Bioreactors in coffee micropropagation

Hervé Etienne[1,*], E Dechamp[1], D Barry-Etienne[2] and Bernóit Bertrand [1]

*[1]Centre de Coopération Internationale en Recherche Agronomique pour le Développement - Département des Cultures Pérennes (CIRAD-CP). UMR-DGPC, Résistance des Plantes, IRD, 911 Av. de Agropolis, BP 64501, 34394, Montpellier, France ; [2]ALCINA, Incubateur d'entreprises de l'AgroM. 2, place Viala. 34060 Montpellier Cedex.*Corresponding author: herve.etienne@cirad.fr*

In coffee, bioreactors are the most promising way for scaling-up micropropagation processes, particularly somatic embryogenesis. The availability of an efficient somatic embryogenesis process would allow the rapid mass production of heterozygous materials such as selected *Coffea canephora* clones and F_1 Arabica hybrid varieties. For the last fifteen years, bioreactors (mechanically or pneumatically agitated bioreactors, temporary immersion bioreactors) have mostly been used on coffee to optimize the mass regeneration of somatic embryos from embryogenic tissues. This review presents the main results, obtained with several bioreactor models, concerning the different steps of the micropropagation process : i) the multiplication of embryogenic tissues, ii) the somatic embryo mass regeneration and iii) the production of pre-germinated embryos and plantlets in bioreactors. The literature shows that scaling-up can be successful, since very efficient embryo production has been achieved for both *C. arabica* and *C. canephora.* Moreover, it was proven that the pre-germinated coffee embryos – i.e. embryonic axis elongation (10-12 mm), root tip formation, cotyledon expansion and greening - obtained in temporary immersion bioreactors were photoautotrophic and able to regenerate vigorous plantlets after sowing under nursery conditions. The feasibility to apply the bioreactor technology in an industrial micropropagation procedure is also discussed in the particular socio–economic context of coffee growing.
Key words: *Coffea*, breeding, liquid medium, mass propagation, somatic embryogenesis, temporary immersion.

Bioreatores na micropropagação do café: Em café, o uso de bioreatores é a mais promissora maneira de aumentar o processo de micropropagação, particularmente a embriogênese somática. A disponibilidade de um eficiente processo de embriogênese somática poderia aumentar a rápida produção em massa de materiais heterozigotos, tais como clones selecionados de *Coffea canephora* clones e variedades híbridas F_1 de *Coffea arabica*. Nos últimos 15 anos, bioreatores (bioreatores mecanicamente ou pneumaticamente agitados, bioreatores de imersão temporária) têm sido predominantemente usados em café para otimizar a regeneração em massa de embriões somáticos, a partir de tecidos embriogênicos. Esta revisão apresenta os principais resultados obtidos com vários modelos de bioreatores, no que diz respeito aos vários passos do processo de micropropagação: i) a multiplicação de tecidos embriogênicos, ii) a regeneração em massa de embriões somáticos e iii) a produção de embriões pré-germinados e plântulas nos bioreatores. A literatura mostra que o escalonamento do processo de micropropagação pode ser útil, desde que uma produção muito eficiente de embriões seja atingida para *C. arabica* e *C. canephora.* Além disso, foi demonstrado que embriões pré-germinados de café – i.e. com alongamento do eixo embrionário (10-12 mm), formação de ponta de radícula, expansão do cotilédone e esverdeamento – obtidos em bioreatores de imersão temporária eram fotoautotróficos e capazes de regenerar plântulas vigorosas depois de semeados em viveiro. A possibilidade do uso da tecnologia de bioreatores em escala industrial de micropropagação é também discutida, particularmente no contexto sócio-econômico do cultivo do café.
Palavras-chave: *Coffea*, embriogênese somática, imersão temporária, meio líquido, melhoramento, propagação em massa.

INTRODUCTION

Bioreactors are the most promising way of scaling-up micropropagation processes, particularly somatic embryogenesis, as it is possible to work in large containers. They also enable a high degree of control over culture conditions (pH, aeration rate, oxygen, ethylene and carbon dioxide concentrations) and are compatible with the automation of micropropagation procedures, particularly using robots, and a reduction in production costs. According to Ibaraki and Kurata (2001), the embryo production steps in bioreactors that could be automated are the qualitative evaluation of embryogenic cultures, embryo development and harvesting. Medium renewal, which is labour-intensive with conventional culture vessels can easily be simplified in bioreactors.

