*International Journal of Plant Developmental Biology* ©2007 Global Science Books

# Bioreactors for Coffee Mass Propagation by Somatic Embryogenesis

**Jean-Paul Ducos*** • **Charles Lambot** • **Vincent Pétiard**

Nestlé R&D Centre Tours, 101 Avenue Gustave Eiffel, Notre Dame D'Oé, BP 49716, 37097 Tours Cedex 2, France

*Corresponding author*: * **jean-paul.ducos@rdto.nestle.com**

## ABSTRACT

Coffee somatic embryogenesis in liquid medium is a powerful alternative to other vegetative propagation techniques for mass propagation of selected *Coffea canephora* (Robusta) clones and $F_1$ *Coffea arabica* hybrids. This review presents the different types of bioreactors used for coffee somatic embryogenesis by Nestlé R&D Centre-Tours and by other scientific teams. Mechanically agitated bioreactors were used for the production of torpedo-shaped embryos. Critical parameters are the inoculation density (0.5 to 1.0 g FW $L^{-1}$), medium renewing and the initial oxygen transfer rate ($K_La$: 5 $h^{-1}$). In this system, Robusta embryo concentrations range between 200,000 to 400,000 $L^{-1}$ within 2 months. Maturation from the torpedo to the cotyledonary-stage embryos was achieved in various temporary immersion bioreactors (TIB): in 1-L RITA® system (up to 1,000 cotyledonary embryos per system), in 10-L glass bottles (up to 20,000) and in 10-L flexible disposable bags. The latter one, the so-called "Box-in-Bags", insures a higher light transmittance to the biomass due to its horizontal design. At the end of the maturation phase, the somatic embryos are green and able to photosynthesize: these pregerminated embryos can be directly transplanted to the greenhouse to get fully germinated plantlets. More recently, a temporary root immersion bioreactor (TRI) has been described for the growth of individualized Arabusta plantlets in photoautautrophic conditions, i.e. in sugar-free medium with enriched $CO_2$ and high light intensity. The pros and cons of these different bioreactors will be discussed considering how they can be integrated in a mass propagation process. We present a "state of the art" by describing a pilot scale process for the production of pregerminated Robusta embryos and some examples of diffusion of coffee selected genotypes. These last years, two major trends have been developed for industrial micropropagation: i) bulk-cultivation of small propagules in photomixotrophic conditions (with sugar) followed by their selection and transfer to the greenhouse for their conversion to plant, ii) production of singulated and fully developed plantlets in the laboratory under photoautotrophic conditions. Next development in coffee mass propagation by somatic embryogenesis will probably originate from the combination of these two approaches. The usage of the "micro-environment" method, combined with media releasing $CO_2$, is well adapted for the *ex vitro* germination of coffee embryos. Particularly, this method can be a relevant alternative to the conventional one, consisting on insufflating $CO_2$ in the culture rooms or in the greenhouses.

**Keywords:** Box-in-Bags, breeding, $CO_2$ enrichment, *Coffea*, mass propagation, scaling-up, photoautotrophy, somatic seedling, temporary immersion
**Abbreviations: BA**, benzylaminopurine; **FW**, fresh weight; **TIB**, temporary Immersion Bioreactor

## CONTENTS

INTRODUCTION ..... 2
COFFEE SOMATIC EMBRYOGENESIS IN LIQUID MEDIA ..... 3
Multiplication phase ..... 3
Embryogenic phase ..... 3
STIRRED BIOREACTORS FOR PRODUCTION OF TORPEDO-STAGE EMBRYOS ..... 5
Robusta and Arabusta ..... 5
Arabica ..... 6
Limits ..... 6
TEMPORARY IMMERSION BIOREACTOR FOR PRODUCTION OF COTYLEDONARY-STAGE EMBRYOS ..... 6
Temporary immersion cultures ..... 6
1-L RITA® ..... 6
10-L glass bottle ..... 7
Horizontal disposable 10-L bags ..... 7
Limits ..... 7
TEMPORARY ROOT IMMERSION BIOREACTOR FOR PRODUCTION OF PLANTLETS ..... 8
Photoautotrophic cultures ..... 8
TRI bioreactor ..... 8
Limits ..... 8
COMMERCIAL APPLICATION ..... 8
Agronomic performance and trueness to type ..... 8
Pilot scale process at Nestlé R&D-Tours ..... 9
Somatic seedling production in coffee producing countries ..... 10
PROSPECTS ..... 10
Pregermination phase under photoautotrophic conditions ..... 10
*Ex vitro* germination with $CO_2$ enrichment ..... 10
Automation and artificial seeds (as a micro-bioreactor) ..... 10
CONCLUSION ..... 11
REFERENCES ..... 11

Received: 4 March, 2007. Accepted: 9 April, 2007.

Special Feature

## INTRODUCTION

Somatic embryogenesis is a type of vegetative propagation based on plant cell totipotency which offers a powerful alternative to other vegetative propagation methods, i.e. cuttings or grafting. In the case of coffee, one of the most important crops, it can allow the rapid propagation of selected clones of the self incompatible species *C. canephora* (Robusta) and the Arabusta hybrid (*C. canephora* X *C. arabica*). For the autogamous *C. arabica species*, its main use is for $F_1$ hybrid propagation, thereby avoiding manual hybrid seed production and cuttings which are costly and difficult on Arabica.

Since the first report by Staritsky in 1970, a large number of papers have been published on coffee somatic embryogenesis using solid medium culture (for review, see Söndhal and Loth 1985). The distinction between low, i.e. directly from the explant, and high, i.e. indirectly after a callus phase, frequencies of somatic embryo induction from mature leaves of *C. arabica* was described by Söndhal and Sharp (1977). Somatic embryos obtained by direct embryogenesis are less numerous than embryos derived from indirect embryogenesis. Thus the second type is generally preferred for large-scale propagation.

Regardless of the plant species, the various somatic embryogenesis protocols generally follow a similar process flow: a) induction of embryogenic calli followed by their identification and selection by physical isolation; b) multiplication of the embryogenic cells (undifferenciated phase); c) regeneration of large numbers of embryos from these cells (embryogenic phase); d) conversion of these embryos into mature embryos able to regenerate a plant.

In the early 1990s, significant progress was made toward commercial implementation by achieving coffee somatic embryogenesis in liquid medium (Zamarripa *et al.* 1991a, 1991b; Neuenschwander and Baumann 1992; Zamarripa *et al.* 1993; van Boxtel and Berthouly 1996). This advance led to the possibility for the scaling-up in large vessels, i.e. bioreactors. Although the term "bioreactor" may refer to any container to grow cells or tissues, it generally designs a fermentation vessel for the culture of microorganisms in liquid culture. Such a device allows the scaling-up to automate easily operations like collecting embryos, medium renewal, monitoring different parameters such as dissolved oxygen and pH. Such systems were implemented initially for the production of carrot somatic embryos in our laboratory (Ducos *et al*. 1993a). Various adaptations of large-scale vessels for plant micropropagation were recently reviewed (Takayama and Akita 1994; Ziv 2005; Paek *et al.* 2005; Jimenez-Gonzales 2005; Adelberg 2006; Takayama and Akita 2006; Teixeira da Silva 2006).

This paper presents an overview of this technology applied to coffee somatic embryogenesis, mainly based on the experience acquired on Robusta at Nestlé R&D Centre, Tours, France. As the main reason to use bioreactors is to reduce the number of operations, we will point out the number of singulations (individual handling), where they take place and at which developmental stage the embryos are transferred to the greenhouse (**Fig. 1**).

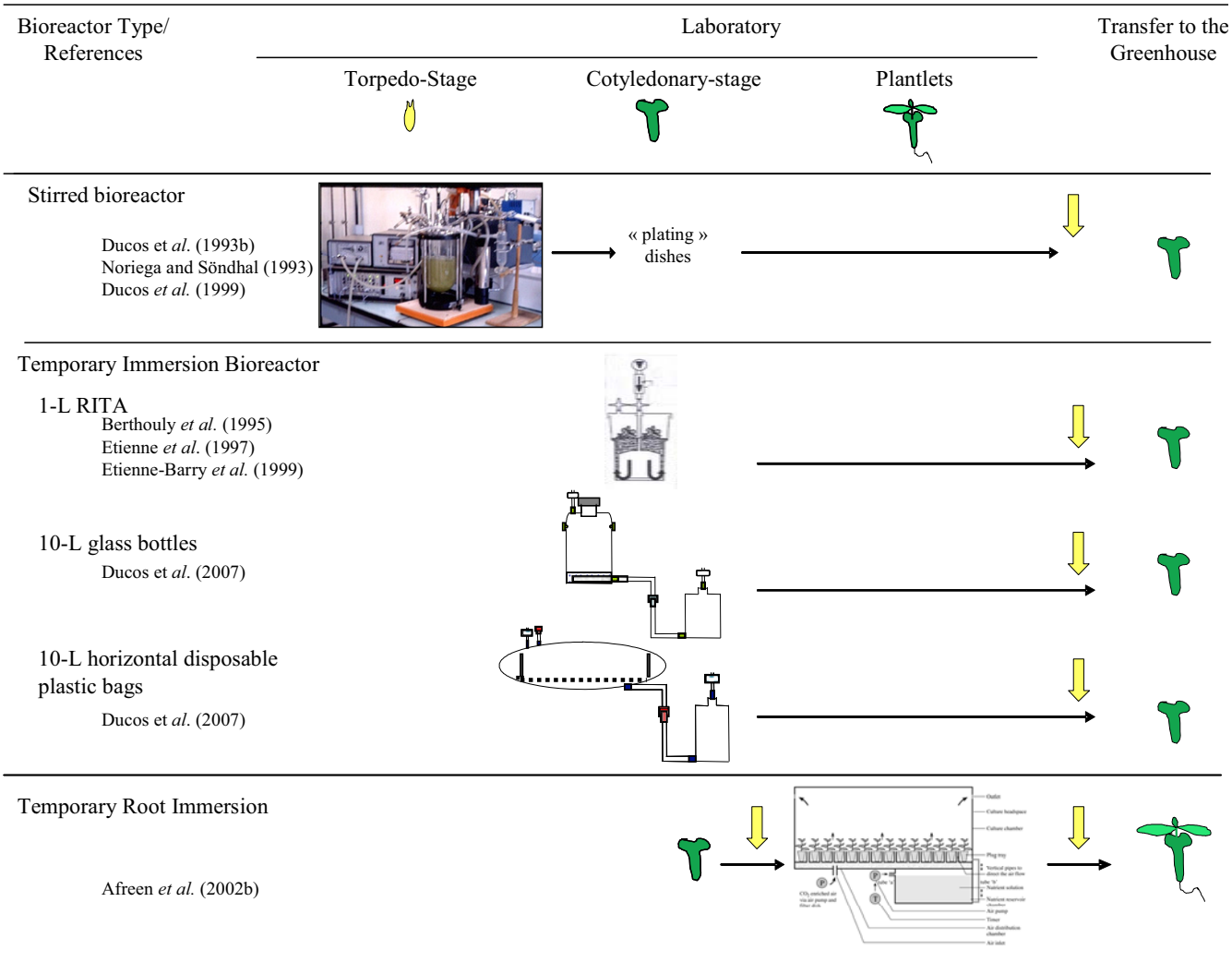


**Fig. 1 Bioreactor types used to growth coffee somatic embryos at different stages.** Individual handling (singulation) are symbolised by a yellow arrow.

