Temporary Immersion: A Technique for Mass Propagation of Heterozygous *Coffea spp.* Genotypes through Somatic Embryogenesis

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SUMMARY

In many countries *Coffea arabica* breeding creates and selects hybrids that combine vigour, high yields, cup quality and genes of resistance to the major diseases and pests. Somatic embryogenesis has been considered to evaluate these heterozygous genetic structures on a large scale, and later for mass propagation. Somatic embryogenesis may also replace horticultural cuttings for *Coffea canephora*. Finally this technique would allow fast establishment of clonal seed gardens. The mass propagation technique through somatic embryogenesis developed by CIRAD is based on the use of temporary immersion bioreactors and on direct sowing *ex vitro* of the embryos. This process was successfully used in various situations: a) mass propagation of selected hybrid genotypes in Central America and Tanzania, b) propagation of the progenitors of the Nemaya variety (rootstock resistant to nematodes in Central America). A large-scale multilocational trial and Nemaya seed gardens have been established in five Central American countries, totalling 50,000 plants. About 200,000 plants produced with this technique should be established in Tanzania and Central America within two years. This work allowed us to optimise the various phases of the process and to evaluate on a large scale the conformity of the plants obtained.

RÉSUMÉ

L'amélioration variétale de *Coffea arabica* s'oriente, dans de nombreux pays, vers la production et la sélection d'hybrides vigoureux, productifs, donnant un produit de qualité et possédant des gènes de résistance aux principales maladies et ravageurs.

Pour évaluer en grandeur nature ces structures hétérozygotes et les propager à grande échelle, le CIRAD a développé un procédé de propagation de masse par embryogenèse somatique basé sur l'utilisation de bio réacteurs à immersion temporaire et le semis direct des embryons *ex vitro*. Ce procédé a été utilisé avec succès dans trois situations différentes: i) multiplication des géniteurs de la variété porte-greffe 'Nemaya' tolérante aux nématodes en Amérique Centrale; ii) propagation d'hybrides sélectionnés en Amérique Centrale et en Tanzanie. 50.000 plantes – champs semenciers et réseau d'essais d'évaluation multilocale – ont été mis en place dans cinq pays centre-américains. 200.000 plantes issues de ce procédé doivent mises au champ en Amérique Centrale et en Tanzanie. Ces productions ont permis d'optimiser les différentes phases du procédé et d'évaluer à grande échelle la conformité génétique du matériel régénéré.

INTRODUCTION

Coffea arabica traditional breeding aiming at resistance, productivity and quality uses genealogic selection, and ends up with the release of fixed varieties that are multiplied and dispatched through seeds.

Recent Coffee breeding programmes (Montagnon et al., 2000; Sondhal et al., 1984), especially for *Coffea arabica* use more and more the creation and selection of vigorous, high yielding hybrids that produce quality coffee and that show some resistance to the major pests and diseases (Nyange et al., 2000).

Selected individual trees can only be multiplied and disseminated through vegetative propagation. Therefore one major objective associated with the breeding strategy is to elaborate efficient and economical processes to propagate selected individual trees of the cultivated species (Aitken-Christie and Davies, 1998; Etienne et al., 1997b; Lorenzo et al., 1998).

Cirad developped a mass propagation process using Somatic Embryogenesis based on the use of temporary immersion bioreactors (Alavard et al., 1993; Berthouly et al., 1995; Teisson et al., 1999) and direct sowing of embryos obtained.

Work and achievements presented below are the result of a multi-organisation collaboration between CIRAD and IICA/Promecafe, CATIE, Tanzania Coffee Research now TACRI. They represent two practical examples of the use of this technique.

These results allow considering new breeding strategies making an enhanced use of the genetic variability and giving faster answers to parasitic risks by developing F1 hybrids that can be easily multiplied (Etienne et al., 1997a).

