more mature surrounding cells and tissues on the juvenile potential of the SAMs. Rare, but meaningful observations in the field demonstrate the existence of cells within SAMs of mature trees that have remained juvenile and organogenic (Figure 2).

Figure 2*. Demonstration of natural rejuvenation: juvenile-like shoot produced by a SAM from a mature Eucalyptus sp. (courtesy of Franclet).*

Ideally, such juvenile cells or tissues must be excised during the most appropriate time window to be set in culture. This time window has been assumed to be more and more restricted as the age of the donor tree increases (Krenke 1940, Monteuuis 1989). Practically, at present, it is still difficult to identify the more juvenile SAM cells or tissues to be excised in viable condition at the most suitable period from mature *in situ* selected individuals for SE induction. All these constraints plus the stress caused by the excision of the selected tissues make such operations hazardous. The smaller the size of tissue removed for placement in culture, the stronger the negative influence of stress on explant survival and further development (Bonga and Von Aderkas 1992).

Ex-vitro preconditioning procedures aimed at stimulating SE induction capacity

***Figures 3**. a) Shoots from proventitious buds exhibiting a juvenile morphology for the first leaves produced in Acacia mangium and b) stimulation of interfascicular buds giving rise to shoots with euphylls (arrows) after BA sprays on grafted mature Pinus pinaster scions.*

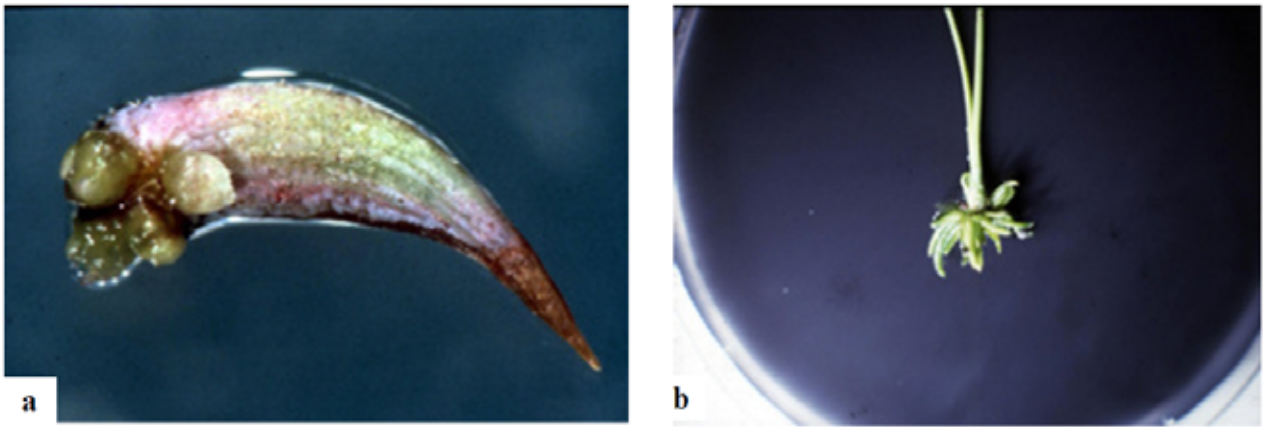
Different treatments have rejuvenated SAMs, resulting in the production of juvenile-like leaves from mature genotypes. These arose from quiescent proventitious buds after the suppression of apical dominance by operations such as pinching, pruning, hedging or even felling. In this latter case, coppicing shoots with juvenile-like traits can be produced from the stumps of the felled trees (Monteuuis *et al.* 1995a). A more conservative and as such a more recommendable method is to cut branches into longitudinal parts with at least one axillary bud, and to place the sticks thus obtained vertically under mist and shade (Monteuuis *et al.* 1995b). The first leaves produced by the very soft shoots starting to elongate usually exhibit a juvenile morphology, as is illustrated in Figure 3a. Another option consists in applying cytokinins, in the form of sprays of BA in aqueous solutions, on grafted scions collected from mature selected ortets (Franclet 1983). It has been very efficient when applied at the end of winter, just before budbreak, to mature *Pinus pinaster* auxiblast shoot tips grafted onto seedling rootstocks (Figure 3b).