## COFFEE SOMATIC EMBRYOGENESIS IN LIQUID MEDIA

### Multiplication phase

Either on semi-solid medium (calli) or in liquid medium (cell suspensions), the multiplication of embryogenic cells is a key step because it greatly and rapidly scales up the number of potential embryos to be produced. For most of the Robusta genotypes, embryogenic calli can be induced and multiplied in a medium containing 6-benzylaminopurine (BA) as the only growth hormone. This medium described by Yasuda *et al.* (1985) is composed of ¼-strength macro salts and ½-strength micro salts of MS medium (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al.* 1968), supplemented with 1.0 mg $L^{-1}$ BA and 30 g $L^{-1}$ sucrose.

The Robusta embryogenic cell lines are cultured in Erlenmeyer flasks by repeated transfer of the cell suspensions to fresh media at a high initial cell density (10 g FW $L^{-1}$). An example of growth kinetics of a cell line is shown in **Fig. 2**. Growth occurred without exponential phase and maximum cell density is achieved after 45 days when the carbohydrates have been consumed. Typically, these cell lines are characterized by a linear growth and by a low conversion rate from carbohydrate to dry biomass (DW) which is only 20%. They consist of clumps which have a size ranging between 500 to 2000 µm and are composed of two cell types (**Fig. 3B**). In the centre of the clumps, the cells are vacuolated and highly loaded with voluminous starch grains (**Fig. 3C**). At the periphery, cells present a high nucleoplasmic ratio, a slightly vacuolated cytoplasm containing small starch granules and frequent mitotic figures. These cells demonstrate a set of characters generally associated with embryogenic cells (Lu and Vasil 1985), except the size of their nucleoli which are not particularly large (average diameter = 1.1 µM) as it is the case for typical embryogenic cells (Michaux-Ferriere *et al.* 1989). These cells, which can be defined as "embryogenic potential" cells, are clustered in peripheral nodules and give rise to new nodules via their mitotic activity. Mechanical agitation of the culture led to the fragmentation of the aggregates.

One important possible limitation to the commercial application of this process is the loss of embryogenic potential observed in the course of regular subcultures. The embryogenic potential of 38 cell lines was recorded every 3 months (Ducos *et al.* 1999; Ducos and Pétiard 2003a): over a one-year period, the average number of embryos which can be regenerated per g of cells decreased from 200,000 to 65,000, and the average number of plantlets from 56,000 to 13,000. Even if some cell lines maintain a high embryogenic potential beyond a year, cryoconservation of embryogenic tissues must be integrated into the whole process to overcome this limitation. A simplified method was developed by Florin *et al.* (1995): it is based on the induction of freezing tolerance

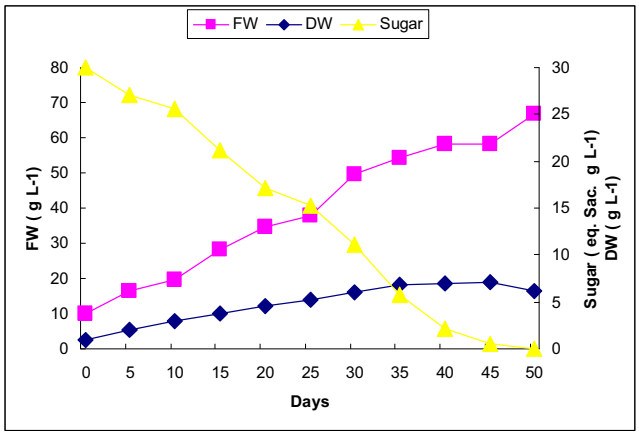


**Fig. 2 Growth kinetics of a Robusta embryogenic cell line in 0.25 L Erlenmeyer flasks.**

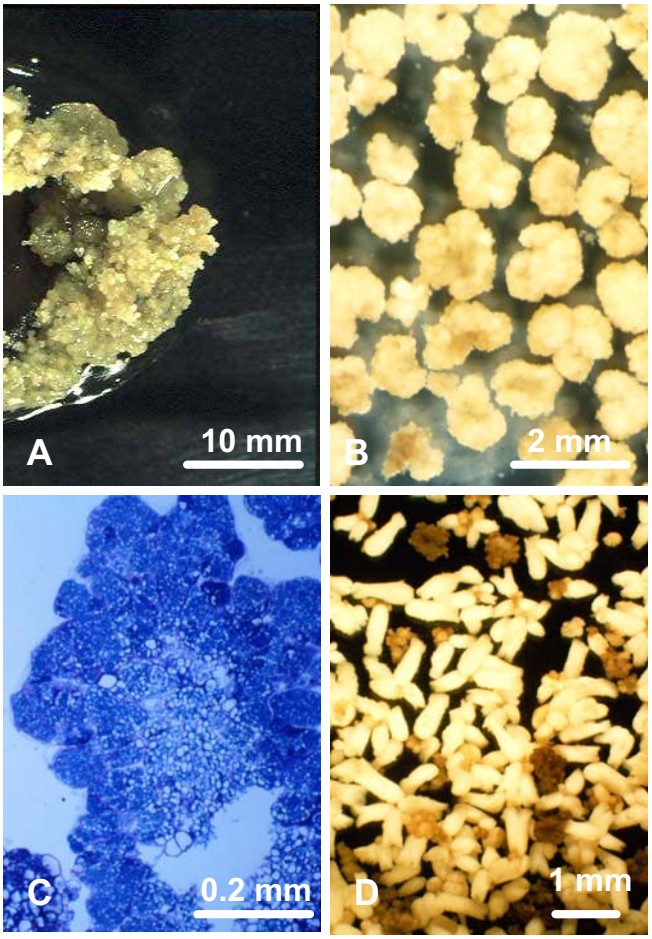


**Fig. 3 Robusta somatic embryogenic tissues at different stages.** (**A**) primary embryogenic callus on a leaf explant; (**B**) embryogenic clumps in liquid medium; (**C**) histological view of an embryogenic clump; (**D**) torpedo-stage embryos at the end of the embryogenic phase.

during a pre-treatment phase, without any addition of cryoprotectants. After this pre-treatment phase, the samples are placed in a freezer at -20°C for 20 h before transfer to liquid nitrogen. Using this protocol, the cell suspensions of coffee species maintain their viability and regeneration capacity.

Embryogenic cell suspensions provide a very reactive material in quantities sufficient to inoculate large vessels. Typically, the multiplication rate of the biomass ranges between 2 to 3 within 2 weeks. At this date, 1 L of suspension of undifferentiated tissues allows to inoculate 20 to 30 L of embryogenic medium with an inoculation density of 1.0 g FW $L^{-1}$. As an example, a 3-month multiplication duration in liquid medium is sufficient, in terms of productivity, to achieve a large scale propagation, considering the following data: a) 60 g of embryogenic cells are obtained after 3 months of multiplication starting from 1.0 g of primary callus; b) 1.0 g of a 3 month-old cell line has the potential to produce 50,000 plantlets.

So, one run starting from 1 g of calli should allow the production of about 3 million plantlets. Consequently, the establishment of embryogenic cell lines in flasks is a prerequisite but their culture in a bioreactor is not mandatory for the scale-up of the process.

### Embryogenic phase

This phase is initiated by transferring the Robusta embryogenic cells into medium composed of macro and micronutrients of MS medium (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al.* 1968) and 30 g $L^{-1}$ sucrose. For some clones, the medium is supplemented with BA at 1.0 mg $L^{-1}$. Probably due to the absence of auxin, no isolated proembryogenic masses (PEMs) can be observed, as it is

**Table 1** Effect of the initial oxygen transfer rate (Kla) on Robusta somatic embryo production. Embryos are counted after 4 weeks of culture in flasks inoculated with a cell density of 1.0 g $L^{-1}$ and shaken at 110 rpm.

| Flask volume ( L) | Medium volume (L) | Kla ( $h^{-1}$) | Total embryos (Number X $10^3$ $L^{-1}$) |
|---|---|---|---|
| 0.25 | 25 | 6.8 | 57 |
| | 100 | 5.3 | 77 |
| | 125 | 4.9 | 98 |
| 1.0 | 300 | 3.5 | 18 |
| | 400 | 3.5 | 27 |
| | 500 | 3.1 | 28 |

**Table 2** Effect of inoculation density and medium renewing once a week on Robusta somatic embryo production. Embryos are counted after 6 weeks of culture in 0.25 L flaks at 110 rpm (4 replicates) (reproduced from Zamarripa *et al.* 1991a).

| Inoculation density (g FW $L^{-1}$) | Total embryos (Number X $10^3$ $L^{-1}$) | | Torpedo-stage embryos (%) | |
|---|---|---|---|---|
| | Without renewing | With renewing | Without renewing | With renewing |
| 0.1 | 31 | 21 | 48 | 25 |
| 0.5 | 233 | 240 | 37 | 40 |
| 1.0 | 269 | 382 | 8 | 31 |
| 1.5 | 369 | 484 | 4 | 25 |
| 3.0 | 5 | 5 | 0 | 0 |

the case for carrot (Halperin 1964). Consequently, embryogenesis is initiated without size selection of calli.

Key factors have been identified and optimized in flasks agitated on a gyratory shaker before the scaling-up in bioreactors.

One of the key factors is the oxygen transfer rate ($K_La$). Its initial optimal value is about 5.0 $h^{-1}$ which corresponds to the $K_La$ measured in 0.1 L of culture medium contained in a 0.25 L Erlenmeyer flask agitated at 110 rpm (**Table 1**). Either higher $K_La$ values, obtained for instance by decreasing the medium volume, or lower values, obtained in 1 L flasks, both inhibit embryo production. The initial cell density is another key factor which plays an essential role in leading cell suspension to produce embryos, showing that low densities of 0.5-1.0 g FW $L^{-1}$ are optimal (**Table 2**). On the contrary, high inoculation densities (3 g $L^{-1}$) strongly inhibit embryogenesis. The same observation can be made for the embryo development, expressed by the percentage of torpedo-stage embryos. Renewing the medium limits this inhibition in the case of inoculation densities higher than 0.5 g $L^{-1}$. On the contrary, for the lowest density (0.1 g $L^{-1}$), renewing the medium has a detrimental effect. These observations suggest that coffee embryogenesis depends on a balance of promoting as well as inhibiting factors released by cells into the medium as described for carrot (de Vries *et al.* 1988). However, inhibition appears to be stronger when the cell density is high.

Several studies showed that extracellular compounds released from embryogenic tissues can affect somatic embryogenesis. Most of these studies emphasized the role of extracellular glycoproteins or cell wall arabinogalactan proteins. Extracellular specific endochitinases and peroxidases stimulate carrot and Norway spruce embryogenesis (de Vries *et al.* 1988; de Jong *et al.* 1992; von Arnold *et al.* 1995). A few glycoproteins were also reported to suppress stages of embryo development, for example in gravepine (Coutos-Thevenot *et al.* 1993). Arabinogalactan proteins isolated from conditioned media were shown to re-induce embryogenesis in old non embryogenic cell lines of carrot and *Picea abies* (Kreuger and van Holst 1993; Egertsdotter *et al.* 1995).

