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Current Applications of Coffee (*Coffea arabica*) Somatic Embryogenesis for Industrial Propagation of Elite Heterozygous Materials in Central America and Mexico

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Abstract

Of all the possible micropropagation techniques, it is widely accepted that vegetative propagation by somatic embryogenesis is by far the most promising for rapid, large-scale dissemination of elite individuals. Yet, to date, examples of somatic embryogenesis processes applied on an industrial scale are very few and far between. There are many complications. They usually involve a major genotypic effect, particularly for obtaining embryogenic tissues, or are related to the quality of regenerated somatic embryos, the incidence of somaclonal variation and, more generally, a lack of reproducibility and efficiency at certain stages of the process, leading to production costs that are prohibitive. Research on coffee somatic embryogenesis began at the end of the 1970s at various institutes, including CIRAD. Between 1995 and 2001, CIRAD moved the technique forward from a research laboratory scale to a technique enabling industrial dissemination of extremely promising *Coffea arabica* F1 hybrids. Over that period, two technological innovations made technology transfer economically feasible: mass production of somatic embryos in temporary immersion bioreactors and the possibility of sowing them directly in the nursery. At the same time, reassuring data were obtained on the genetic conformity of regenerated plants (somaclonal variation frequency < 3%). In 2002, in partnership with the ECOM group, CIRAD decided to transfer the somatic embryogenesis method on an industrial scale to Central America so that four Arabica hybrid clones, that were selected for agroforestry-based farming systems, could be disseminated throughout that part of the world. This article describes the different stages and the difficulties we had to overcome before successful technology transfer could occur in 2010. It describes one of the first examples of somatic embryogenesis technology applied on a commercial scale.
Keywords: Somatic embryogenesis, micropropagation, technological transfer, coffee tree, production costs, clonal conformity, somaclonal variations, *in vitro* plantlet, nursery

Introduction

Somatic embryogenesis, a long-awaited technology!

Somatic embryogenesis enables rapid and massive vegetative propagation of elite genotypes by doing away with lengthy and costly pedigree selection processes. Still, to date, examples of somatic embryogenesis processes applied on an industrial scale are few and far between. Nonetheless, a few examples can be mentioned, such as loblolly pine (*Pinus taeda*) [Gupta PM, pers. comm.], oil palm (Khaw *et al*. 1999) , *Coffea arabica* (Menéndez-Yuffá *et al*. 2010, present communication] and *C. canephora* (Sampote *et al*. 2006, Ducos *et al.* 2010), for which annual production now exceeds one to several million plants annually. Yet, this vegetative propagation technique is widely accepted as being by far the most promising for capturing genetic gain quickly through rapid and largescale dissemination of elite individuals. This is all the more true with woody species for which biological cycles are long. In the 1980s, there was great enthusiasm for developing this technology and expectations were running high, which explains why research was undertaken on a large number of species, without any immediate justification in some cases. There are many complications in developing this technology. They usually involve a major genotypic effect, particularly for obtaining embryogenic tissues, or they are related to the mediocre quality of regenerated somatic embryos, the incidence of somaclonal variations, and more generally a lack of reproducibility and efficiency at some stages, leading to production costs that prove prohibitive.

Quickest possible dissemination of genetic progress in the Arabica species

Research on coffee somatic embryogenesis began in the early 1980s at various institutes, including CIRAD, without any clear objective. At the beginning of the 1990s, CIRAD, in partnership with the Central American research network, PROMECAFE, set out to create *Coffea arabica* intraspecific F1 hybrids, by crossing the varieties traditionally grown in Latin America with wild individuals originating from Ethiopia and Kenya. The resulting hybrids proved to be extremely promising as they displayed a high level of heterosis, producing an average

40% more than the best cultivated varieties, with some of them producing coffee exhibiting better sensory qualities than those of the reference varieties (Bertrand *et al.* 2005). The co-breeders of these new varieties soon found the need for a somatic embryogenesis process capable of massively propagating Arabica F1 hybrid clones. However, moving from a technique developed in a research laboratory to an industrial process enabling the annual production of several million plants is a major leap forward. The cobreeders decided to go ahead and fund the research required to achieve this first change of scale. It took place under CIRAD management at CATIE, a regional research centre in Costa Rica.