Concomitant to the elongation of the terminal bud, the numerous needle fascicle buds of the mature scion started to increase in size to give rise soon to shoots with primary leaves or euphylls (Dumas 1987). This attested that the quiescent interfascicular buds had been rejuvenated by the application at the right time of BA sprays. Instead of only one terminal bud at the top of the mature scion, this treatment induced the formation of plenty of rejuvenated shoots with buds at the tip end of each. The advantage of using primary leaves or euphylls instead of brachyblasts with pseudophylls or needle fascicles that characterize the mature phase for pines is explained hereafter.

Serial or “en cascade” grafting has been reported to induce “rejuvenation”, especially on Douglas fir, as the number of grafting cycles of the selected mature scions onto the young seedling rootstocks increases (Franclet 1983). However, it seems more proper to talk about reinvigoration when a regaining of vigor is the main effect resulting from these serial graftings.

Prospects of serial *in vitro* subcultures for inducing SE

Subculturing, sometimes for several years, of microcuttings on appropriate *in vitro* culture media has induced in different species a degree of rejuvenation, at least for certain traits, in comparison with juvenile controls exposed rigorously to the same experimental conditions. More or less pronounced reversions to juvenile characteristics were observed as the number of subcultures increased with regard to traits such as: leaf morphology, growth, multiplication by axillary budding and adventitious rooting rates, and even the capacity for adventitious budding (Mullins *et al.* 1979, Fouret *et al.* 1986, Walker 1986). Usually the subculture media used contained cytokinins, but this was not always the case. In *in vitro* cultures of giant sequoia, analogies between a 100-yr-old mature clone and the juvenile control became more and more obvious for different traits as the number of microcutting transfers onto a MS-derived medium with activated charcoal and free of cytokinin increased. However, replacing the MS salt composition by a Knop-derived macroelement formulation had a totally opposite effect (Monteuuis 1988). Subculture-induced



Figures 4. *de novo formation of adventitious buds from euphylls (a) and pseudophylls (b) produced by long-time subculture of microcuttings from mature selected Pinus pinaster.*

rejuvenation has been particularly obvious for heteroblastic species like *Acacia mangium* where SAMs from mature trees can produce after several subcultures juvenile-like leaves instead of the phyllodes that characterize the mature phase (Baurens *et al.* 2004, Monteuuis 2004). Meaningful responses (Figures 4) were also observed for *Sequoia sempervirens* and *Pinus pinaster* where *de novo* formation of buds and shoots could be obtained from juvenile-like leaves produced by meristems of microcuttings from mature selected ortets, after that these microcuttings had been for a long time subcultured (Walker 1986, Dumas and Monteuuis 1991). This demonstrates the possibility of using well adapted subculture protocols for physiologically rejuvenating SAMs from mature trees, even if most of such rejuvenation has been limited to the tissue culture episode and has reverted to the mature phase after acclimatization under *ex vitro* conditions (Mullins *et al.* 1979, Fouret *et al.*