In the case of Robusta, we could check that the medium conditioned by a 2-year old cell line (CM) cultured

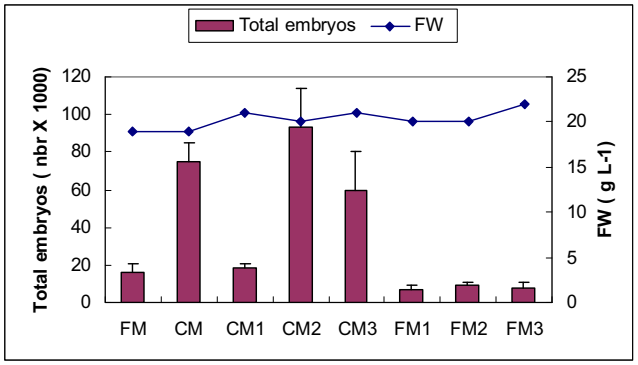


**Fig. 4 Glycoprotein(s) related to the stimulatory activity of a medium conditioned by Robusta embryogenic cells cultured with a low initial cell density.** Biological activities of the conditioned medium (CM) and related sub-fractions (CM1 to CM3) are compared to the ones of the fresh medium (FM) and its sub-fractions (FM1 to FM3). Medium from a low cell density suspension is collected after 5 weeks, concentrated and dialyzed (8 Kda), then separated on a Con A-Sepharose column. The gel-bound proteins are eluted step-wise using 0.01 M (CM2) and 0.3 M methyl-D-mannoside (CM3), respectively. For the biological test, 5 ml of fresh medium (10 X concentrated) is added to 45 ml of the conditioned medium and to its fractions. The total number of embryos are counted after 4 weeks of culture in 0.1-L flasks (inoculation density: 1.0 g $L^{-1}$). Data are the means of 3 replicates.

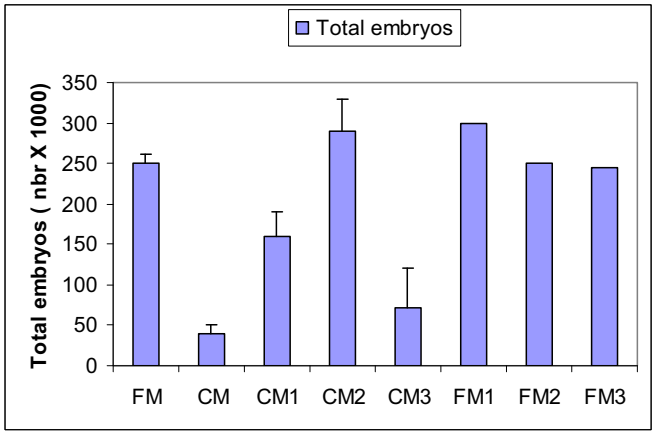


**Fig. 5 Small hydrophobic molecule(s) related to the inhibiting activity of a medium conditioned by Robusta embryogenic cells cultured with a high initial cell density.** Biological activities of the conditioned medium (CM) and related sub-fractions (CM1 to CM2) are compared to the ones of fresh medium (FM) and its sub-fractions (FM1 to FM3). Medium from a high cell density suspension is collected after 3 weeks then dialysed (1 Kda). The diffusat is fractionated on a C-18 column (Waters Sep-pack): CM1: aqueous, CM2 aqueous fraction collected after rinsing the column; CM3: 50% methanol fraction. Each fraction was evaporated to dryness in vacuum then dissolved in 10 ml distilled water and added to 90 ml of fresh medium. The total number of embryos are counted after 4 weeks of culture in 0.25-L flasks (inoculation density: 1.0 g $L^{-1}$). Data are the means of 3 replicates.

with a low cell density stimulates the embryo production of the same line. This medium has to be previously dialysed (<8 KDa), probably due to the removal of low molecular weights endogenous inhibitors. When fresh media is supplemented with CM, the embryo production increased from 16,000 to 78,000/L (**Fig. 4**). The embryogenic-stimulating activity was recovered by affinity chromatography on Concanavalin A-Sepharose into main fractions (CM2, CM3) containing both secreted glycoproteins. These fractions were resolved by ion exchange chromatography into two subfractions each. In both cases, the somatic embryogenesis-stimulation was mainly preserved in one of the two sub-fractions. Peroxidase activity was always associated with active fractions, but it was not possible to prove that this enzyme activity was related to the stimulation of so-

matic embryogenesis. These actives fractions only stimulated the embryogenesis of its mother cell line but did not modify the behaviour of cell lines of other genotypes.

On the other hand, the inhibiting activity of a medium conditioned by cells cultured with a high cell density was mainly recovered from its hydrophobic fraction (**Fig. 5**). This inhibiting activity looks to be related to low molecular weight factor(s), below 1 Kda, having a hydrophobic characteristic. Eventually, this endogenous inhibitor is similar to the one purified from carrot cell suspensions and identified as 4-hydroxybenzyl alcohol (Kobayashi *et al.* 2000).

Histological investigations showed that most of the peripheral cells of the aggregates have a very large and intensely stained nucleolus (diameter = 2.0 µM) after 6 days of culture in no-inhibiting conditions. This change in nucleolar activity, in comparison to embryogenic potential cells, expresses an increase in RNA metabolism. These cells, having highly meristematic characters, are entirely similar to embryogenic cells observable in the initial stages of zygotic embryogenesis (Jones and Rost 1989). As a result of their rapid divisions, these cells establish special areas which evolve relatively synchronously around the clump. When the expression is inhibited by high initial cell density, the cytological appearance remains identical to the one of cells in the multiplication phase. Increased nucleolar activity can thus be considered as an early marker of the embryogenic expression.

Using a similar procedure (high density for multiplication of cells, low density for the differentiation into embryos), several authors reported large production of Robusta as well as Arabica somatic embryos in liquid medium (Neuenschwander and Baumann 1992; van Boxtel and Berthouly 1996; Etienne *et al.* 1999; de Feria *et al.* 2003; Santana *et al.* 2004). However, on the contrary of Robusta, auxin is generally required for *Coffea arabica* to block the tissues at the undifferentiated stage by inhibiting precocious embryo development during the multiplication step.

## STIRRED BIOREACTORS FOR PRODUCTION OF TORPEDO-STAGE EMBRYOS

### Robusta and Arabusta

Critical parameters for the success of mass regeneration of coffee somatic embryos were investigated using a mechanically stirred fermentation apparatus operating at 3-L working volume (Setric SGI, model SET4CV) (Ducos *et al.* 1993b).

Although the agitation was provided by a 4-blade "cell-lift" propeller to minimize the shear stress, preliminary experiments showed that an initial agitation of 100 rpm had a detrimental effect on the growth. Consequently, agitation was kept at the lowest level (50 rpm) until day 21 and then gradually increased to 100-120 rpm. Darkness also suppres-

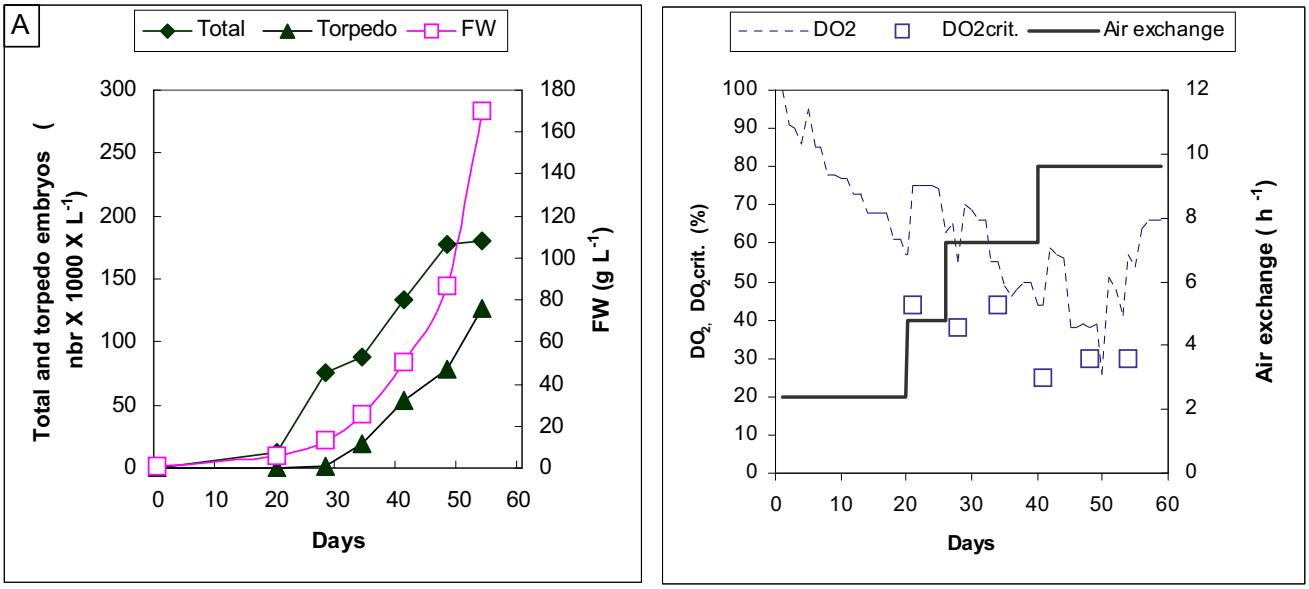


**Fig. 6 Production of Robusta somatic embryos in a 3-L stirred bioreactor.** (**A**) Growth kinetics of somatic embryo development, (**B**) Changes in $DO_2$, $DO_2$ crit. and aeration rate (Reproduced from Ducos *et al.* 1993b).