Results

Situation prior to technological transfer (1995-1996): identification of points for improvement

Several limitations were identified that were an obstacle to technological transfer of the somatic embryogenesis process developed by CIRAD at that point in time. First of all, production costs. With the development of a software package capable of estimating a range of production costs under different culturing conditions, the verdict was announced: 1.52 USD/plant, whereas a conventional seedling cost 0.25-0.35 USD! There was a further handicap, because the planting densities practised with Arabica in Latin America were between 6 and 8,000 trees/hectare. This is a large number that is only possible because of the dwarfism of the varieties used, and that thus allows intensification of production. The additional cost of planting *in vitro* plantlets needed to be limited, even though significant added value was expected with hybrid material. The software also proved useful for precisely identifying the stages in the process responsible for the high cost of production; it involved some later stages, including germination and the development of weanable plantlets, i.e. possessing at least two pairs of leaves to withstand the shock of acclimatization to *ex vitro* conditions. This *in vitro* growth period was classically labourintensive because of the required subculturing and manufacturing of nutrient media and because it took up a great deal of space in the culture rooms. The second limitation was a risk that had so far been overlooked, namely that the somatic embryogenesis process could lead to a high frequency of somaclonal variation. These "photocopy errors" are undesirable since "variant" plants do not display all the agricultural qualities of the selected "mother-plant". Somaclonal variation is a recurring problem in *in vitro* cultures, particularly with somatic embryogenesis systems, which used relatively high concentrations of auxins, such as 2,4-D and IAA to induce the formation and multiplication of embryogenic cells. These growth regulators have often been shown to be implicated in the induction of somaclonal variation (Karp 1994).

Technological innovations and reassuring information on genetic conformity (1996-2001)

Over this period, two technical innovations made technological transfer economically feasible: i) mass production of pregerminated somatic embryos in temporary immersion bioreactors (Albarran 2005) and ii) the possibility of sowing them directly on horticultural substrate to achieve the regeneration of photoautotrophic plantlets in the nursery Barry-Etienne *et al.* 1999, Etienne *et al.* 2002a, Barry-Etienne *et al.* 2002b). These two technological leaps made it possible to transfer most of the late stages (germination, embryo conversion into plants) from the laboratory to the nursery and this considerably reduced production costs. The cost price per plantlet was thus estimated at 0.5 USD. It was wagered that costs could be reduced further by moving on to industrial production conditions.

At the same time, reassuring data were obtained on the genetic conformity of regenerated plants. Firstly, the frequency of somaclonal variation in the field proved relatively low (less than 3%). Secondly, the only variations observed over five years were qualitative, i.e. easily identifiable on a phenotypic level, and not quantitative. For example, the quantity of coffee produced or the amount of a given biochemical contained in the beans were not modified (Etienne and Bertrand 2001). Seven types of phenotypic variants were thus described: the Angustifolia (narrow leaves), Variegata (variegated leaf colouring) and Dwarf variants were the most frequent (Etienne and Bertrand 2003). In addition, multiplication conditions were specified for embryogenic material in cell suspensions, whereby the regeneration of somaclonal variants could be controlled. In 2001, the process (Fig. 1) was therefore considered transferrable to the industrial level, particularly as it had functioned on all nineteen of the F1 hybrids tested.

Establishing the partnership (2003)

In 1999-2000, CIRAD decided to go all the way in commercially developing this somatic embryogenesis process for large-scale multiplication of F1 hybrids, but also endeavoured to acquire useful experience for other tropical species for which application of this technology was being considered. It sought a partner interested in technological transfer for *C. arabica*. A contract was signed with the ECOM group in 2003. The Swiss group, which is a trader of quality coffees and well established in Latin America, notably in Mexico and Central America, proved to be greatly interested, as it was keen to secure its top-of-the-range coffee supplies in that zone. At the time, agronomy trials involving F1 hybrid clones were revealing their remarkable adaptation to agroforestry conditions and confirming the excellence of certain clones in sensory terms (Bertrand *et al.* 2010). The ECOM group was logically very interested, as the majority of coffee plantations in Latin America are managed as agroforests. The adoption of F1 hybrids might make it possible to increase the quantity and quality of coffee produced. The partners chose Nicaragua as the technological transfer site to disseminate Arabica hybrids throughout Central America.