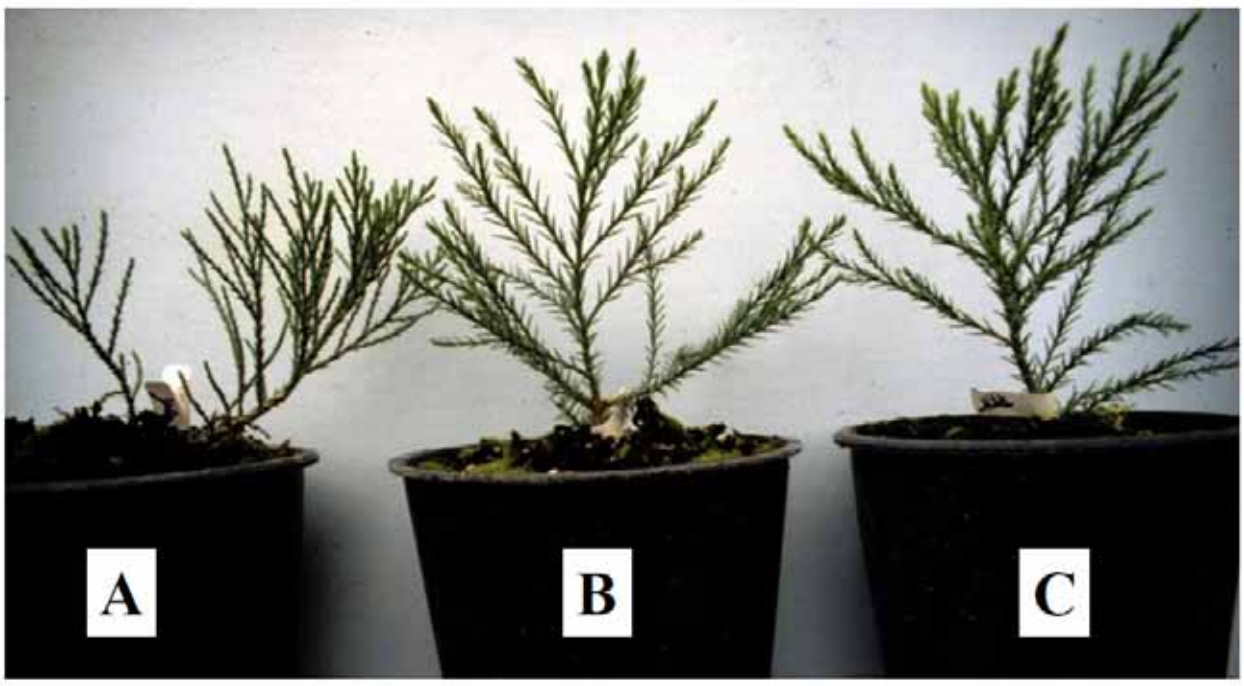