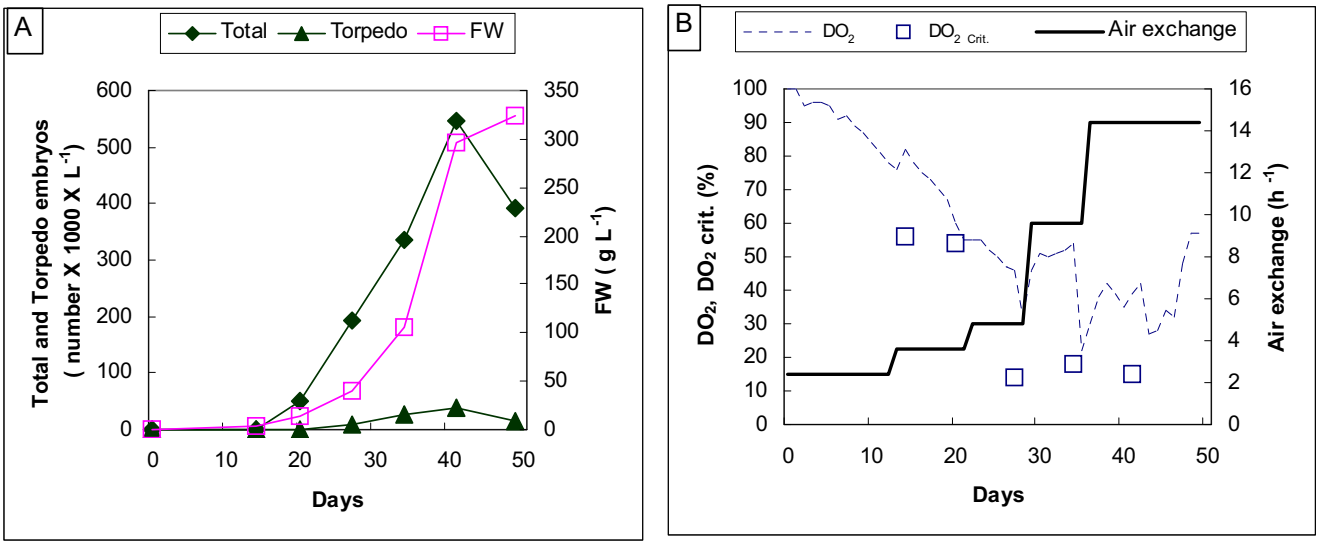


**Fig. 7 Production of Arabusta somatic embryos in a 3-L stirred bioreactor.** (**A**) Growth kinetics of somatic embryo development, (**B**) Changes in $DO_2$, $DO_2$ crit. and aeration rate. (Reproduced from Ducos *et al.* 1993b).

sed somatic embryo production. The optimal initial aeration rate is 2.4 $h^{-1}$ (air volume per medium volume per hour) which corresponds to an oxygen transfer rate of 5 $h^{-1}$. As in flasks, higher and lower $K_La$ values had a detrimental effect during the first weeks of the culture. Detrimental effect of higher $K_La$ values possibly results from the removal of positive volatile compounds such as $CO_2$. Consequently, the air flow rate was maintained at the lowest level sufficient to maintain the dissolved oxygen concentration ($DO_2$) above the critical level ($DO_2$ crit.) throughout the cultures. Measurements taken during different runs showed that $DO_2$ crit. was higher than 40% of the saturation during the first 3 weeks. Such a high $DO_2$ crit. value can be explained by the large size of the clumps: the liquid film resistance to the oxygen transfer around the clumps may be significant.

The bioreactor was charged with Robusta or Arabusta embryogenic cells at a rate of 0.5 g FW $L^{-1}$ (**Figs. 6, 7**). Spent medium was removed once a week by applying air pressure, after stopping agitation and tissues sedimentation, and replaced by fresh medium. Production of Robusta embryos began on day 21 and was completed on day 58: at this date, the embryo concentration reached a maximum of 180,000 embryos $L^{-1}$. The first torpedo-shaped embryos appear at day 28 and 70% of the embryos reach this stage at the end of the culture. For Arabusta, a population of about 400,000 $L^{-1}$ embryos was obtained within 50 days. The concentrations in $CO_2$ and $C_2H_4$ in the outlet gas stream reached a maximum values at day 28, respectively 0.5% and 0.06 µL $L^{-1}$, then decreased. It was observed that specific oxygen uptake and the specific production rates of $CO_2$ and ethylene (expressed on a g basis) decreased as a function of culture time, indicating a possible lower metabolic rate in the more mature embryos.

### Arabica

Mass production of *C. arabica* cv 'Catuai' somatic embryos in a 5-L stirred bioreactor were reported by Noriega and Söndhal (1993). A total yield of 45,000 embryos were recovered within 3 months. More recently, de Feria *et al.* (2003) produced *C. arabica* cv 'Catimor' embryos in 2-L bioreactors with a rate of 70,000 embryo per g FW of inoculated calli.

### Limits

Within 2 months, the biomass reaches such a high density in the stirred bioreactors that it is impossible to ensure sufficient mixing and aeration of the cultures. To extend the development of the embryos beyond the torpedo-stage, it is necessary to dilute the embryo populations. Moreover, the conversion from the torpedo to the cotyledonary-stage is mainly characterized by the greening. Anomalous morphogenesis, when green propagules are grown in liquid media, results in hyperhydricity which is well known as a major problem in micropropagation because it affects plant survival after transplanting.

So, the torpedo embryos must be collected in sterile conditions from the bioreactor, then individually subcultured onto gelose medium for their development into plantlets suitable for acclimatization (Zamarripa *et al*. 1991b). The conversion to the cotyledonary-stage lasts 4 weeks on a semi-solid medium containing BA. At the end of this culture (pregermination), the embryos are green and present a pair of well developed cotyledons. Then the germination step is conducted by transferring the embryos onto a similar medium, but without BA. With this two-media sequence, up to 80% of embryos may be developed into plantlets bearing two to three pairs of leaves. The survival rate is higher than 90% when these plantlets are acclimatized. However, this two-stage procedure constitutes a major source of costs which had to be reduced for the economical viability of the process. Consequently, a new protocol was developed in which the embryos are simply dispersed on gelose medium ("plating") for the cotyledon expansion and then directly transferred, at the cotyledonary-stage, from the plating dishes to *ex vitro* conditions (Ducos *et al*. 1999). This simplified protocol, in which the germination occurs in *ex vitro* instead of *in vitro* conditions, led to a drastic reduction of the labor at the laminar flow because the only individual embryo handling takes place at the transfer time to the greenhouse. A cost assessment showed that the estimated price of a somatic seedling, at the plantation time to the field, could be considered as competitive compared to the one of a cutting, all costs evaluated in a coffee-producing country environment (Ducos *et al*. 1999).

## TEMPORARY IMMERSION BIOREACTOR FOR PRODUCTION OF COTYLEDONARY-STAGE EMBRYOS

### Temporary immersion cultures

Temporary immersion method involves placement of plant tissues on solid supports which are periodically perfused with nutrients solutions. It offers the advantages of cultures in liquid medium, so reducing labor cost, without the disadvantages of a liquid environment. Etienne and Berthouly (2002) described the various temporary immersion bioreactors (TIB) which have been applied for plant micropropagation since the 1980s by some pioneers as Tisserat and Vandercook (1985) and Aitken-Christie *et al.* (1985). According to these authors, the advantages compared to conventional bioreactors are: a) limited hyperhydricity due to avoidance of continuous immersion; b) limited shear stress due to the lack of mechanical agitation or permanent aeration; c) provision of an adequate oxygen transfer (because the tissues are not permanently immersed in liquid media in which oxygen is poorly soluble); d) control of the growth by manipulating the frequency and duration of immersions; e) provision of a sufficient mixing.

In 1993, Alvard *et al.* applied this method to grow meristems of bananas by using a standard autoclavable filtration unit with two compartments. A similar apparatus was commercialized, so-called Recipient for Automated Temporary Immersion (RITA®, Vitropic, France). It has a volume of 1 L and contains 0.2 L of liquid medium. A fine screen is placed in the bottom of the upper part to hold the explants. The system is charged with tissues in the top part and fresh medium in the bottom part, which is connected to a small air pump. When the pump is turned on, the air enters the lower section and, as the pressure builds up, the liquid medium is pushed to the upper part. When the pump is switched-off, the medium flows back down merely with the effect of gravity.

### 1-L RITA®

This small bioreactor has been used to grow embryogenic calli (Berthouly et *al.* 1995) and torpedo-stage embryos of ten *C. arabica* F1 hybrids (Etienne *et al.* 1997). Depending on genotype, yields ranging from 15 to 50,000 somatic embryos per g of embryogenic cells were recorded starting with a cell density of 1 g $L^{-1}$ (i.e. 200 mg per system). The biomass must be divided into several RITA® during the process. Modifications of immersion frequencies and durations affect the efficiency of the somatic embryo regeneration (Albarran *et al.* 2005). Short immersions frequently repeated, 1 min immersions every 4 h, led to the largest quantities of torpedo-shaped embryos without hyperhydricity.

However, according Etienne-Barry *et al.* (1999), the main advantage of this bioreactor concerns the pregermination phase: the cotyledonary embryos produced in RITA® are able to regenerate plantlets after their direct sowing in the greenhouse. According to these authors, it was the first time that coffee embryos could be directly transferred from a liquid culture system to *ex vitro* conditions. This approach reduced handling time to 13% of the values obtained with conventional acclimatization of plantlets issued from gelose medium. Inoculation densities between 50 to 12,000 torpedo

embryos per RITA® were compared. The pregermination was triggered by applying liquid medium for 2 months at an immersion frequency of 5 min twice a day. The optimal initial density phase was 1,600 embryos: it led to 47% of cotyledonary-stage embryos able to develop plantlets in the greenhouse with 50% success. In another study, the same team obtained about 800 embryos per RITA®, 86% of which reached the cotyledonary stage but with morphological heterogeneity (Barry-Etienne *et al.* 2002). This population was sub-divided into three categories according to cotyledon area: "small", "medium" and "large". Somatic embryos with large cotyledons had only a 25% plantlet conversion rate, whereas somatic embryos with small to medium-sized cotyledons had conversion rates of 47% and 63%, respectively. From these different studies, it can be estimated that 300 to 400 coffee plants can be produced per RITA® system.

According to Etienne and Berthouly (2002), new simplifications are possible and highly desirable to reduce the price of the TIB or to increase their efficiency. In fact, different authors described modifications to improve this method, mainly by increasing the volume of the device for commercial scale-up. The twin flask system consisting of a pair of bottles connected by a silicone tube is generally preferred because it allows larger vessels, up to 10 L. It was used for culture of pineapple shoots (Escalona *et al.* 1999), *Phalaenopsis* shoots (Hempling and Preil 2005), potato microtubers, banana embryos and various other tropical crops in Cuba (Jimenez-Gonzalez 2005).

## 10-L glass bottle

At Nestlé R&D Centre-Tours, we implemented the scaling-up of the pregermination of Robusta somatic embryos in a 10 L-glass TIB. This TIB consists of two glass jars (Ducos *et al.* 2007): a 10-L jar containing the somatic embryos (20-cm diameter × 30-cm height) and a 5-L bottle containing the medium and placed below the 10-L jar.

The two recipients are connected with a silicone tubing, which is fixed to the ports located at their basis. A connector is placed on this tube to change the 5-L bottle during the medium renewal operations. The 10-L jar has a cover which can be attached by a metal clamp. On this cover, there is a 6-cm port by which the biomass is aseptically introduced under a laminar flow hood.

Overpressure (0.5 bar) is applied to the vent filter of the medium bottle twice a day to immerse the embryos during 5 min. The TIB are autoclaved with 5 L of medium consisting of half strength of macro and micronutrients of MS medium, B5 vitamins, 0.1-1.0 mg $L^{-1}$ of BA and 20 g $L^{-1}$ sucrose.

By comparison with the twin flask systems, this bioreactor is characterized by: a) a polyurethane foam disk on the bottom of the 10-L jar. This disk isolates the embryos from the thin liquid medium layer remaining in the vessel between the immersion periods. It retains about 1L of liquid medium inside the vessel, therefore it maintains a sufficient relative humidity (85 to 90%). Moreover, this disk has the function of an air sparger during the immersion periods and facilitate a good ventilation of the headspace. b) good accessibility. The embryos can be easily bulk-harvested after removing the cover. c) a simplicity of use as all the operations related to medium renewal can be made without moving the apparatus out of the culture room.