However, both partners were aware of the difficulty of

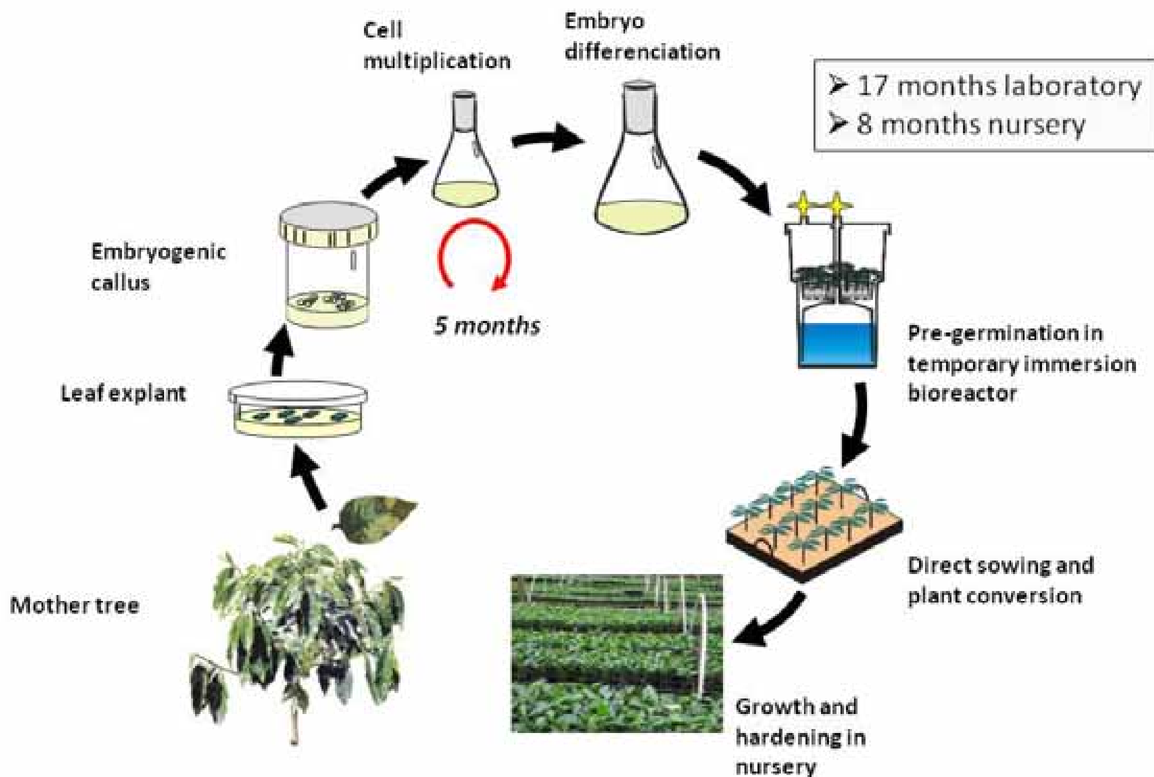

Figure 1: *Schematic representation of the industrial scale coffee somatic embryogenesis process that was transferred.*

such a technological transfer, though probably for different reasons. CIRAD focused on technical difficulties linked to the actual technological transfer itself, and the major change of scale to be achieved (increasing from an annual production of 50,000 plants to several million). For its part, ECOM's main concern was to be able to sell *in vitro* plantlets, as it was justifiably worried about the dual particularity of this new planting material: F1 hybrid and *in vitro* plantlet. Indeed, at that time, there were no known examples of coffee tree breeding programs leading to the commercial distribution of F1hybrids or *in vitro* plantlets. The market had to be created from scratch and there was likely to be a lot of hesitation on the part of coffee growers.

Construction of infrastructures and first adjustments (2004-2006)

The choice was made to construct a small operational 300 m^2 laboratory (Fig. 2), a facility small enough not to increase production costs with unavoidable expenses (fluids, work surfaces, etc.). It was also decided to locate it on the same site as a large coffee processing factory ('beneficio') at Sebaco, a small town 100 km away from the capital Managua, so that the many producers bringing their de-pulped coffee to be processed could also discover the hybrid material and familiarize themselves with this new *in vitro* propagation method. A collection of "mother-plants" (horticultural cuttings or graftings of selected hybrids) was set up near the laboratory. It was to provide the basic material required for *in vitro* propagation. Six to eight clonal copies of each selected tree were maintained at the site under stringent phytosanitary conditions to encourage plant reactivity once placed under *in vitro* conditions. Acclimatization structures were also installed near the laboratory because acclimatization, which is a very delicate stage of the process, calls for meticulous preparation and monitoring after completion of the *in vitro* phase. The acclimatization, hardening and development nurseries were installed at one farm of the ECOM group ('La Cumplida'), located near Matagalpa in the coffee growing zone 30 km from the laboratory.