Bioreactor use was firstly geared towards microbial technology and almost totally limited to stirred tank reactors (STR). Bioreactors were then applied to cell culture and various types of bioreactors with mechanical or gas-sparged mixing were used to provide mixing and aeration (Ziv, 1995). Mass production in bioreactors was subsequently used for micropropagation by organogenesis – i.e. multiplication of clusters of meristems and buds – and by somatic embryogenesis. Micropropagation in liquid culture media increases nutrient uptake and promotes growth; however the advantages of *in vitro* culture in a liquid medium are often counterbalanced by technical problems such as asphyxia, hyperhydricity, shear forces and the need for complex equipment. To solve these problems, bioreactors have evolved considerably and numerous models have been proposed.

Different types of bioreactors were tested on coffee for different micropropagation processes. A few assays were carried out on microcuttings in temporary immersion bioreactors, but the multiplication rates obtained, whilst higher than those achieved on solid medium, were not sufficient to consider mass propagation (Berthouly et al., 1995; Dufour et al., 1995). Our group utilizes temporary immersion bioreactors for rapid mass multiplication of coffee roots genetically modified with *Agrobacterium rhizogenes* (‘hairy roots’). For the last fifteen years, bioreactors have mostly been used on coffee to optimize the mass regeneration of somatic embryos from embryogenic tissues. Bioreactors have also proved in these works to be efficient for carrying out basic studies on somatic embryogenesis, and more widely for cell biology studies, as they offer the possibility of monitoring or even controlling a large number of culture parameters. We decided to present studies on coffee undertaken in bioreactors as followed: 1) Multiplication of embryogenic tissues in bioreactors, 2) Somatic embryo mass regeneration in bioreactors, 3) Production of pre-germinated embryos and plantlets in bioreactors.

Need for new mass propagation techniques for coffee breeding

With *C. canephora*, a cross-fertilizing species, horticultural cuttings can be used for mass propagation of selected trees, resulting in better productivity when compared to varieties produced from seed (Capot, 1975). However, the number of orthotropic cuttings that a coffee tree can produce is limited, and reproduction by cuttings requires the installation of clonal budwood gardens (Deuss and Descroix, 1984). For large-scale propagation, these two constraints may mean a considerable time lapse between the creation of a variety and its subsequent dissemination.

Horticultural vegetative propagation is still not available for *C. arabica*, probably due to the greater difficulty in achieving satisfactory multiplication rates (Van der Vossen, 1985). Moreover, difficulties to transport the cuttings and the risks of disease propagation are real and have discouraged the use of cuttings on a commercial level. Likewise, male sterility is still not available for the propagation of heterozygous materials. Arabica varieties (allotetraploid self-fertilizing) are sold in seed form as more or less fixed “pure lines” (F5-F6) after a relatively lengthy pedigree selection process, taking at least 20 years. Micropropagation techniques may be applicable for rapid mass production of selected *C. canephora* clones, and for interspecific hybrids such as Arabusta, but they are of particular interest in the case of *C. arabica,* for which F_1 hybrid superiority over varieties has been largely demonstrated in Kenya (Van der Vossen and Walyaro, 1981), Ethiopia (Ameha, 1983) and Central America (Bertrand et al., 2005).

Current limitations of micropropagation

Micropropagation regroups all the techniques of *in vitro* cloning. At the moment, most of the micropropagation procedures used commercially are based on the multiplication of axillary and apical meristems. Whilst offering true advantages over conventional propagation techniques for rapid cloning of selected planting materials, micropropagation remains a tricky and costly production technology. Current techniques require a large number of small containers, agarose media and aseptic division of plant tissues by hand.