The TIB are inoculated with 20 to 30 g of torpedo stage embryos. When most of the embryos turn green, generally within 2 to 4 weeks, the medium is replaced by fresh media of same composition but without BA. After 2 to 3 months, the top of the biomass reached the cover in some vessels. The embryos present a large heterogeneity in size, from precocious (1 mm) to fully expanded cotyledon stage (20 mm). Only cotyledonary embryos having well developed cotyledons and a hypocotyl larger than 5 mm are taken into consideration. These pregerminated embryos represent around 50% of the total number. These morphological criteria characterize the embryos evaluated sufficiently developed to be sown in the greenhouse. Depending on the clones, between 3,700 to 10,700 pregerminated embryos are collected per TIB. From some vessels, the quantity is as high as 20 to 25,000.

Obviously, light becomes a rate-limiting factor during the culture, as it can only penetrate the first few centimeters into the biomass. Moreover, the embryos with large cotyledons settle last at the end of each immersion period, thus cover the biomass underneath. A non-uniform light distribution inside the TIB may be responsible for differences in growth and quality among the embryos.

## Horizontal disposable 10-L bags

Ziv *et al.* (1998) described the first disposable system, so-called LifeReactor®, suitable for the proliferation of meristematic clusters. This apparatus, based on airlift bioreactor, has a vertical and conical shape. However, when the aim is to produce micro-plants ready to be transplanted to the greenhouse, a horizontal design is more convenient than a vertical one by providing a higher illumination of the biomass.

Therefore it was decided to look for containers offering a greater surface-to-volume ratio to overcome light limitation. So far, the most simple solution looks to place a rigid box inside a plastic bag (Ducos *et al.* 2007). The bottom of the rigid box is bored. The system is gamma-sterilized then connected to the medium bottle. Preliminary experiments showed that the embryos cultured in this new TIB, so-called "Box-In-Bag", are bigger than in the 10-L glass jar TIB, probably because the area is 1,260 $cm^2$, i.e. fourfold the one of the 10-L glass jars for a similar volume.

## Limits

For the production of micro-plants ready for transplantation in the soil, high illumination is required in a bioreactor. However, among the technologies involved in bioreactor industry for plant propagation, the most important but difficult one is the introduction of light into the bioreactors (Takayama and Akita 1994). Although various illuminated bioreactors have been designed (photo-bioreactors), the same authors came recently to the conclusion that light introduction remains inefficient (Takayama and Akita 2006). The "Box-in-Bags" disposable TIB can overcome this problem due to its horizontal design ensuring a higher light transmittance. This disposable TIB combines the advantages provided by the two kinds of plastics, the flexible and the rigid one. The flexible plastic confers the advantages of a disposable device (low cost, simple to operate), a high process security and versatility by allowing diversity in designs and sizes. The rigid plastic box maintains a culture headspace between the immersion periods and a horizontal distribution of the biomass, allowing better oxygenation and illumination. Moreover, considering the possibility to stack several boxes one top of another, it makes this system easy to transport. It will even be possible to send *in vitro* plants keeping them inside the bioreactor in which they have grown. The international exchanges of sterile plant material would be greatly facilitated.

However, further experiments are required to assess different designs for optimizing aeration and mixing throughout the cultures.

Recently, Roels *et al.* (2006) reported that the frequent headspace renewal by surrounding air is responsible for the quality increase of plantain shoots cultured in a small TIB. In its headspace, $CO_2$ and $C_2H_4$ concentrations reach a maximum of 5% and 0.06 µm $L^{-1}$ respectively, instead of 12% $CO_2$ and 0.45 µm $L^{-1}$ $C_2H_4$ in a control vessel containing semi-solid medium. Such studies, by monitoring the composition of the headspace under different immersion regimes, will certainly lead to the optimization of the temporary immersion method.

Heterogeneous distribution of embryos in TIB remains a

sticky problem. During the first weeks, the embryos are uniformly dispersed in a 1 to 2-mm layer on the support. Later, whatever the TIB type and even in a small one as the RITA® system, it was observed that some areas occasionally accumulate embryos which form compact aggregates. Nevertheless, the operator can easily move disposable TIB, considering their low weight, to disperse the immersed embryos on the support when the bags are inflated.

Adelberg (2006) remarks that combining rigid multiple-use and flexible single-use components will allow further innovations in vessel construction. That position looks totally relevant and the "Box-in-Bag" disposable TIB can be an example of such innovations but other ones will certainly be proposed. Unfortunately, as also mentioned by this author, such containers are not commercially available yet. Scale-up of micropropagation may probably be facilitated by the commercialization of large disposable bioreactors, but the opportunity of plant propagation as a new market for the plastic industry remains questionable (Sluis 2006).

## TEMPORARY ROOT IMMERSION BIOREACTOR FOR PRODUCTION OF PLANTLETS

### Photoautotrophic cultures

Plant conversion or seedling development involves a transition from the heterotrophic embryos to an autotrophic plant. Kosai *et al.* (1992, 1997) and Teixeira da Silva *et al*. (2005) demonstrated the feasibility of photoautotrophic micropropagation that uses a sugar-free medium on different plant species. The advantages over the conventional method that utilizes a sugar-containing culture medium are the following: a) microorganisms do not grow significantly on the sugar-free medium, so large vessels can be used; b) photosynthesis and growth of plants *in vitro* are promoted considerably by increasing the $CO_2$ concentration in the vessel; c) quality of plants *in vitro* can be improved by ventilation; d) *in vitro* growth of plantlets are faster and more uniform; e) *in vitro* rooting in vitro is enhanced as callus formation at the shoot base is reduced in the absence of sugar; f) few physiological abnormalities are observed; g) thus, acclimatization can be simplified with nearly 100% survival.

This method was applied to grow Arabusta plantlets from cotyledonary embryos in *in vitro* conditions. In the multi-stage coffee somatic embryogenesis, cotyledonary stage is the earliest stage embryo, capable of photosynthesizing due to a high chlorophyll contents (Afreen *et al*. 2002a). Once they developed chlorophyllous cotyledons exhibiting active photosynthesis, coffee embryos could be successfully cultured in photoautotrophic conditions as it was previously demonstrated by direct transfer to the greenhouse (Ducos *et al.* 1999; Etienne-Barry *et al.* 1999). The main issue of these studies is that high $CO_2$ concentrations (0.50%) strongly stimulate leaf and root development, under photoautotrophic conditions, comparing to ambient air (Uno *et al.* 2003). Elevating $CO_2$ might compensate the lack of cotyledon development in immature embryos but photosynthesis is probably not the sole explanation for this $CO_2$ effect. The growth promoting effect of additional $CO_2$ could also be explained for example by a stimulation of the non-autotrophic $CO_2$ fixation involving PEP carboxylases, as in batch cultures of *Catharanthus Roseus* cells (Ducos and Pareilleux 1986).

### TRI bioreactor

A bioreactor, so-called temporary root zone temporary immersion system (TRI), was developed by Afreen *et al.* (2002b) to grow photoautotrophically coffee plantlets from cotyledonary embryos. The TRI-bioreactor consists mainly of two chambers as the TIB described above: the lower chamber was used as a reservoir for the nutrient solution, and the upper one for culturing embryos. The 2.6-L culture chamber contains an autoclavable cell tray. A narrow air distribution chamber is located between the two chambers. Two air-inlet tubes open into the air distribution chamber and are directly connected to an air pump via a filter disc to prevent microbes entering the culture vessel. The top of the air distribution chamber had several narrow tubes fitted vertically in between the rows of the cell tray an opened in the headspace of the culture chamber. The $CO_2$-enriched air enters the culture chamber by means of these tubes.

Each TRI contains 54 cotyledonary embryos selected from semi-solid medium and transplanted onto miniplugs composed of vermiculite and paper pulp. The embryos are cultured under photoautotrophic condition with $CO_2$ enrichment at 0.1% and high PPF (100 to 150 μmol $m^{-2}$ $s^{-1}$). The nutrient solution immerse the root zone temporarily for a total of 15 min every 6 h. The solution consists of MS nutrients without hormones, vitamins and sucrose, to ensure the phototoautrophic conditions and to limit the growth of micro-organisms. Forced ventilation is introduced with an initial aeration rate of 50 ml $min^{-1}$ (1.6 air exchange par hour) then it is gradually increased every 2 or 3 days to maintain the $CO_2$ concentration at 0.1 %. After 45 days, 84% on the cotyledonary embryos produced plantlets.

### Limits

Zobayed *et al.* (2004) and Kozai and Xiao (2006) made a very detailed report on a commercial laboratory for the micropropagation of ornamental species using TRI bioreactors. They gave a cost analysis, for a yearly production capacity of 150,000 plants, to demonstrate its superiority on the conventional sugar-containing photomixotrophic cultures. However, in the case of coffee, our opinion is that it is preferable to conduct the plantlet production in traditional greenhouses, for the following reasons: a) the heat generated by the numerous lamps required by the photoautotrophic method could be a problem in tropical countries; b) the use of plugs must be carefully considered for coffee because this species requires a perfect tap root system which must be checked at the transfer time to the polyethylene bags; c) to reduce the transportation costs, the plantlet production should be located as close as possible to field plantation. The facilities should therefore be easy to implement.

## COMMERCIAL APPLICATION

### Agronomic performance and trueness to type

For large-scale practical used, this technology had to be validated by confirming the true-to-type status of the regenerated plants at the field level.

From 1996 to 2000, large-scale Robusta field trials have been set up in five coffee-producing countries: Philippines, Thailand, Mexico, Nigeria and Brazil. These trials represent a total of 12,000 somatic seedlings from 10 clones. In the Philippines and in Thailand, a total of 5,067 trees originating from 5- to 7-month-old embryogenic cell lines were compared with control trees derived from *in vitro* axillary budding (microcuttings). They have not shown major undesired somaclonal variation (Ducos and Pétiard 2003a; Ducos *et al.* 2003b). For the observed morphological traits and the yield characteristics, no significant differences were seen between the somatic seedlings and the microcutting-derived trees. The following points were also checked: a) that the somatic seedlings which displayed a different growth rate in the nursery present identical traits in the field indicating that this initial size heterogeneity is not related to somaclonal variation; b) with results obtained in Nigeria and Brazil trials, that the cryopreservation of the embryogenic cells in liquid nitrogen has not any incidence on the agronomic performance of the trees.

In the case of Arabica, somaclonal variations could be observed. Based on several somatic embryogenesis procedures and a large number of varieties, Söndhal and Lauritis (1992) estimated the overall variability found in 12,000 *in vitro*-derived plants to 10%. Somaclonal variation frequency was highly genotype-dependent: 3 to 30% depending on the

varieties (Söndhal and Baumann 2001). Etienne and Bertrand (2001) confirmed this last point: among 30,000 plants belonging to 20 clones of *C. arabica* $F_1$ hybrids, the number of off-types ranged between 3 to 10%, depending on the genotype. The variant percentage drastically increases when embryogenic suspensions proliferate beyond the sixth month (Etienne and Bertrand 2003). Among plants produced after 3 months of callus multiplication in liquid media, the frequency of variants is only 1.3%; after 12 months, this percentage increases to 25%. Apart from a dwarf type, the majority of off-types can be detected and eliminated in the nursery.

These studies show that this propagation method can be used for large-scale commercial applications without any negative unforeseen consequences for the grower, even if the occurrence of some somaclonal variations at the DNA level cannot be excluded. Obviously, the duration of the cell multiplication phase must be restricted and cryopreservation of young coffee embryogenic cell suspensions must be routinely integrated in the process to reduce the risk of somaclonal variations.