MATERIAL AND METHODS

From leaf explants a highly embryogenic tissue can be easily obtained on a solid medium (Berthouly, 1996). Then this tissue can be amplified or multiplied by cell suspension (Van Boxtel and Berthouly, 1996) either in a liquid medium, or by temporary immersion (Berthouly et al., 1995; Etienne et al., 1997a); Cirad has been developing the latter technique for ten years.

This technique is used for regeneration and for germination (Photos a, b). It allows direct acclimatisation of the germinated embryos (Etienne and Berthouly, 2001; Berthouly and Etienne, 1999). Results presented below were obtained by using this technique for pilot productions of 100,000 embryos in both Central America (CATIE) and France (CIRAD), the latter for multiplying Tanzanian coffee hybrids.

In Central America Somatic Embryogenesis was used to multiply two types of material, *Coffea canephora* and *Coffea arabica.*

Coffea canephora

A hybrid variety was selected for its resistance to nematodes *Meloidogyne* and *Pratylenchus.* It is used as rootstock for *C. arabica.* T3751 (1-2) et T 3561 (2-1), the parent clones of this variety had to be multiplied rapidly in order to establish seed gardens in the regions affected by nematodes. The somatic embryos were regenerated directly from the high frequency

embryogenic callus, without amplification in liquid medium. Germinated embryos were acclimatised directly.

Coffea arabica

Many F1 hybrid progenies have been created between commercial varieties, Ethiopians accessions, and Catimor lines. Twenty high yielding individual trees were selected for yield, vigour, and cup quality. They were multiplied using somatic Embryogenesis in order to validate the technique, and to establish multilocational clone trials.

Photo a.

Photo b.

After culturing leaf explants using the method developed by CIRAD (Berthouly and Michaux-Ferrière, 1996) high frequency embryogenic callus was produced on solid medium. This callus was then

- either amplified in liquid medium by cell suspensions in erlens for 5 months. Somatic embryos were regenerated every 3 months in 600 bioreactors. The effect of the culture duration on the frequencies and types of variants could thus be studied;
- or regenerated directly without amplification, so as to limit the apparition of variants. Pre-germinated embryos obtained by this method were dispatched directly to 5 Central American countries for direct sowing, acclimatisation and hardening.

In both cases regeneration and germination (Photos a, b) took place in temporary immersion devices (RITA). The germinated embryos were transferred into simple trays for acclimatisation (Photo c) until plantlets develop. The latter were transplanted into normal nurseries for six-month hardening (Photo e).

Photo c.

Tanzania / CIRAD

Based on the selection within selected progenies of twelve hybrid trees (Table 1), leaf explants were cultured from the beginning of 1999 on Lyamungu Research Station (Tanzania), and transferred to Cirad (France) for multiplication. They belong to various, more or less complex, crosses between commercial lines susceptible to Leaf Rust and to CBD, and progenitors like Rume Sudan and Hybrid of Timor, that are resistant to either or both diseases (Kilmambo et al., 2000; Nyange et al., 2000). The transfer of fresh leaves from Tanzania to Montpellier for culturing was not successful.

After 6 months on solid medium high frequency embryogenic callus (Berthouly and Michaux-Ferrière, 1996) was amplified in liquid medium using temporary immersion (Berthouly et al.,

1995). Regeneration medium (Van Boxtel and Berthouly, 1996) was used for 2 to 3 cycles of 6 weeks. Unlike at CATIE regeneration did not use cell suspension (Van Boxtel and Berthouly, 1996).

Then the embryos were dispatched into more RITAs in order to get a density suitable for good development and germination (Photos a, b). Embryos at the right stage were then stored in sterile plastic containers at 20°C before being sent to Tanzania for acclimatisation (Photo c), and after six weeks transferred in nurseries (Photo d).