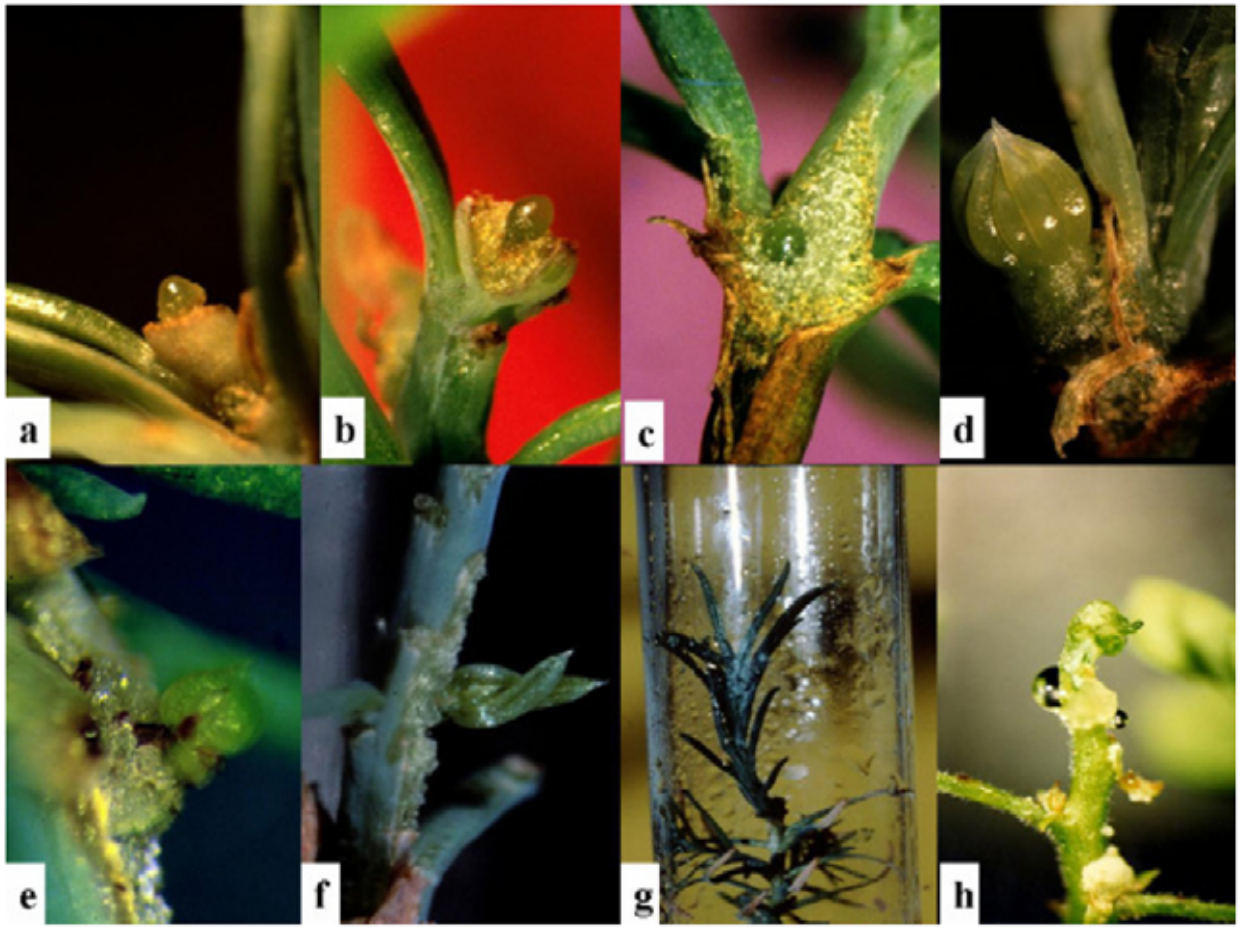