## Pilot scale process at Nestlé R&D-Tours

Based on the different technologies described above, a process for large-scale production of pregerminated Robusta somatic embryos was implemented at Nestlé R&D-T (Ducos *et al.*, submitted). Seventeen Robusta clones selected from the Coffee Core Collection (Pétiard *et al.* 2004) were propagated during 3 years. The whole process consists of 8 phases (**Fig. 8**):

At the end of the induction phase (1), embryogenic calli are selected using visual criteria (yellowish color, friable and granulous aspect). They are sub-cultivated on semi-solid medium every 8 weeks using the same criteria (2). Regularly, new cell lines are initiated from this pool of calli to multiply the embryogenic cells in liquid medium (3). A batch production is started every month starting from embryogenic cell lines. Due to the large number of clones to propagate, flasks were preferred to stirred bioreactors for the embryo development phase (4).

Each run batch consists of the phases 4, 5 and 6 and requires 4 to 6 months to produce the pregerminated somatic embryos. Typically, a batch production can be summarized as follows: a) torpedo-stage embryo production (4) is initiated by inoculating sixty g FW of embryogenic cells into six hundred 0.25-L flasks. After 3 weeks of culture, when the embryos started to appear (globular stage), the cultures are grouped into one hundred fifty 1.0-L flasks to reduce the number of handlings for the medium renewal at $T_{5W}$ and $T_{7W}$. b) after 6 to 8 weeks, the cultures are transferred into a total 30 to 35 10-L glass TIB for the conversion from the torpedo to the cotyledonary stage embryos (5). Within 2 to 3 months, about 200,000 cotyledonary embryos having a hypocotyl axis larger than 5 mm are collected per batch; c) then the embryos are spread out in layers onto commercial coconut fibers to keep them in stock in the greenhouse for 2 to 8 weeks (6).

After storage, the pregerminated embryos are sent to coffee producing countries where they are sown in *ex vitro* conditions during 4 to 6 months to develop plantlets bearing true pairs of leaves (7). Finally, the plantlets are grown in polyethylene bags in the nurseries during 6 to 12 months before their transplantation to the field (8).

Initially, the phase 6 was implemented for storage purpose, but it seems that a hardening process occurs when the embryos are kept in such conditions. At the harvest time from the TIB, the embryos look brittle and shiny. After a few weeks under storage conditions, the appearance of the embryos changes: they take on a light green color and look hardy and healthy. It was checked that the storage phase does not have a detrimental effect on the aptitude of the embryos to develop plantlets. On the opposite, the germination rate tends to be higher after 4 or 8 weeks of storage.

To estimate their ability to develop plantlets, germination tests are organized in the Nestlé R&D-T greenhouse. They are sown on coconut fibers supplemented with fertilizer at 1.0 g $L^{-1}$. They are maintained under protective conditions inside a plastic tunnel. Depending on the clone, the embryo-to-plantlet conversion rate varies from 25 to 67%, 41% on average. In 2005, a total of 2.5 M pregerminated embryos were produced. Considering the respective percentage for each clone, this quantity was enough to potentially regenerate about 1.1 M coffee trees.

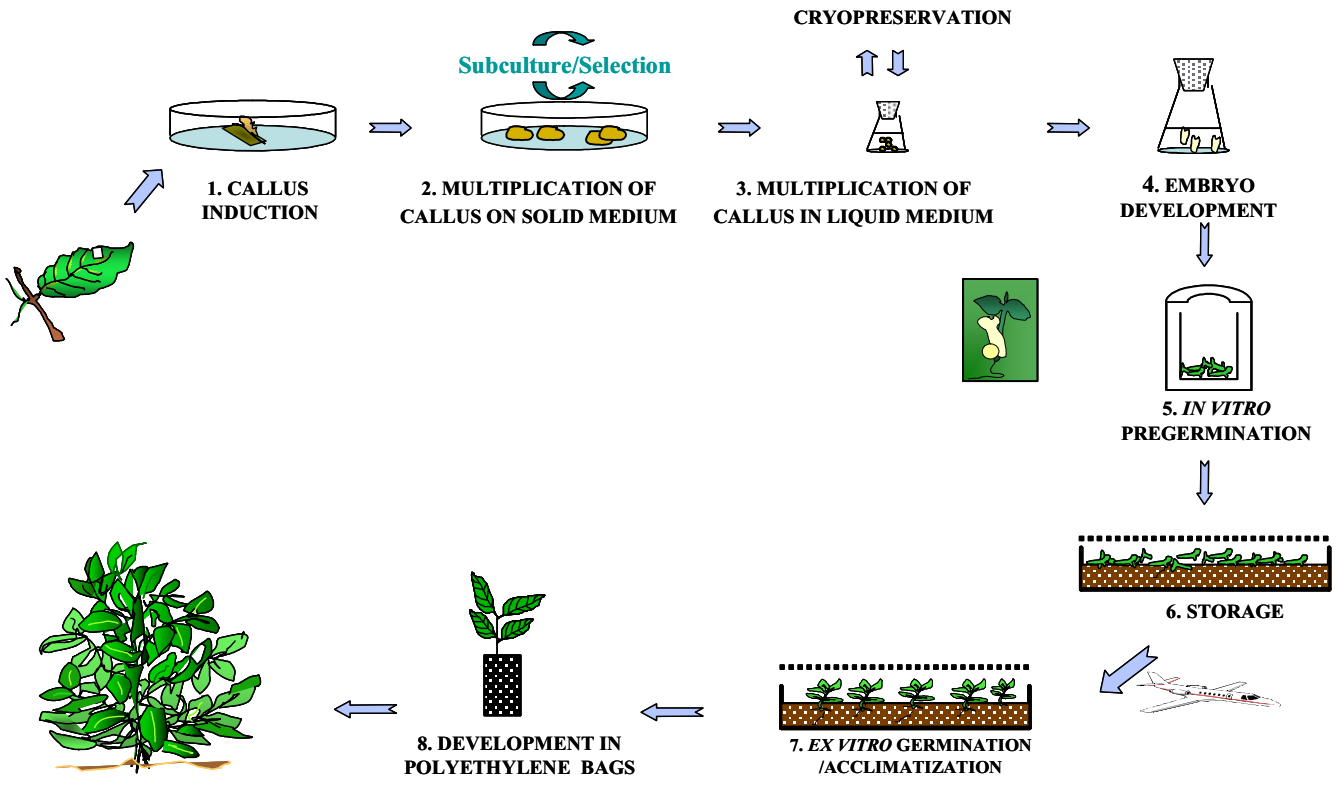


**Fig. 8 Diagram of propagation of Robusta selected clones by somatic embryogenesis in liquid medium at Nestlé R&D Centre - Tours.**

Recently, it was observed that a microenvironment, achieved by placing a transparent cover at 2 to 3 cm above the embryos, significantly increases the *ex vitro* germination rate. With this improvement, an annual production of 1.6 to 1.8 M somatic seedlings can be expected from this pilot production.

### Somatic seedling production in coffee producing countries

Pregerminated embryos of Robusta clones were sent to coffee producing countries, mainly Thailand and Mexico. In total, in April 2006, about 600,000 somatic seedlings were already distributed to the farmers or growing in polyethylene bags (550,000 in Thailand, 50,000 in Mexico).

The process of Robusta somatic embryogenesis has been transferred to the Department of Agriculture (DOA) of Thailand. The aim is to improve the yield of old Robusta plantations by replacing the low performing trees with selected clones (Sanpote *et al*. 2006). The Chumphon Horticultural Research Center (CHRC) started to produce embryogenic calli during 2002 and the first plants were delivered to the farmers in 2003. The technique of temporary immersion in liquid media is currently implemented to increase the laboratory capacity. The local scientific team was the first to scale-up the *ex vitro* germination phase, up to 100 to 200,000 plants per year. Nestlé R&D-T also transferred this technique to Viet Nam (WASI), Mexico (INIFAP) and Indonesia (ICCRI).

Considering Arabica, more than 100,000 somatic seedling of $F_1$ hybrids are currently in commercial plots in four Central America countries (Etienne and Bertrand 2003).

## PROSPECTS

This review illustrates two major trends which have been implemented in commercial laboratories during these last years: a) bulk-cultivation of small propagules in photomixotrophic conditions (with sugar) followed by their selection and transfer to the greenhouse for their conversion to plant. b) production of fully developed and individualized (singulated) plantlets in photoautotrophic conditions (in sugar-free medium with $CO_2$-enrichment and a high light intensity) followed by their transfer to the greenhouse.

Next progress on coffee somatic embryogenesis will probably originate from the combination of these two approaches, both in the laboratory for the pregermination and in the greenhouse for the germination.

### Pregermination phase under photoautotrophic conditions

Bulk-cultivation of *in vitro* plants ready for the greenhouse still remains difficult in large vessels because of poor light illumination of the plant tissues. Takayama and Akita (2006) came to the conclusion that new technology is required for high illumination efficiency for bulk-production of transplantable propagules. Such advances can be provided by the development of horizontally designed bioreactors, facilitated by the plastic bags technology. Investigations are in progress to check if increasing the light transmittance in such a manner will lead to more homogeneous coffee embryo populations. Nestlé R&D-T is currently testing “Box-in Bags” TIB manufactured by Hegewald-Medical (Germany).

Moreover, horizontal TIB could lead to a compromise between the two alternatives mentioned above, by allowing a double step culture in the same container: a) conversion from torpedo to cotyledonary stage embryos with sugar; b) then a maturation phase of the cotyledonary stage embryos under photoautotrophic conditions before their acclimatization.

Obviously, this second step, conducted under photoautotrophic conditions, will only be efficient if the coffee embryos are well illuminated, i.e. enough dispersed on the support. It can improve the autotrophic characters of the pregerminated embryos as described by Afreen *et al.* (2002a), therefore their ability to develop plantlets in the greenhouse.

### *Ex vitro* germination with $CO_2$ enrichment

Concerning the *ex vitro* germination protocol, the reports on the effects of $CO_2$-enrichment in sugar-free cultures (Kozai *et al.* 1992) suggest beneficial promotion on coffee embryo growth in the greenhouse. Most probably $CO_2$-enrichment beyond 0.04% will have a positive effect on cotyledonary embryos. Teixeira da Silva and the research group in the Tanaka lab have shown that a number of plants (strawberry, sweet potato, eucalyptus, *Cymbidium*, papaya, among others; e.g. Teixeira da Silva *et al*. 2007) can be effectively acclimatized *in vitro* through the use of $CO_2$-enrichment in special vessels, the Vitron™; subsequently no special acclimatization step is required, and plantlets are directly introduced into the *ex vitro* environment or greenhouse. Undoubtedly the system would be highly applicable for coffee cultures, too.

Recently, it was observed that a microenvironment stimulates the *ex vitro* germination rate and that this positive effect is linked to the release of $CO_2$ by commercial horticultural substrates, as peat or coconut fibers (Ducos *et al*. 2007; Positive effect of $CO_2$ released by commercial horticultural media on the *ex vitro* germination of coffee somatic embryos. *3rd International Symposium on Acclimatization and Establishment of Micropropagated Plants*. Faro. Portugal 12-15 September, 2007, submitted). The usage of the “micro-environment” method, combined with media releasing $CO_2$, is well adapted for the acclimatization of very small *in vitro* plants. Particularly, this method can be a relevant alternative to the conventional one, consisting on insufflating $CO_2$, for large-scale coffee plantlet productions in tropical greenhouses.