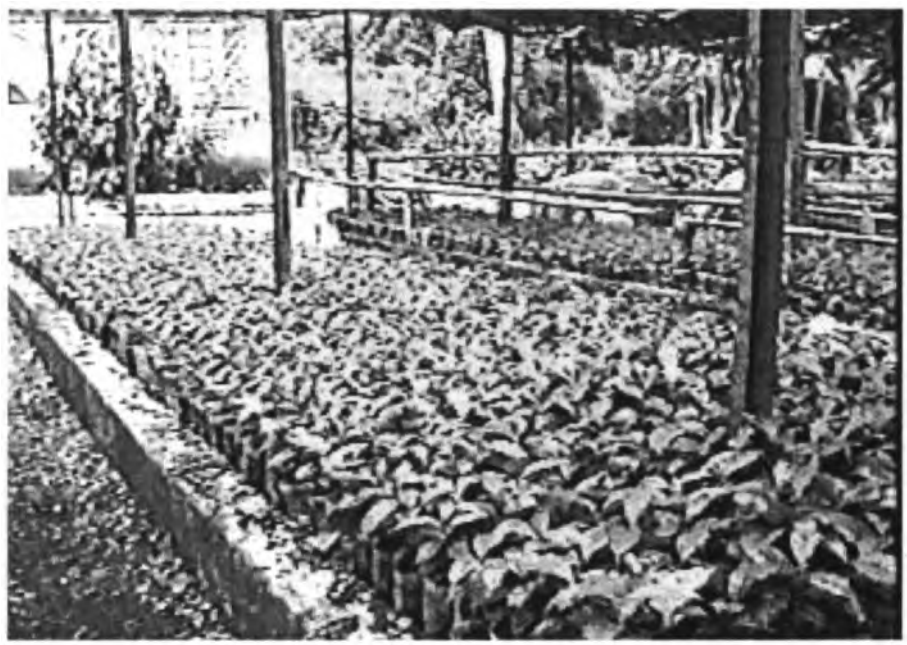

Photo d.

They were dispatched in 4 batches:

- March 2000: 43,000 germinated embryos
- July 2000: 39,000 germinated embryos
- October 2000: 8,000 germinated embryos
- February 2001: 22,000 germinated embryos

These various batches will allow to study the effect of the duration of the culture on somaclonal variation.

RESULTS

Central America

C. canephora

Over 20,000 plants of the parent clones of the hybrid variety have been produced. No variants have been detected up to now. The plants are being established in seed gardens in various countries in Central America.

C. arabica

Somaclonal variation was assessed on these plants issued from cell suspensions in a trial field (Photo f). Indeed it is well known that the culture duration influences the apparition, type (Photo g) and proportions of variants.

The types of variants observed in this material are described in Table 2 show some of the variants.

Table 1. Mother trees used for multiplication, Tanzania

Clone	Yield kg gb/ha (1)	Availability of leaves (2)	Stem Diameter (mm)	Parentage
1	1630	++	108	(N39 x Hdt) x RS
2	2170	+	125	(N39 x Hdt) x RS
3	1988	++	115	Kaffa x ('N39 x Geisha) x Hdt)
4	2464	+++	117	(N39 x kaffa) x (RS x Hdt)
5	1963	+++	110	(N39 x kaffa) x (RS x Hdt)
6	1979	++	107	((N39 x OP729)xHdt) x (H66 x Hdt)
7	1973	+++	118	((N39 x OP729)xHdt) x (H66 x Hdt)
8	2020	+++	129	(N39 x Hdt) x (N39x(N39 x Geisha) x Hdt)
9	2078	++	99	N39 x Hdt
10	2020	+	N/A	H66 x Hdt
11	2468	+	126	N39 x RS
12	2020	++	N/A	KP423 x Hdt

(1)Average yield (5 years) of the mother tree
(2)Presence of leaves at the right stage for culturing explants