Plant micropropagation involves periodic transfers of plant material to fresh media, after subcultures of 4 to 6 weeks, due to exhaustion of the nutrients in the medium and also because of continuous tissue growth and proliferation, which is rapidly limited by the size of the culture container (Maene and Debergh, 1985). High production costs generally limit the commercial use of micropropagation to markets with a very high unit value, such as ornamentals, foliage plants and selected fruit crops (Sluis and Walker, 1985; Simonton et al., 1991). Labour generally accounts for 40 to 60 % of production costs. Although tissue handling is the major part of the work and the most technical, there is also the cleaning, filling and handling of a large number of containers (Maene and Debergh, 1985). Other major costs come from losses occurring during acclimatization in greenhouses and stem and root vitrification (Reuther, 1985). It has been concluded for various species that extensive expansion of micropropagation would only take place if new technologies became available to automate procedures, and if acclimatization protocols were improved (Kitto, 1997).

Advantages of liquid media for plant micropropagation

Using liquid media in micropropagation processes is considered to be the ideal solution for reducing plantlet production costs and for considering automation (Debergh, 1988; Aitken-Christie, 1991). Indeed, liquid culture systems provide much more uniform culturing conditions, the media can easily be renewed without changing the container, sterilization is possible by ultrafiltration and container cleaning after a culture period is much easier. In addition, with liquid culture media, much larger containers can be used and more of the container volume can be used, whereas agar media necessitate flat culturing. Transfer times can be reduced since explants are no longer positioned, but in many cases merely placed in contact with the liquid medium.

Moreover, plant tissues from numerous species have multiplied better when cultured in liquid medium rather than on a semi-solid medium. The development of scaled-up liquid bioreactor cultures was considered from the outset for both embryogenic and organogenic regeneration pathways (Ziv, 1995). However, somatic embryogenesis appears to be the least labour-intensive, hence the most appropriate for an automated system in liquid medium.

State of the art: coffee tree micropropagation

The microcutting technique has been developed for coffee but cannot be considered for mass propagation as it involves too much work for low multiplication rates. Among the micropropagation techniques, somatic embryogenesis has the greatest multiplication potential, enables numerous technical simplifications (i.e. use of liquid nutrient media), and should consequently entail the lowest production costs (Etienne et al., 1997; Berthouly and Etienne, 1999). Several teams are working on scaling-up of somatic embryogenesis, with a short-term objective of producing several million *in vitro* plantlets per year and per industrial laboratory. Somatic embryogenesis in several *Coffea* species and genotypes is well documented. Two types of processes have generally been described using leaf sections as explants. Direct somatic embryogenesis: somatic embryos are obtained quickly (approximately 70 days) on only one medium with the production of limited callusing. This procedure is particularly suited to *C. canephora*. Indirect somatic embryogenesis based on the use of two media: an induction medium for primary callogenesis, and a secondary regeneration medium to produce friable embryogenic callus regenerating several hundred thousand somatic embryos per gram of callus (Söndahl and Sharp, 1977; Dublin, 1984; Berthouly and Michaux-Ferrière, 1996). This indirect procedure works well on *Coffea arabica* and *C. canephora*. Both procedures are currently being used by different groups to develop commercial micropropagation methods for both cultivated species that systematically include one or more steps in liquid medium.

Micropropagation systems with bioreactors

In 1994, Takayama and Akita classified the bioreactors into three types according to agitation methods and vessel construction. However, over the last fifteen years an increasing amount of work has led to the emergence of a fourth type corresponding to temporary immersion bioreactors. All the bioreactors used for micropropagation can therefore be classed in the following categories:

- Mechanically agitated bioreactors, including aeration-agitation bioreactors, rotating drums and spin-filter bioreactors.
- Pneumatically agitated bioreactors, including air-lift bioreactors, bubble column bioreactors and simple aeration bioreactors.
- Non-agitated bioreactors, including gaseous phase (mist) bioreactors, oxygen permeable membrane bioreactors, overlay aeration bioreactors and perfusion bioreactors.
- Temporary-immersion bioreactors, including systems with temporary complete immersion by pneumatic-driven transfer of liquid medium (RITA® and BIT® systems). It can

be considered that they belong to a new category because they generate a weak agitation during a reduced immersion time which is limited most of the time to only a few minutes daily (Etienne and Berthouly, 2002).