### Automation and artificial seeds (as a micro-bioreactor)

The combination of semi-automated sterile fermentor production with propagules that can be separated and planted in the greenhouse has already been implemented for comercial micropropagation (Sluis 2006). Progress towards a complete automation, by avoiding any individual manipulation, will bring micropropagation to the next level of commercialization.

Because they are relatively small and uniform in size, torpedo somatic embryos look more suitable for automation than larger embryos. Approaches were described where torpedo embryos are embedded in sterilized plugs moistened with medium containing sucrose, then maintained in an aseptic environment until they become photoautotrophic (Timmis *et al*. 1992; Dupuis *et al.* 1994, 1999). Nevertheless, automation of embryo distribution from liquid medium onto such plugs provokes problems of agglutination and choking inside the delivery pipes. It was investigated whether carrot and coffee somatic embryos can be handled after their dehydration by using seed sowing and sorting technologies (Ducos *et al.* 2005).

Approaches toward automation have also been focused on production of "artificial seeds” involving encapsulation of hydrated torpedo somatic embryos which should allow direct sowing into the soil (Redenbaugh *et al.* 1987). The need for a sugar supply in the capsules causes the beads to rapidly become contaminated with microbes when they are transferred in a non-sterile environment (Molle *et al*. 1993). This approach has only been continued by the Weyerhaeuser Compagny which has several patents on “manufactured seeds” describing oxygen carrier emulsions, hard seed coat and a cotyledon restraint system to ensure emergence of coniferous germinating embryos (Gupta and Timmis 2005).

To our knowledge, studies on methods for culturing autotrophic plants from heterotrophic plant material in “manu-

factured seeds" or sterile plugs have never considered the possible effect of $CO_2$ to facilitate this transition. In the case of coffee, Uno *et al.* (2003) reported that even torpedo embryo growth, not only cotyledonary one, may occur in optimal photoautotrophic conditions. Artificial seed technology can possibly be improved a lot by further studies on the transition from heterotrophic to autotrophic status. Such studies, dealing with gas exchange characterization, will have to consider an "artificial seed" as a "mini-bioreactor".

## CONCLUSION

Coffee somatic embryogenesis can be industrially used for large scale and rapid propagation of selected heterozygous varieties, as Robusta clones or $F_1$ hybrid Arabica. Pilot units, up to 1 M somatic seedlings per year, are currently being implemented by various teams. It is clear that the enormous embryogenic potential of this species is far from being fully exploited and that several improvements could be expected to reduce the cost production. Particularly, rapid next progress are expected for the *ex vitro* germination step, its scaling-up efficiency in large greenhouses and its flexibility to be rapidly adapted to various environments.

Coffee is a perfect plant model for applied research on the development of new bioreactors and on strategies for large-scale production of somatic seedlings. It is also a relevant model species for fundamental research on somatic embryogenesis, particularly for the investigations on: a) the release of inhibitors as well as stimulating compounds in the medium; b) QTL for the aptitude to somatic embryogenesis; c) the transition from heterotrophic to autotrophic metabolism.

Considering its economical importance and its ability to produce easily somatic embryos, there are evidences that coffee will therefore join the group of other important crops, like coniferous and oil palm, to be largely propagated by somatic seedlings.

## REFERENCES


**Adelberg J** (2006) Agitated, thin-films of liquid medium for efficient micropropagation. In: Gupta SD, Ibaraki Y (Eds) *Plant Tissue Culture Engineering*, Springer, Dordrecht, The Netherlands, pp 101-115

**Afreen F, Zobayed SMA, Kozai T** (2002a) Photoautotrophic culture of *Coffea arabusta* somatic embryos: ability and growth of different stage embryos. *Annals of Botany* **90**, 11-19

**Afreen F, Zobayed SMA, Kozai T** (2002b) Photoautotrophic culture of *Coffea arabusta* somatic embryos: Development of a bioreactor for the large scale plantlet conversion from cotyledonary embryos. *Annals of Botany* **90**, 20-29

**Aitken-Christie J, Jones C, Bond S** (1985) Wet and waxy shoots in radiate pine micropropagation. *Acta Horticulturae* **166**, 93-100

**Albarran J, Bertrand B, Lartaud M, Etienne H** (2005) Cycle characteristics in a temporary immersion bioreactor affect regeneration, morphology, water and mineral statusd of coffee (*Coffea arabica*) somatic embryos. *Plant Cell, Tissue and Organ Culture* **81**, 27-36

**Alvard D, Cote F, Teisson C** (1993) Comparison of methods of liquid medium culture for banana micropropagation. *Plant Cell, Tissue and Organ Culture* **32**, 55-60

**Barry-Etienne D, Bertrand B, Schlönvoigt A, Etienne H** (2002) The morphological variability within a population of coffee somatic embryos produced in a bioreactor affects the regeneration capacity and the development in the nursery. *Plant Cell, Tissue and Organ Culture* **68**, 153-162

**Berthouly M, Dufour M, Alvard D, Carasco C, Alemano L, Teisson C** (1995) Coffee micropropagation in a liquid medium using the temporary immersion technique. In: ASIC (Ed) *Proceeding of 16th Colloquium of International Coffee Science Association*, ASIC, Vevey, Switzerland, pp 514-519

**Coutos-Thévenot P, Jouenne T, Maes O, Guerbette F, Grosbois M, le Caer JP, Boulay M, Deloire A, Kader JC, Guern J** (1993) Four 9 kDa proteins excreted by somatic embryos of gravepine are isoforms of lipid transfer protein. *European Journal of Biochemistry* **217**, 885-889

**de Feria M, Jimenez E, Barbon R, Capote A, Chavez M, Quiala E** ( 2003) Effect of dissolved oxygen concentration on differenciation of somatic embryos of *Coffea arabica* cv. Catimor 9722. *Plant Cell, Tissue and Organ Culture* **72**, 1-6

**de Jong AJ, Cordewener J, Lo Schiavo F, Terzi M, Vanderkerckhove J, van Kammen A, de Vries SC** (1992) A carrot somatic mutant is rescued by chitinase. *The Plant Cell* **4**, 452-433

**de Vries SC, Booij H, Janssens R, Vogel R, Saris L, Lo Schiavo F, Terzi M, van Kammen A** (1988) Carrot somatic embryogenesis depends on the phytohormone-controlled presence of correctly glycosylated extracellular proteins. *Genes and Development* **2**, 462-476

**Ducos JP, Pareilleux A** (1986) Effect of aeration rate and influence of $pCO_2$ in large-scale cultures of *Catharanthus roseus. Applied and Microbiology Biotechnology* **25**, 101-105

**Ducos JP, Bollon H, Pétiard V** (1993a) Production of carrot somatic embryos in a bioreactor. *Applied and Microbiology Biotechnology* **39**, 465-470

**Ducos JP, Zamarripa A, Eskes A, Pétiard V** (1993b) Production of somatic embryos of coffee in a bioreactor. In: ASIC (Ed) *Proceeding of 15th Colloquium of International Coffee Science Association*, ASIC, Vevey, Switzerland, pp 89-96

**Ducos JP, Gianforcaro M, Florin B, Pétiard V, Deshayes A** (1999) A technically and economically attractive way to propagate elite *Coffee canephora* (Robusta) clones: *in vitro* somatic embryogenesis. In: ASIC (Ed) *Proceeding of 18th Colloquium of International Coffee Science Association*, ASIC, Vevey, Switzerland, pp 295-300

**Ducos JP, Pétiard V** (2003a) Propagation de clones de Robusta (*Coffea canephora* P.) par embryogenèse somatique en milieu liquide. In: El Hadrami I, Daayf F (Eds) *Biotechnologies Végétales: De la Structure des Génomes à l'Amélioration des Plantes*, El Watanya, Marrakech, Marocco, pp 142-159

**Ducos JP, Alenton R, Reano JF, Kanchanomai C, Deshayes A, Pétiard V** (2003b) Agronomic performance of *Coffea canephora* P. trees derived from large-scale somatic embryo production in liquid medium. *Euphytica* **131**, 215-223

**Ducos JP, Florin B, Dupuis J, Pétiard V** (2005) A new approach for automation: sorting and sowing dehydrated somatic embryos using seed technologies. In: Hvoslef-Eide AK, Preil W (Eds) *Liquid Culture Systems for In Vitro Plant Propagation*, Springer, Dordrecht, The Netherlands, pp 415-424

**Ducos JP, Chantanumat P, Vuong P, Lambot C, Pétiard V** (2007) Mass propagation of Robusta clones: Disposable plastic bags for pre-germination of somatic embryos by temporary immersion. *Acta Horticulturae*, in press

**Dupuis JM, Roffat C, Derose R, Molle F** (1994) Pharmaceutical capsules as a coating system for artificials seeds. *Bio/Technology* **12**, 385-389

**Dupuis JM, Millot C, Teufel E, Arnault A, Freyssinet G** (1999) Germination of synthetic seeds: new substrates and phytoprotection for carrot somatic embryos to plant. In: Soh WY, Bhojwani SS (Eds) *Morphogenesis in Plant Tissue Cultures*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 418-441

**Egertsdotter U, Mo LH, von Arnold S** (1995) Extracellular proteins in embryogenic suspensions cultures of Norway spruce (*Picea abies*). *Physiologia Plantarum* **88**, 315-321

**Escalona M, Lorenzo JC, Gonzales B, Daquinta M, Gonzales JL, Desjardins Y, Borotto CG** (1999) Pineapple (*Ananas comosa* L. Merr) micropropagation in temporary immersion systems. *Plant Cell Reports* **18**, 743-748

**Etienne H, Bertrand B, Anthony F, Cote F, Berthouly M** (1997) L'embryogenèse somatique : un outil pour l'amélioration genetique du caféier. In: ASIC (Ed) *Proceeding of 17th Colloquium of International Coffee Science Association*, ASIC, Vevey, Switzerland, pp 457-465

**Etienne H, Berthouly M** (2002) Temporary immersion systems in plant micropropagation. *Plant Cell, Tissue and Organ Culture* **69**, 215-231

**Etienne H Bertrand B** (2001) Trueness-to-type and agronomic characteristics of *Coffea arabica* trees micropropagated by the embryogenic cell suspension technique. *Tree Physiology* **21**, 1031-1038

**Etienne H, Bertrand B** (2003) Somaclonal variation in *Coffea arabica*: effects of genotype and embryogenic cell suspension age on frequency and phenotype of variants. *Tree Physiology* **23**, 419-426

**Etienne-Barry D, Bertrand B, Vasquez N, Etienne H** (1999) Direct sowing of *Coffea arabica s*omatic embryos mass-produced in a bioreactor and regeneration of plants. *Plant Cell Reports* **19**, 111-117