Many problems were encountered over this period, preventing routine production. Firstly, on a technical level, the water used proved to be extremely hard and heavy limescale deposits covered the heating elements of the autoclaves, the stills, the bioreactors and the leaves of the mother-plants. There were numerous power-cuts which considerably hindered laboratory operations. High contamination levels were recorded during the wet season. Access to locally unavailable manufactured products proved to be a complication. The impossibility of finding staff trained in *in vitro* culture was a major difficulty and meant that the personnel had to be fully trained in the different tasks involved in production operations.

Figure 2: *Coffee in vitro propagation laboratories of Sebacco, Nicaragua (A, view of the building; B, culture room for cell suspension; C, sub-culturing room; D, culture room for bioreactors) and Xalapa, Mexico (E, view of the building; F, room for nutrient media preparation).*

Industrial production and change of scale (2007-2010)

By 2007, most of the technical problems mentioned above had been ironed out and a team of 25 people, eleven of whom were working in the laboratory, had been trained and organized. Several people were trained for each specialized job (medium preparation, autoclaving, preparing cell suspensions, data reporting, acclimatization, etc.), so that there was a replacement for anyone leaving their post. Production started at the beginning of 2007 and rose steadily over 3 years: 30,000 plants sold in 2007, 280,000 in 2008, 650,000 in 2009, and 1,300,000 so far in 2010 with a forecast of 2,500,000 plants by the end of 2011. Eventually, the production target for this laboratory is 5 million plants per year, without any modifications or additional facilities. As we shall see later, it will be possible to achieve this increase in