Continuous contact of plant tissues with the liquid medium, be it total or partial, is a source of hyperhydricity (Debergh et al., 1981; Ziv et al., 1983; Hussey, 1986). It is characterized by different degrees of morphological and physiological disorders including a glassy, waterlogged-tissue appearance, disordered growth in the shoot system, and more specifically in the leaves (Ziv, 1995). Hyperhydricity is responsible for poor growth and substantial losses during and after *in vitro* culture. For the organogenic pathway, culturing clusters of buds and meristems has been shown to be an alternative propagation system for bioreactors and to overcome hyperhydricity, providing a biomass with limited leaf elongation (Ziv, 2000). Temporary immersion bioreactors have also been designed to limit hyperhydricity, based on a principle similar to that of mist bioreactors, preferring temporary contact between the plants and the liquid medium rather than permanent contact (Alvard et al., 1993).

Multiplication of coffee embryogenic tissues in bioreactors

In coffee, cell proliferation is usually obtained easily by establishing embryogenic cell suspensions in Erlenmeyer flasks. Using bioreactors to multiply embryogenic tissues is therefore not warranted. The suspensions, which are established after transferring embryogenic calli to liquid culture medium, are maintained in the long term by agitation at 100 rpm. Protocols describing the establishment of embryogenic suspensions for different coffee species, and their efficient regeneration, have been described by several teams. Embryogenic cell suspensions are the material most frequently used to inoculate bioreactors with a view to achieving mass production of somatic embryos (Zamarripa et al. 1991, Noriega and Söndahl 1993, Van Boxtel and Berthouly 1996, Etienne et al., 1997). They provide large quantities of a very reactive material. In the presence of auxin, the material is often uniform and blocked at the meristematic or embryogenic stage, whereas in the absence of auxin it is more heterogeneous, and at more advanced regeneration stages (mixture of embryogenic aggregates and embryos at the globular and heart stages) requiring frequent sorting by sieving.

Nevertheless, some cell multiplication assays in bioreactors have been reported. Dubuis et al. (1995) studied *Coffea arabica* cell growth and alkaloid (caffeine) production rates in a 5−litre bubble-free loop fluidized bed reactor (LFBR). They observed different growth rates using inoculum consisting of small (diameter 710 μm) and large (diameter 1 mm) aggregates. The growth of large aggregates was no longer exponential and less than that of small aggregates. Fast-growing and dividing cells at the top of the bed produced little or no caffeine (“dividing cells’) compared to differentiated ‘producing cells’ at the bottom. The critical dissolved oxygen level for *Coffea* culture was determined as 25 % of oxygen saturation. Determination of that parameter was important for optimizing bioreactor operation and guaranteeing that the metabolic activity of the plant tissues was not affected by a lack of oxygen. These authors also showed in coffee that growth was not stimulated by CO_2. Dubuis et al. (1995) concluded that bubble-free loop fluidized bed reactors led to better growth than shaken flasks, a very high culture density and high secondary metabolite productivity. According to the authors, among the advantages of this bioreactor were the absence of agitation shear stress and that bubble-free aeration protected cells from the bursting of air bubbles. De Feria et al. (2003) showed that, when compared with a 50 % dissolved oxygen (DO) concentration, a concentration of 80 % DO stimulated the multiplication of *C. arabica* embryogenic cell aggregates in CMF-100 (CHEMAP AG) aeration-agitation bioreactors.

Working with temporary immersion bioreactors, Dufour et al. (1995) observed for different *Coffea arabica* genotypes that embryogenic callus growth was greater than in a stirred liquid medium in an Erlenmeyer flask. In their work, the immersion times played an important role in morphogenesis. An immersion of 1 min every 6 h proved to be optimum conditions for cell proliferation, whilst longer immersions (15 min) applied at the same frequency (every 6 h) led to complete regeneration of somatic embryos.

Somatic embryo mass regeneration in bioreactors

Generally speaking, as explained by Ibaraki et Kurata (2001), although there has been a large number of publications describing somatic embryo production in different species, including coffee, there does not exist any well-established protocol. Coffee, like carrot (Ammirato and Styer, 1985), sandalwood (Bapat et al., 1990) and *Eschscholtzia californi*a (Archambault et al., 1994), belongs to the species for which successful production of somatic embryos in bioreactors has been reported.