**Florin B, Ducos JP, Firmin L, Meschine C, Thierry C, Pétiard V, Deshayes A** (1995) Preservation of coffee somatic embryos through desiccation and cryopreservation. In: ASIC (Ed) *Proceeding of 16th Colloquium of International Coffee Science Association*, ASIC, Vevey, Switzerland, pp 542-547

**Gamborg OL, Miller RA, Ojima K** (1968) Nutrients requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**, 151-158

**Gupta PK, Timmis R** (2005) Mass propagation of conifer trees in liquid cultures: Pregress towards commercialization. In: Hvoslef-Eide AK, Preil W (Eds) *Liquid Culture Systems for In Vitro Plant Propagation*, Springer, Dordrecht, The Netherlands, pp 389-402

**Halperin W** (1964) Morphogenetic studies with partially synchronized cultures of carrot embryos. *Science* **146**, 408-410

**Hempfling T, Preil W** (2005) Application of a temporary immersion system in mass propagation of *Phalaenopsis*. In: Hvoslef-Eide AK, Preil W (Eds) *Liquid Culture Systems for In Vitro Plant Propagation*, Springer, Dordrecht, The Netherlands, pp 231-242

**Jimenez-Gonzales E** (2005) Mass propagation of tropical crops in temporary immersion systems. In: Hvoslef-Eide AK, Preil W (Eds) *Liquid Culture Systems for in Vitro Plant Propagation*, Springer, Dordrecht, The Netherlands, pp 197-211

**Jones TJ, Rost TL** (1989) Histochemistry and ultrastructure of rice *(Oriza sativa*) zygotic embryogenesis. *American Journal of Botany* **76**, 504-520

**Kobayashi T, Higashi K, Sasaki K, Asami T, Yoshida S, Kamada H** (2000) Purification from Conditioned Medium and chemical purification of a factor

that inhibits Somatic Embryogenesis in Carrot. *Plant Cell Physiology* **41**, 268-273

**Kozai T, Fujiwara K, Hayashi M, Aitken-Christie J** (1992) The *in vitro* environments and its control in micropropagation. In: Kurata K, Kosai T (Eds) *Transplants Production System*, Kluwers Academic Publishers, Dordrecht, The Netherlands, pp 247-282

**Kosai T, Kubota C, Jeong BR** (1997) Environmental control fort the large-scale production of plants through in vitro techniques. *Plant Cell, Tissue and Organ Culture* **51**, 49-56

**Kosai T, Xiao Y** (2006) A commercialized photoautotrophic micropropagation system. In: Gupta SD, Ibaraki Y (Eds) *Plant Tissue Culture Engineering*, Springer, Dordrecht, The Netherlands, pp 355-371

**Kreuger M, Van Holst GJ** (1993) Arabinogalactan proteins are essential in somatic embryogensis of *Daucus carota* L. *Planta* **189**, 243-248

**Lu CY, Vasil IK** (1985) Histology of somatic embryogenesis in *Panicum maximum* (Guinea grass). *American Journal of Botany* **72**, 1908-1913

**Michaux-Ferrière N, Biyesse D, Alvard D, Dublin P** (1989) Etude histologique de l'embryogenèse somatique chez *Coffea arabica*, induite par culture sur milieux uniques de fragments foliaires de génotypes différents. *Café, Cacao Thé* **33**, 207-217

**Molle F, Dupuis JM, Ducos JP, Anselm A, Crolus-Savidan I, Pétiard V** (1993) Carrot somatic embryogenesis and its application to synthetic seeds. In: Redenbaugh K (Ed) *Synseeds: Applications of Synthetic Seeds to Crop Improvement*, CRC Press, Boca Raton, USA, pp 257-287

**Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

**Neuenschwander B, Baumann TW** (1992) A novel type of somatic embryogenesis in *Coffea arabica*. *Plant Cell Reports* **10**, 608-612

**Noriega C, Söndhal MR** (1993) Arabica coffee micropropagation through somatic embryogenesis via bioreactors. In: ASIC (Ed) *Proceeding of 15th Colloquium of International Coffee Science Association*, ASIC, Vevey, Switzerland, pp 73-81

**Paek KY, Chakrabarty D, Hahn EJ** (2005) Application of bioreactor systems for large-scale production of horticultural and medicinal plant. In: Hvoslef-Eide AK, Preil W (Eds) *Liquid Culture Systems for In Vitro Plant Propagation*, Springer, Dordrecht, The Netherlands, pp 96-116

**Pétiard V, Lepage B, Lambot C, Crouzillat D, Florin B, Brulard E, Alvarez M, von Rutte S, Leloup V, Gangel C, Liardon R, Jung ML, Rytz A, Labbe D** (2004) Establishment, preliminary agronomic and qualitative evaluation of collections of *Coffea arabica* and *Coffea canephora* cultivated varieties. In: ASIC (Ed) *Proceeding of 20th Colloquium of International Coffee Science Association*, ASIC, Vevey, Switzerland, pp 834-841

**Redenbaugh K, Viss P, Slade D, Fujii JA** (1987) Scale-up: artificial seeds. In: Green CE, Somers DA, Hackett WP, Biesboer DD (Eds) *Plant Tissue and Cell Cultures,* A.R. Liss, New York, USA, pp 473-493

**Roels S, Noceda C, Escalona M, Sandoval J, Canal MJ, Rodriguez R** (2006) The effect of headspace renewal in a Temporary Immersion Bioreactor on Plantain (*Musa* AAB) shoot proliferation and quality. *Plant Cell, Tissue and Organ Culture* **84**, 155-163

**Sanpote S, Ducos JP, Lambot C, Kunasol T, Kasinkasaempong K, Chantanumat P, Pétiard V** (2006) First industrial massive propagation of *Coffea canephora* through somatic embrygenesis organized in Thailand. In: ASIC (Ed) *Proceeding of 21st Colloquium of International Coffee Science Association*, ASIC, Vevey, Switzerland, in press

**Santana N, Gonzales ME, Valcarcel M, Canto-Flick A, Hernandez MM, Fuentes-Cerda CF, Barahona F, Mijantos-Cortez J, Loyola-Vargas VM** (2004) Somatic embryogenesis: a valuable alternative for propagating selected Robusta coffee (*Coffea canephora*) clones. *In Vitro Cellular and Development Biology - Plant* **40**, 95-101

**Sluis CJ** (2006) Integrating automation technologies with commercial micropropagation. In: Gupta SD, Ibaraki Y (Eds) *Plant Tissue Culture Engineering*, Springer, Dordrecht, The Netherlands, pp 231-248

**Söndhal MR, Sharp WR** (1977) High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. *Zeitschrift für Pflanzenphysiologie* **81**, 395-408

**Söndhal MR, Loth WHT** (1985) Coffee biotechnology. In: Clarke RJ, Macrae R (Eds) *Coffee: Agronomy* (Vol 4), Elsevier Applied Science, London, UK, pp 235-262

**Söndhal MR, Lauritis JA** (1992) Coffee. Biotechnology of perennial fruit crops. In: Hammerschalg FA, Litz RE (Eds) *Biotechnology in Agriculture*, CAB international Wallingford, UK, pp 401-420

**Söndhal MR, Baumann TW** (2001) Agronomy II: developmental and cell biology. In: Clarke RJ, Witzthum OJ (Eds) *Coffee: Recent Developments*, Blackwell Science, Oxford, UK, pp 202-220

**Staritsky G** (1970) Embryoid formation in callus cultures of coffee. *Acta Botanica Neerlandica* **19**, 509-514

**Takayama S, Akita M** (1994) The types of bioreactors used for shoots and embryos. *Plant Cell, Tissue and Organ Culture* **39**, 147-156

**Takayama S, Akita M** (2006) Bioengineering aspects of bioreactor in plant propagation. In: Gupta SD, Ibaraki Y (Eds) *Plant Tissue Culture Engineering*, Springer, Dordrecht, The Netherlands, pp 83-100

**Teixeira da Silva JA** (Ed) (2006) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol II), Global Science Books, London, 571 pp

**Teixeira da Silva JA, Giang DTT, Tanaka M** (2005) Micropropagation of sweet potato (*Ipomoea batatas*) in a novel $CO_2$-enriched vessel. *Journal of Plant Biotechnology* **7**, 1-8

**Teixeira da Silva JA, Giang DTT, Chan M-T, Sanjaya, Norikane A, Chai M-L, Ruíz JC, Penna S, Granström T, Tanaka M** (2007) The influence of different carbon sources, photohetero-, photoauto- and photomixotrophic conditions on protocorm-like body organogenesis and callus formation in thin cell layer culture of hybrid *Cymbidium* (Orchidaceae). *Orchid Science and Biotechnology* **1**, 15-23

**Timmis R, Kreitinger M, Yancey MJ** (1992) Methods and apparatus for culturing autotrophic plants from heterotrophic plant material. Weyerhaeuser. US Patent No. 119588

**Tisserat B, Vandercook CE** (1985) Development of an automated plant culture system. *Plant Cell, Tissue and Organ Culture* **5**, 107-117

**Uno A, Ohyama K, Kosai T, Kubota C** (2003) Photoautotrophic culture with $CO_2$ enrichment for improving micropropagation of *Coffea arabusta* using somatic embryos. *Acta Horticulturae* **625**, 271-277

**Van Boxtel J, Berthouly M** (1996) High frequency somatic embryogenesis from coffee leaves. Factors influencing embryogenesis and subsequent prolixferation and regeneration in liquid medium. *Plant Cell, Tissue and Organ Culture* **44**, 7-17

**von Arnold S, Egertsdotter U, Mo LH** (1995) Importance of arabinogalactan proteins for the development of somatic embryos in Norway spruce (*Picea abies*) *Physiologia Plantarum* **93**, 334-345

**Yasuda T, Fujii Y, Yamaguchi T** (1985) Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. *Plant Cell Physiology* **26**, 595-597

**Zamarripa A, Ducos JP, Bollon H, Dufour M, Pétiard V** (1991a) Production of coffee somatic embryos in liquid medium: effects of inoculation density and renewal of the medium. *Café Cacao Thé* **35**, 233-244

**Zamarripa A, Ducos JP, Tessereau H, Bollon H, Eskès AB, Pétiard V** (1991b) Developpement d'un procédé de multiplication en masse du caféier par embryogenèse somatique en milieu liquide. In: ASIC (Ed) *Proceeding of 14th Colloquium of International Coffee Science Association*, ASIC, Vevey, Switzerland, pp 392-402

**Zamarripa A** (1993) Study and development of somatic embryogenesis in liquid medium of coffee. PhD Thesis, Ecole Nationale Supérieure Agronomique, Rennes, France, 191 pp

**Ziv M, Ronen G, Raviv M** (1998) Proliferation of meristematic clusters in disposable presterilized plastic bioreactors for the large-scale micropropagation of plants. *In Vitro Cellular and Development Biology - Plant* **34**, 152-158

**Ziv M** (2005) Simple bioreactor for mass propagation of plants. In: Hvoslef-Eide AK, Preil W (Eds) *Liquid Culture Systems for In Vitro Plant Propagation*, Springer, Dordrecht, The Netherlands, pp 79-93

**Zobayed SM, Afreen F, Xiao Y, Kozai T** (2004) Recent advancement in research on photoautotrophic micropropagation using large culture vessels with forced ventilation. *In Vitro Cellular and Developmental Biology- Plant* **40**, 450-458