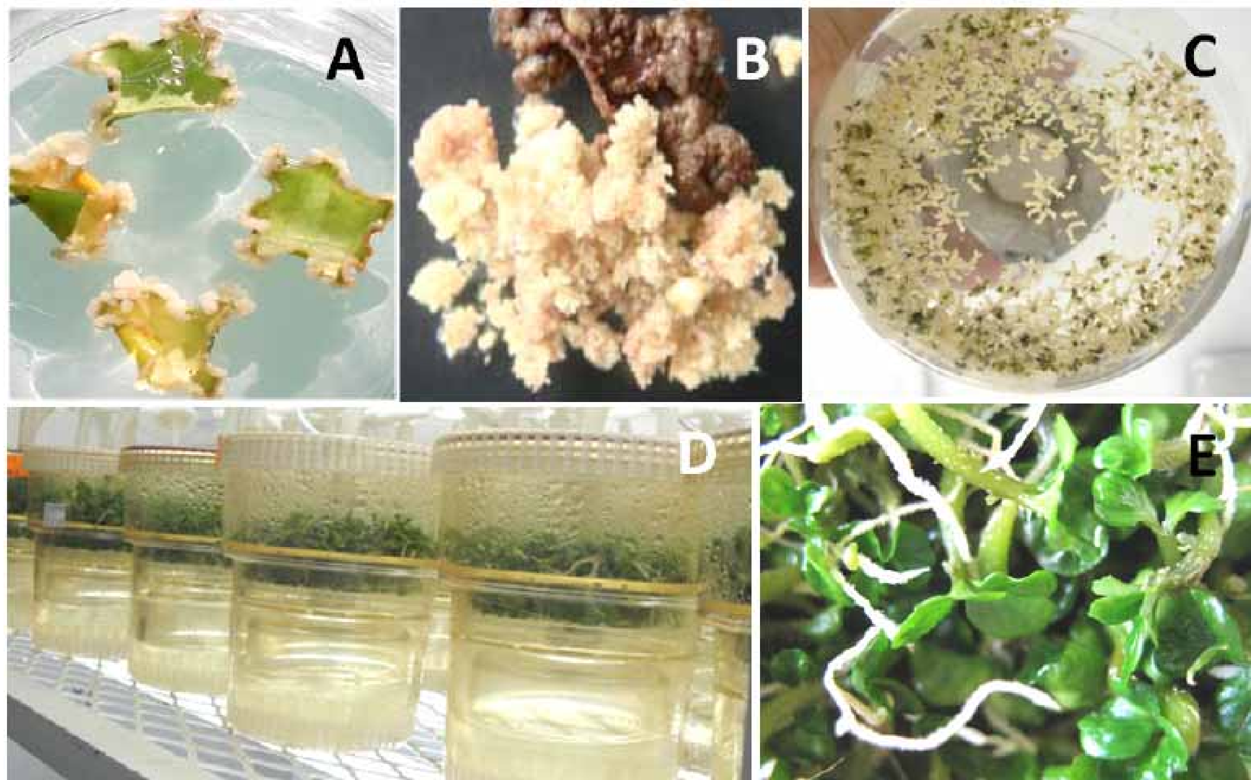

Figure 3: *Development stages of coffee tissues during the in vitro steps of the somatic embryogenesis process. A, leaf explants after one month in culture ; B, embryogenic callus regenerated 8 months after in vitro introduction; C, somatic embryo differentiation in Erlenmeyer flasks ; D, embryo pre-germination in 1 L-RITA® temporary-immersion bioreactors ; E, pre-germinated embryos ready for nursery transfer*

production by optimizing the process. Around ten F1 hybrids were cloned and used to establish a network of several hundred thousand plants grown under agroforestry conditions in Meso-America and Mexico. The first pre-industrial output provided an opportunity to test each stage of the somatic embryogenesis process, identify trouble spots and implement major optimizations. This experience is detailed below, stage by stage.

Industrial feasibility of the different stages of the process

The different stages of the somatic embryogenesis process are diagrammatically represented in Figure 1. Cloning, from the culturing of leaf fragments to the production of plants transferrable to the field, takes 2 years, of which 8 months are spent in the nursery.

Commercial production was launched once the genetic conformity of all the mother-plants had been checked by microsatellite molecular markers (SSR). Three mother trees did not conform to the expected genotype and were discarded.

- *Embryogenic tissue production*. This stage does not raise any problems in Arabica, apart from the fact that it is relatively long (8-10 months). All the explants react by producing a primary scar callus (Fig. 3A) and between 10 and 40% of them, depending on the genotype, produce a secondary embryogenic callus (Fig. 3B). These frequencies are enough for large-scale production, particularly as the embryogenic tissues are subsequently multiplied in the form of suspended cell aggregates. A genotype effect exists but it is easily taken into account by adapting the quantities of leaf explants used.
- *Multiplication of embryogenic tissues and embryo differentiation*. These two stages are carried out in a stirred liquid medium in Erlenmeyer flasks (Fig. 2B), which drastically reduces manpower and laboratory space requirements. For example, 5 million embryos are produced annually on 4 m^2 of stirring tables. This does not raise any problems industrially, but it requires major technical know-how in relation to the other stages, particularly for initially establishing the suspensions. These stages also enable important synchronization of plant material development, which will subsequently persist and help to reduce the work involved in sorting acclimatizable embryos. Consequently, each of these stages corresponds to a single development stage, i.e. embryogenic aggregates, then fully developed embryos at the torpedo stage at the end of the differentiation stage (Fig. 3C).
- *Pre-germination of somatic embryos in bioreactors*. The scaling-up at this stage has been successfully achieved over the last 3 years; 4 million pre-germinated embryos were produced in 2010 in