Zamarripa et al. (1991) were the first to report the production of coffee somatic embryos in bioreactors. The fermentation system used was a 3-litre stirred bioreactor (model SET4CV, Setric SGI). They found embryogenesis kinetics similar to those obtained in Erlenmeyer flasks. Embryo production began 20 days after bioreactor inoculation and after culturing for 49 days the embryo concentration was 200 x 10^3 embryos per litre. Torpedo embryos amounted to 20 % of the total embryos. In their work, contrary to observations by Noriega and Söndahl (1993), embryos were clustered together. However, 4 weeks after transfer to solid germination medium, the embryos initially transferred in clusters could be easily separated.

With the same bioreactor, Ducos et al. (1993) defined very comprehensively the optimum culture conditions for somatic embryo production in *C. canephora* and Arabusta. They showed that shear stress, caused by an agitation speed of 100 rpm, had a detrimental effect on the induction of the embryogenic process and that 50 rpm was an optimum agitation speed. Darkness also suppressed somatic embryo production. To optimize their culture parameters, they systematically compared the embryo yields obtained in the bioreactor with those obtained in Erlenmeyer flasks (Bprod/ Fprod). The highest ratio was obtained with an initial aeration rate of 0.04 VVM (volume of air per medium volume per minute). The agitation speed and air flow rate were then gradually increased (from 50 to 100 rpm and from 0.04 to 0.16 VVM, respectively) to keep the dissolved oxygen concentration (DO) always over 30 % and always over the critical concentration throughout the culture time. Similarly to results with shaken flasks (Zammaripa et al., 1991), embryo concentrations were between 200,000 and 500,000 embryos l^{-1} after 40 to 50 days of culturing in the bioreactor. Plant conversion frequencies were similar to those obtained with embryos in flasks, i.e. 47 % for *C. canephora* and 35 % for Arabusta. Seventy percent of the embryos were at the torpedo stage for *canephora*, as opposed to only 7 % for Arabusta, but the same results were obtained in Erlenmeyer flasks.

Noriega and Söndahl (1993) using a 5-litre magnetic bioreactor model (K. Ray Ono Biotech Instruments, Elmer, NJ, USA) reported high frequency embryo regeneration (45,000 embryos/ 5-liter bioreactor). They inoculated embryogenic tissues maintained in liquid cultures into the bioreactor at a density of 1–5 ml PCV (Pack Cell Volume) of embryogenic tissues/litre of regeneration medium. The bioreactor was maintained at 70-120 rpm, at 25°C in darkness. In the second and third weeks, the embryogenic tissues multiplied, with biomass doubling each week. The PCV was maintained at the initial level by removing excess tissue when changing the medium. Regeneration began in the fourth week (globular stage) and was virtually complete by the fifth week. After 13 weeks, the embryo populations in the bioreactor were as follows: 25 % torpedo, 45 % heart, 30 % globular. Germination was obtained by plating bioreactor samples onto semi-solid germination medium. Full germination (cotyledon expansion and radicle emission) was noted after 10 weeks.

De Feria et al. (2003) achieved mass regeneration of *C. arabica* (cv Catimor 9722) embryos in CMF-100 (CHEMAP AG) aeration-agitation bioreactors. These authors used 2–litre bioreactors equipped with a gas blending unit (air, O_2, N_2, CO_2) to control the DO and compared the effects of 50 and 80 % DO on somatic embryo regeneration. They observed that the number of somatic embryos was larger (71,072 embryos l^{-1}) with 80 % DO but the major proportion was globular and heart shaped embryos and only 6.6 % of the total embryos were torpedo shaped. A 50 % DO led to the production of the highest percentage (20 %) of torpedo shaped embryos. De Feria and coll. concluded that higher DO induced globular and heart shaped embryo differentiation, but for production of torpedo shaped embryos lower DO were required. These conclusions agree with data reported by Ducos et al. (1993) which showed that specific oxygen uptake and the specific CO_2 and C_2H_4 production rates reached a maximum after 21 days, when embryo regeneration started, and afterwards decreased as a function of culture time, probably because more mature embryos exhibited a lower metabolic activity.