Figure 5. *(A) a 3-yr-old graft from a 100-yr-old Sequoiadendron giganteum ortet; (B) a representative of the rejuvenated line from the same 100-yr-old ortet as in (A), 6 months after acclimatization, self rooted and exhibiting an orthotropic growth pattern and juvenile morphology like the control (C); this rejuvenated line was obtained from a SAM in vitro (see Monteuuis, 1991 for more details).*



***Figures 6**. SAM micrografting of Pseudotsuga menziesii"top-grafting" (a), "cleft-grafting" (b), "side-grafting" (c) and a more advanced stage of development (d), of Pinus pinaster, "side-grafting" (e),and (f) for a more advanced stage of development, of Sequoiadendron giganteum exhibiting a morphologically rejuvenated scion (g), and first stages of development of an Acacia mangium micrografted shoot apex (h).*

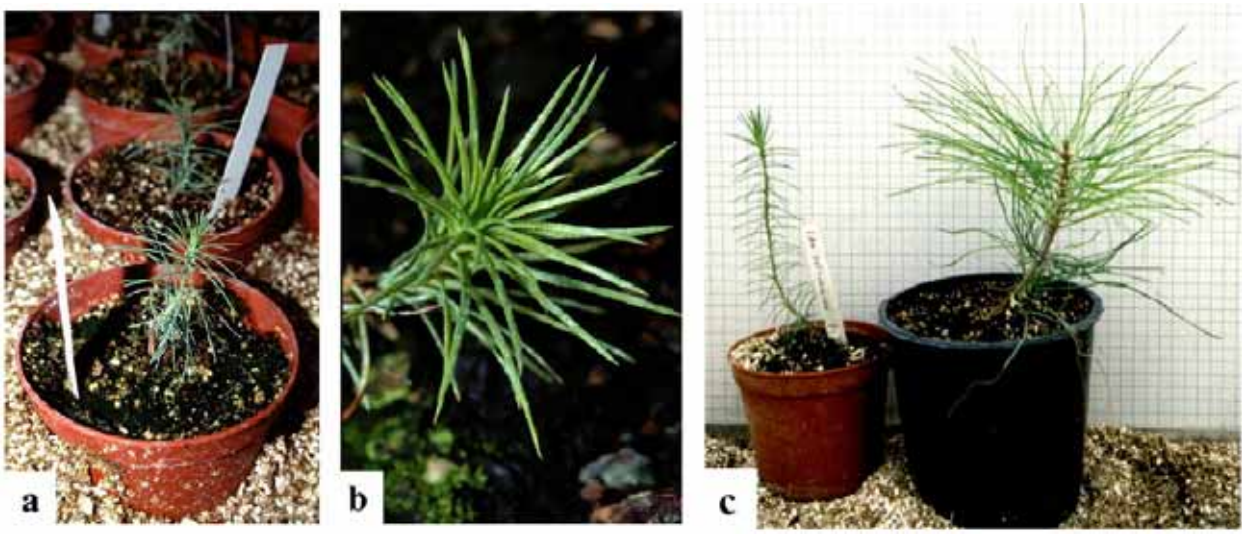
1986).

Prospects of SAM culture and micrografting for inducing SE

In addition to phytosanitary advantages, *in vitro* culture of SAMs can induce physiological rejuvenation (Bonga and Von Aderkas 1992). This is a far more immediate and straightforward strategy than serial grafting or subcultures. These latter involve bigger vegetative structures, with more mature tissues left attached below the SAM, and aim at obtaining increasingly less differentiated cells as the number of successive transfers increases.

In 1986, one SAM out of a few hundreds excised from a 100-yr-old giant sequoia gave rise to a truly rejuvenated line *in vitro* and its juvenile traits persisted after transfer to *ex vitro* (Figure 5). This rejuvenated line has been maintained in culture in a juvenile state ever since (Bon and Monteuuis 1991, Monteuuis 1991, Monteuuis *et al.* 2008). Such rejuvenated materials could profitably be used for SE induction.

SAM micrografting offers all the advantages of SAM culture (Monteuuis 1987b, Monteuuis and Dumas 1990). In addition, it avoids the limitations associated with the composition of the synthetic media used for SAM culture. It should be noted that SAMs become increasingly more medium-sensitive as the donor tree from which they are taken ages (Monteuuis 1987a). A young seedling is undoubtedly a more natural and suitable culture support for excised SAMs than a synthetic medium. Moreover, the hypothetical beneficial influence of rejuvenating substances that may be produced by the juvenile seedlings used as rootstocks on the micrografted SAMs cannot be ruled out (Monteuuis 1987b, Bon 1988, Huang *et al.* 1992). For SAM micrografts the required minimum size of the tissue grafted again appears to be about 100 µm. In addition to size of the graft, success also depends on the species, the age and the organogenic stage of the SAM or shoot apices. Shoot apices include more differentiated cells and tissues and these are liable to have a negative influence on the expected rejuvenation.



Figures 7. *In vivo rejuvenated shoots resulting from SAM micrografting of mature Pinus pinaster ortets (a and b), exhibiting a juvenile morphology compared to a cutting of the same mature ortet that had rooted with difficulty (c).*

SAM micrografting has been successfully applied for initiating tissue culture from mature selected trees of various species (Figures 6) that could not or only hardly be multiplied by SAM culture on synthetic *in vitro* media. These include *Sequoiadendon giganteum* (Monteuuis 1986, 1987b), *Pinus pinaster* (Dumas *et al.* 1989), *Pinus strobus* (Goldfarb *et al.* 1992), *Picea abies* (Monteuuis 1994) and *Pseudotsuga menziesii* (Monteuuis 1995). In contrast, the 200µm long scions used for *Acacia mangium* (Monteuuis 1996) were not SAMs *per se* but shoot apices, the SAMs being too tiny to be micrografted. Different grafting techniques can be used (Figures 6).