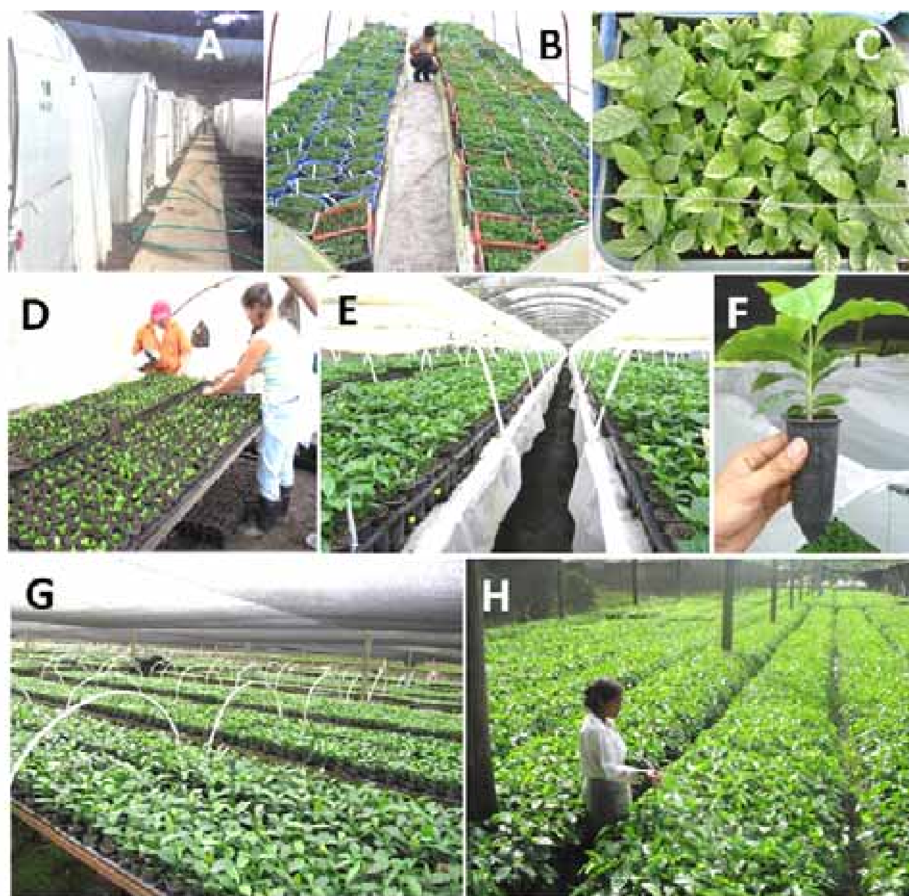

Figure 4 : *Nurseries of somatic embryo-derived coffee plants in Nicaragua. A and B, acclimatization tunnels ; C, plantlets obtained by direct sowing of pre-germinated embryos ; D and F, transfer of plantlets in 'tubetes' and hardening (E) ; G and H, growth nurseries (farm 'La Cumplida', Matagalpa, Nicaragua).*

one-litre RITA® temporary immersion bioreactors (Teisson and Alvard 1995) (Figs. 2C,D). The contents of these bioreactors had to be harvested 2 to 3 times to collect all the pre-germinated embryos (Fig. 3E) capable of continuing their development into nursery plantlets. However, we found that the volume of the bioreactors (Fig. 3D) was too small for industrial production and prevented any further change of scale by negatively affecting several production parameters. Tests with larger bioreactors (3 litres) revealed several advantages. The reduction in the total number of bioreactors led to greater efficiency, i.e. a larger number of acclimatizable embryos produced for the same amount of work involved. Fewer bioreactors also mean less investment and less cleaning work for the different constituent parts. In addition, the embryo stirring achieved in a larger volume is much more effective and makes for better synchronization during the initial germination stages. Thanks to this optimization all the embryos suitable for transfer to the nursery can be harvested in one go

- *Direct sowing of pre-germinated embryos on horticultural substrate and conversion into plantlets*. This is the trickiest stage of the process during which embryos have to be left to adapt to non-sterile *ex vitro* conditions, which are more subject to variations in temperature and relative humidity than under laboratory conditions. The transfer is made under conditions of saturated relative humidity in plastic tunnels (Fig. 4A). Somatic embryos are grown in the tunnels at high density in an inert, peat-based substrate (Fig. 4B). At the moment, this stage is an industrial bottleneck, as only 60% of embryos regenerate plantlets (Fig. 4C), on average, for all 11 genotypes propagated. Quite a strong genotype effect is found between the propagated hybrid clones. The average time taken for conversion into plants after sowing is relatively long (22 to 24 weeks) compared to seedlings (14 to 15 weeks after seed sowing). Conversion into plants is asynchronous and two to three successive harvests are needed. These observations illustrate that there is major room for improvement at this stage.
- *Growth in the nursery*. Plantlets with two to three pairs of leaves are transferred to more traditional nursery conditions where they are "hardened" to outside conditions by gradually reducing relative humidity and increasing light intensity (Figs 4D, E, G,

Figure 5. *A, 'variegata' somaclonal variant ; B, 'angustifolia' somaclonal variant ; C, Arabica F1 hybrid clone in the field ; D, transport of hybrid vitroplants to coffee growers ; E, Arabica F1 hybrid clones in agroforestry systems.*