Reproducible production of twenty clones of *C. arabica* F_1 hybrids was successfully achieved using a RITA® temporary immersion bioreactor (Etienne et al., 1997). Depending on the genotypes, yields ranging from 15,000 to 50,000 somatic embryos per gram of embryogenic suspension were recorded. The most spectacular effect has been the degree of improvement seen in the quality of coffee somatic embryos produced by temporary immersion. Whilst the proportion of normal torpedo type embryos is around 20-30 % in a bioreactor or in Erlenmeyer flasks (Zamarripa et al., 1991; Noriega and Söndahl, 1993; De Feria et al., 2003), it was usually over 90 % with temporary immersion. This improvement in quality was reflected in higher plant conversion rates on an agar medium (80 to 90 %) and especially in the successful regeneration of plants after direct sowing on horticultural substrate of somatic embryos

produced in a temporary immersion bioreactor (Barry-Etienne et al., 1999).

The adjustment of immersion times is critical with this type of bioreactors. According to Teisson and Alvard (1995) with 1-litre temporary immersion bioreactors, 15 min' immersion every 6 h led to successive development and germination of coffee embryos, whereas for an identical culture medium, immersions of 1 min every 24 h halted embryo development and stimulated the production of adventive embryos.

Biomass accumulation in bioreactors: Biomass accumulation is the main limitation of the embryo regeneration process in some bioreactors (Scragg, 1995), through excessive density, but also to bioreactor operation. De Feria et al. (2003) observed this phenomenon in coffee with CMF-100 (CHEMAP AG) aeration-agitation bioreactors particularly when an 80 % dissolved oxygen concentration was used. They observed biomass settlement in the bottom of the vessel. According to the authors, this required a gradual agitation increase to 135 rpm to maintain uniform mixing of the suspension and guarantee dissolved oxygen availability in the culture medium. The increased agitation speed, together with the bubble constant and the increased biomass, led to the formation of foam on the liquid medium surface. In order to maintain a sufficient DO, despite the increase in biomass, Ducos et al. (1993) gradually increased the agitation speed to 100 rpm despite the problems associated with shear forces with such agitation. To avoid biomass accumulation, Noriega et Söndahl (1993) maintained a low cell density by removing excess tissue simultaneously with the spent medium. Barry-Etienne et al. (1999) achieved satisfactory conditions for embryo development by dividing up the biomass contained in a RITA®- bioreactor after 8 weeks into several bioreactors, to obtain an optimum density of 4,000 embryos/1-litre bioreactor.

Controlling hyperhydricity: The organs most susceptible to hyperhydricity in bioreactors with permanent immersion are leaves and, to a lesser degree, shoots. The embryogenic tissue multiplication and *sensus stricto* embryogenesis (globular, heart, torpedo/cotyledon stages) steps can be carried out in conventional bioreactors as this material is less exposed to hyperhydricity. As temporary immersion bioreactors make it possible to adjust the time spent by the plant material immersed in the liquid nutrient medium, it is possible to prevent hyperhydricity and thereby obtain coffee embryos with fully developed chlorophyllous cotyledons or leaves.

Albarran et al. (2005) observed that increasing the frequency for short immersions (1 min) in 1-litre RITA® bioreactors stimulated somatic embryo formation and quality in *C. arabia*. Thus, average yields of 480, 2,090 and 3,100 embryos were obtained per 1-litre bioreactor, with 60, 79 and 85 % torpedo embryos, for daily frequencies of 1, 2 and 6 immersions respectively. Hyperhydricity was not observed with such immersion conditions. On the other hand, increasing immersion times by 5 min or more led to a considerable reduction in somatic embryo production and in their quality, becoming all the more critical as the immersion frequencies increased. For example, 15 min immersions applied 2 or 6 times per day led to hyperhydric embryo frequencies of 64 and 90 % respectively. It is likely that each culture step requires adaptation of the immersion length and frequency to obtain optimum results.

Production of pre-germinated embryos and plantlets in bioreactors

In conventional bioreactors (mechanically or pneumatically agitated bioreactors), problems with somatic embryo quality and the difficulty in extending embryo development beyond its torpedo stage in liquid medium have been reported for most species including coffee. To overcome these problems, manual selection of somatic embryos capable of germination and frequent subcultures on semi-solid media are required to obtain plants. These laborious manipulations during the late culture phases greatly increase production costs.