Some of the micrografted SAMs developed juvenile-like leaves, which persisted after transfer to *ex-vitro* conditions, attesting that these SAMs had been physiologically rejuvenated by micrografting (Figures 7). Such rejuvenated leaves, or in the case of *Pinus* pinaster, primary leaves or euphylls, produced adventitious shoots that subsequently rooted and were acclimated (Monteuuis and Dumas 1992, Dumas, unpublished results). This raises the prospect that micrografting of SAMs from mature, selected trees could eventually lead to the formation of tissues that could be used for inducing SE from these trees.

Discussion and concluding remarks

SE is the only way of achieving complete ontogenetic rejuvenation as it resets the ontogenetic process to zero through the formation of embryos. The fact that SE-derived vegetative structures at different stages of development *i.e.* newly formed *in vitro* primary somatic embryos or outdoor growing emblings demonstrate an enhanced capacity for (secondary) SE suggests also some positive physiological influence, as discussed for *Hevea brasiliensis* (Lardet *et al.* 2009). Field planted emblings of different species and more specifically of *Coffea spp* have been observed to flower earlier than seedlings of the same chronological age (Berthouly, unpublished observation). The most rational hypothesis that could account for this premature physiological ageing of emblings could be caused by tissue-culture-induced stress due to non-optimal *in vitro* conditions (Dunstan *et al.* 1995, Von Aderkas and Bonga 2000). These could have a strong negative influence on the few unique isolated cells from which the somatic embryos arose (Berthouly and Michaux-Ferrière 1996). One of the causes could be modification of the DNA methylation status with associated risks of somaclonal variations (Jaligot *et al.* 2004, Xu *et al.* 2004, Leljak-Levanić *et al.* 2009).

SE is still strongly hampered by physiological ageing, and the possibility to clone by SE any mature selected Plus tree remains an ultimate challenge. We deliberately chose to focus our considerations on the prospects of using SAMs for succeeding in SE from mature trees due to the particularity of SAMs of any species to retain a certain degree of juvenility, even if this juvenility becomes more and more time and tissues or cells limited with increasing maturation (Nozeran *et al.* 1982, Monteuuis 1989). The possibility of achieving SE from mature trees or palms, using sporophytic tissues from reproductive organs or leaf portions, although proven quite efficient in certain cases, only works for some genotypes or species, and also might be prone to somaclonal variation or mutation risks (Jaligot *et al.* 2004).

As previously noted, SAMs collected *in situ*, with or without preconditioning rejuvenating treatments prior to their excision, can be used for attempting to induce SE. Such direct introduction into *in vitro* culture conditions could be carried out with minimal if not total absence of disinfection measures. It is interesting to note that the

smaller the shoot tips used - SAMs being the tiniest that can be routinely removed by hand for a limited number of species -, the higher the chances of more stable rejuvenation. This accords with the concept of miniaturization (Nozeran 1978). Subculturing (Fouret *et al.* 1986) or serially grafting longer shoot tips (Huang *et al.* 1992) *i.e.* SAMs with a certain quantity of mature tissues underneath give rise most of the times to *in vitro*-restricted rejuvenations which disappear after transfer to *ex-vitro* conditions. It seems however worth using the juvenile-like leaves formed or even the transitory rejuvenated SAMs for SE induction attempts, either directly or via the adventitious buds or dedifferentiated cells that can arise from these *in vitro* rejuvenations.

A lot of efforts have been devoted during the past years to SE cloning of mature trees of species of economic value, pines especially, including resort to highly sophisticated and advanced technologies (Park 2002, 2010). Given this context and based on the literature available, it is surprising that only limited interest has been shown in the use of preconditioning rejuvenating techniques like subcultures or SAM micrografting that are described in this paper. These techniques, developed some 20 years ago, remain easily applicable, are economical and seem promising for enhancing the SE responsiveness of mature selected trees. This is particularly true for pines considering the heteroblastic features of these species, which are indicators of physiological rejuvenation at the SAM level that should be made use of.

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