H). This stage is well mastered and raises no problems. Although the initial growth of plantlets derived from the somatic embryos is slower and more heterogeneous than that of their seedling counterparts, they catch up by the end of the nursery phase (Fig. 4H) and prove to be even more vigorous than seedlings (Menéndez-Yuffá *et al*. 2010). Losses are minimal, amounting to around 9% of plants, which are discarded in quality checks at the end of the nursery stage. The rejects mostly consist of plants with horticultural defects (lack of vigour, curved stem, etc.); a few plants displaying early symptoms of somaclonal variations (Angustifolia (Fig. 5A) and Variegata (Fig. 5B) are also discarded, but they only amount to 0.3% of total production. The genotype effect is limited or nonexistent during the plant growth stage. The main difficulty for a change of scale at this stage was the choice of containers. If they were too big, very large volumes of horticultural substrate were needed and they took up a great deal of room in the nursery. Moreover, plant transportation to producers and planting out, were subsequently complicated, especially in the predominantly mountainous zones of Central America. A small 200 ml container, called a 'tubete', was chosen (Fig. 4F) because the laboratory has as objective to disseminate *in vitro* plantlets in an inert substrate, free of nematodes and diseases, throughout the Central American zone (Fig. 5D).

Genetic conformity of plants derived from somatic embryos

One of the expectations from this technological transfer was to obtain information about somaclonal variation. We have so far only identified morphological variations (qualitative) in *Coffea arabica* and we have demonstrated that phenotypically normal plants grow and produce normally (Etienne and Bertrand 2001,Etienne and Bertrand 2003). Some variants can be detected and discarded early in the nursery; such is the case with Angustifolia and Variegata variants. As we have seen, these variants only amount to 0.3% of total nursery production. The other phenotypic variants can only be detected at the mature stage, one or two years after planting. The first observations carried out on 100,000 plants established in 2008 in commercial plots in Nicaragua revealed a frequency of around 1% being abnormal, which is commercially perfectly acceptable. The most common abnormalities that were not detected in the nursery were dwarf variants (84%).

Apart from sorting in the nursery, various strategies have been introduced upstream to limit the occurrence of this problem. Firstly, the multiplication time for suspended embryogenic tissues has been shortened, as we

previously showed that the frequency of variation is affected by that parameter Etienne and Bertrand 2003). Likewise, multiplication is carried out in the presence of reduced levels of auxin (2,4-D). Another strategy has consisted in diluting the risk of encountering ahigh frequency of somaclonal variation by grouping together plants regenerated from different cell lines at the end of the nursery phase. To do this, it was necessary to introduce a system of traceability of production batches derived from independent cell lines.

Conclusions on technological transfer

We acquired important experience during this technology transfer. We found that it was unavoidable to move forward by trial and error, as it was impossible to foresee all the possible problems, particularly in a developing country. We learned from this experience that, in the event of problems, it is necessary to analyse these and intervene rapidly, so as to avoid production losses that can quickly become dramatic. In order to do that, it is necessary to establish faultless cohesion and communication beforehand within the team established with the partner. This technology transfer brought together partners from two different worlds, industry/private and research/public. To achieve an efficient partnership based on balanced complementarity, we had to train a joint, multidisciplinary team, which took a while. This partnership was exemplary as it demonstrated that public/private synergy can effectively work for a sustainable agricultural policy under certain conditions. Indeed, providing F1 hybrids to the farmers can serve as a catalyst for them to return to agroforestry (Fig. 5E), after they turned away from it in the 1980s for productivity reasons. At that time they favoured the less ecologically sound practices of the green revolution in vogue during that era.

The technology transfer of somatic embryogenesis is now complete for the Arabica species and demonstrates the feasibility of mass propagation by somatic embryogenesis. A substantial change of scale has been possible for each stage of the process, enabling a continuous flow of production from the laboratory to the nursery. This has given us a precise identification of the strengths and weaknesses of the technical process adopted. The commercial operation was launched in 2008 and the landmark million plants sold in a same year to Central American producers was reached in 2010. In addition, F1 hybrids have confirmed their superiority over traditional lines (Fig. 5C) and are generating such enthusiasm that demand now outstrips production capacity (2 million plants for Nicaragua alone). The aim of the partnership is therefore to respond to demand as quickly as possible through a major change of scale at the production unit in Nicaragua (5 to 6 million plants within 4 years), but also by setting up other units in the region. Indeed, a model is now available for the laboratory and nursery aspects that can be "copied" at other sites. Another production unit was established in May 2010 in the state of Veracruz, Mexico (Figs. 2E, F), to propagate other hybrids; in addition, several industrial nurseries have been established in Mexico, Guatemala and El Salvador.