Effective pre-germination – i.e. embryonic axis elongation (10-12 mm), cotyledon expansion, greening, root tip formation - in a liquid medium was successfully obtained for the first time with coffee using RITA® temporary immersion bioreactors (Barry-Etienne et al., 1999). During the germination step, densities exceeding 1,600 embryos per 1-litre bioreactor had a positive effect on embryo morphology by stimulating elongation of the embryonic axis (+4-5 mm), an increase in fresh weight (+100 %) and a reduction in cotyledon area. It was proven that the pre-germinated embryos obtained were photoautotrophic and able to regenerate plantlets after direct acclimatization under nursery conditions. These three morphological changes were positively correlated to the efficiency of conversion into plants after sowing on horticultural substrate, and to plantlet growth rates. When compared to cultures on a semi-solid medium, the synchronization of development and germination for embryos grown in temporary immersion was spectacular (Barry-Etienne et al., 1999). It was encouraged

by high culture densities (1,500 to 3,000 embryos per 1-litre bioreactor), for which 66 % of the embryos were at the same photoautotrophic pre-germinated stage. The direct sowing of pre-germinated embryos under *ex vitro* conditions, reduced handling times to 13 % and shelving area requirements to 6.3 % of the values obtained with conventional acclimatization of plants developed on semi-solid media and the time spent *in vitro* was reduced by 3 months (Barry-Etienne et al., 1999). Moreover, when compared to plantlets derived from the same embryo population obtained under conventional culturing conditions on semi-solid media, the plantlets obtained *ex vitro* after sowing somatic embryos were more vigorous (Barry-Etienne et al., 2002a).

In another study, Barry-Etienne et al. (2002b) showed that there existed morphological variability within a population of coffee pre-germinated somatic embryos produced in a RITA® bioreactor, which mainly affected the cotyledon area. This heterogeneity affected both plant conversion efficiency in soil and plant growth in the nursery, where it mainly resulted in retarded growth, primarily in plantlets derived from somatic embryos with small cotyledons.

A study by Afreen et al. (2002a) agreed with the results reported by Barry-Etienne et al. (1999, 2002a) confirming, through a physiological approach, that the cotyledonary (pre-germinated) stage is the earliest stage that can be cultured photoautotrophically to ensure plantlet development. Afreen and coll. showed in *Coffea arabusta* that cotyledonary and germinated embryos had photosynthetic ability and could develop into plants if photoautotrophic conditions, in a sugar-free medium, under high photosynthetic photon flux (PPF: 100-150 μmol m^{-2} s^{-1}) and an increased CO_2 concentration (1100 μmol mol-1) were applied.

In another study, Afreen and coll. (2002b) used cotyledonary stage embryos pretreated under a high PPF for 14 days, to compare their ability to achieve plantlet regeneration when grown photoautotrophically in three different types of culture system: Magenta box, a modified RITA® bioreactor and a temporary root zone immersion bioreactor (TRI-bioreactor) with forced ventilation. The TRI-bioreactor was similar to the systems developed by Aitken-Christie and Jones (1987) and Simonton et al. (1991). The embryos were positioned on a culture support and liquid medium was frequently applied then withdrawn so as to immerse only the root area. The highest plant conversion (84 %) was obtained with the TRI-bioreactor. These results confirmed those of Barry-Etienne et al. (1999) who observed that complete plantlet regeneration was not possible using temporary total immersions, but was possible after planting in the nursery pre-germinated embryos produced in a RITA® bioreactor.

Prospects for using bioreactors in an industrial propagation scheme

Coffee growing: a difficult context for micropropagation: Coffee breeding is increasingly relying on biotechnologies (Etienne et al., 2002). Of these, micropropagation, especially somatic embryogenesis, offers the possibility of rapidly and massively multiplying new heterozygous varieties. However, in view of the particular socio–economic context of coffee growing, applying this technology on an industrial scale remains a difficult challenge. Most potential users of *in vitro* plantlets are small coffee farmers with limited resources, and their access to this new material will depend on the added agronomic or technological value of new selected varieties, and on the extra costs arising from the micropropagation stage. Another problem comes from the fact that growing zones are often located in regions with difficult access. Yet it is essential that the propagation laboratories, and even more so the industrial nurseries, be located near those zones, so that farmers have access to this planting material, and so that the problems and costs associated with *in vitro* plantlet transport are avoided. In addition, micropropagation calls for relatively complex technologies in both the laboratory and the nursery, and technology transfer to zones where there is a shortage of qualified manpower. For coffee, it is necessary to achieve a reduction in production costs, in order to be competitive with plants traditionally produced from seed and sold for less than USD 0.10-0.30/plant. In addition, as planting densities are very high for *C. arabica* dwarf varieties (between 5,000 and 10,000 trees/ha) planting costs are substantial.

Constraints associated with the micropropagation technique: In order to be commercially applicable for coffee, somatic embryogenesis procedures need to offer several qualities 1) a substantial multiplication capacity irrespective of the genotype selected, 2) lower production costs, 3) the avoidance of somaclonal variations. Recent studies have shown for the two cultivated coffee species that the occurrence of somaclonal variations has not held back the development of somatic embryogenesis (Etienne and Bertrand, 2001, 2003; Ducos et al., 2003). As we have already seen for the cultivated coffee species and for numerous cultivars, it has been possible to massively produce somatic embryos in liquid

medium. However, there is very little information available about the productivity of the different procedures used and the estimated production costs. Several projects are under way involving pre-industrial scale production by somatic embryogenesis in central America, Mexico, Indonesia and Thailand to test its technical and economic viability when scaled up. It can be wondered what place bioreactors will have in these procedures.

Bioreactors and industrial micropropagation: All the publications on the use of bioreactors for micropropagation have helped to improve our understanding of how physical-chemical parameters affect the different steps of somatic embryogenesis and the physiology of planting material. Moreover, the data obtained have made it possible to optimize the bioreactors and making major technical improvements to the somatic embryogenesis procedures. Using different bioreactor models for somatic embryo production has shown that scaling-up can be successful, since very efficient embryo productions has been achieved for both *C. arabica* and *C. canephora.* Even so, nothing yet guarantees that bioreactors will have a role to play in the industrial procedures used in the future.

It is essential for two steps of somatic embryogenesis to be completed in liquid medium to achieve high productivity, i) multiplication of embryogenic tissues and ii) mass production of somatic embryos. i) As we saw earlier, using bioreactors offers no advantage for embryogenic tissue multiplication and most teams carry out this stage satisfactorily in shaken flasks. ii) Neither is it obvious that bioreactors are essential for mass embryo production, since similar results in terms of quantity and quality to those obtained with conventional bioreactors have been obtained in Erlenmeyer flasks. However, embryos produced in temporary immersion bioreactors offer an advantage in terms of morphological quality (Barry-Etienne et al., 1999, 2002a).

All the bioreactors proposed still involve a sophisticated and expensive apparatus. This problem is even more acute with conventional bioreactors (mechanically and pneumatically agitated bioreactors), which are not adapted to the current situations involving the propagation of a set of selected genotypes. Moreover, culturing in bioreactors proves to be trickier than culturing on agarose media in terms of technicality and contamination risks. These are further difficulties to be taken into account before using bioreactors on an industrial scale. In the coffee growing context, where extremely low production costs will have to be achieved to find a market, the decision will have to be taken after comparing the performance achieved with bioreactors and that obtained with simpler technologies.

Using temporary immersion bioreactors for the late steps is tempting. Trials on a pre–industrial scale to produce *Coffea arabica* F_1 hybrids are under way in central America to validate the technical and economic viability of somatic embryogenesis procedures having this particularity. However, once again, there is nothing to guarantee that the procedures that will ultimately be applied on an industrial scale will include bioreactors for these late steps. For *Coffea canephora*, Ducos et al. (1999) proposed an efficient protocol for clone propagation in which the pre-germination phase was conducted on semi-solid medium in disposable Petri dishes. Similarly to the works of Barry-Etienne et al. (1999, 2002a,b), plant acclimatization and regeneration was achieved by directly sowing pre-germinated embryos under greenhouse conditions. That protocol, which does not envisage the use of bioreactors, is also being validated on a pre-industrial scale by that team (Ducos, personal comm.). Nevertheless, it is likely that this procedure will be too costly and therefore inappropriate for *Coffea arabica* whereas it might be cost-effective for *C. canephora* due to the considerable differences in planting densities (5,000-10,000 trees/ha for *C. arabica* as opposed to 1,200 trees/ha for *C. canephora*). It is therefore seeking to validate bioreactor use for *C. arabica,* to micropropagate heterozygous varieties, that would therefore seem to be the priority.

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