Lastly, the demonstration that it is feasible to propagate *C. arabica* on a large scale by somatic embryogenesis renews the range of possibilities for this species in the field of genetic selection. Indeed, the success of this technological transfer now means we can consider introducing new varieties from hybrid or mutant materials and also the dissemination of GM varieties by somatic embryogenesis, since efficient genetic modification methods are available for this species (Ribas *et al*. 2010).

References

Bertrand, B., Alpizar, E., Llara, L., SantaCreo, R., Hidalgo, M., Quijano, J.M., Montagnon, C., Georget F & Etienne, H. 2010. Performance of Arabica F1 hybrids in agroforestry and full-sun cropping systems in comparison with pure lines varieties. *Euphytica,* in press.

Bertrand, B., Etienne, H., Cilas, C., Charrier, A. & Baradat, P. 2005. *Coffea arabica* hybrid performance for yield, fertility and bean weight. *Euphytica*. 141:255-262

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

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

Barry-Etienne, D., Bertrand, B., Schlongvoïgt, A. & Etienne, H. 2002a. The morphological variability within a population of coffee somatic embryos produced in a bioreactor affects the regeneration and the development of plants in the nursery. *Plant Cell Tiss. Org. Cult.* 68:153-162.

Barry-Etienne, D., Bertrand, B., Vásquez, N. & Etienne, H. 2002b. Comparison of somatic embryogenesis-derived coffee (*Coffea arabica* L.) plantlets regenerated *in vitro* or *ex vitro* conditions : morphological, mineral and water characteristics. *Annals of Botany*. 90:77-85

Ducos, J.P., Gibault, E. & Lambot, C. 2010. Coffee propagation by somatic embryogenesis at Nestle R&D Center-Tours. In: Proceedings of the Conference on Advances in Somatic Embryogenesis of Trees and its application for the future forests and plantations, IUFRO-KFRI 2010 conference, August19-21, 2010, Suwon, Korea.

Etienne, H. & Bertrand, B. 2001. The effect of the embryogenic cell suspension micropropagation technique on the trueness to type, field performance, bean biochemical content and cup quality of *Coffea arabica* trees. *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.

Karp, A. 1994. Origin, causes and uses of variation in plant tissue culture. In: I. Vasil and T. Thorpe, Editors, *Plant Cell and Tissue Culture*, Kluwer Academic Publishers, Dordrecht 1994, pp. 139–151.

Khaw, C.H., Ng, S.K. & Thong, K.C. 1999. Commercial production of clonal palms by tissue culture – prerequisites, constraints and issues. In: Proceedings of the 1999 PORIM International Palm Oil Congress – Agriculture. Emerging Technologies and Opportunities in the Next Millenium. Palm Oil Research Institute of Malaysia (PORIM), Kuala Lumpur, Malaysia, 1-6 February 1999.

Menéndez-Yuffá, A., Barry-Etienne, D., Bertrand, B., Georget, F. & Etienne, H. 2010. A comparative analysis of the development and quality of nursery plants derived from somatic embryogenesis and from seedlings for large-scale propagation of coffee (*Coffea arabica* L.). *Plant Cell Tiss. Org. Cult.* 102:297-307.

Ribas, A., Dechamp, E., Bertrand, B., Champion, A., Verdeil, J.L., Lashermes, P. & Etienne, H. 2010. *Agrobacterium tumefaciens*-mediated genetic transformation of *Coffea arabica* (L.) is highly enhanced by using long-term maintained embryogenic callus. *BMC Plant Biology*, accepted.

Sampote, S., Ducos, J.P., Lambot, C., Kunasol, T., Kasinkasaempong, Y., Chantanumat, P. & Pétiard, V. 2006. First industrial massive propagation of *Coffea canephora* through somatic embryogenesis organized in Thailand. In: Proceedings of the 21st International Conference on Coffee Science, Montpellier (France), 11th - 15th September 2006.

Teisson, C. & Alvard, D. 1995. A new concept of plant *in vitro* cultivation liquid medium : temporary immersion. In : M Terzi *et al*. (eds) Current Issues in Plant Molecular and Cellular Biology (pp 105-110). Kluwer Academic Publishers, Netherlands.