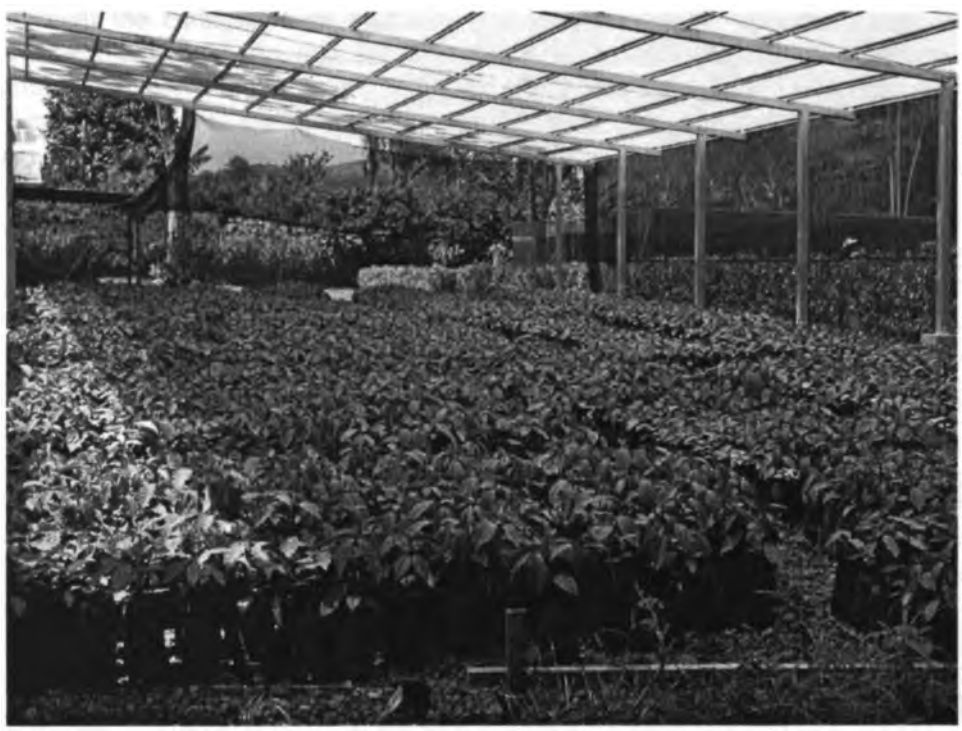

Photo e.

Table 2. Description of variant types observed

Variants types	Phenotypes	Specific weight of leaves $g^{-3} \times m^{-4}$	Stomates density No x m^{-6}	No. of chloroplasts/ Guard Cell
Thick Leaves	Thick leaves Large fruits Star flowers	9,5-10,3 (6,4-9,7)	176-199 (164,3-191,6)	16,4-20,3 (15,3-15,8)
Dwarf	Dwarf Small leaves Small fruit Low viguor	5,8-8,6 (8,7-9,7)	171-277 (172-192)	13,23-14,93 (15,8-16,97)
Dwarf with peaberries	Dwarf Small leaves Small fruit Low vigour >50% peaberries	6,4 (6,4-9,7)	278 (164-191,6)	13,8 (15,3-15,8)

However the results in Table 3 indicate that, although it may depend on the genotype, the percentage of variants as a whole is low.

Also, 100,000 plants have been produced using another process. High frequency callus was produced on solid medium (3) but regenerated directly by temporary immersion without the preliminary amplification phase, in order to minimise the apparition of variants. These plants will be established this year in multilocational trials for final selection. The effect of this process on the rate of variants will be assessed.

Tanzania

At Lyamungu the embryos were transferred to plastic trays for acclimatisation. Various substrates were tested at Lyamungu for acclimatisation; the best results were obtained using a 2:1 mixture of forest soil/rice husks. After six weeks normally plants with 2 to 3 pairs of leaves were transferred to the nursery. Some delays were experienced during the cold season, and fungus attacks were responsible for some mortality.

We expect these plants to be established in 2001 as a large-scale multilocational trial in Arusha and Kilimanjaro regions. This will allow us to assess their conformity thus the somaclonal variation but also their adaptation to various agro-ecological conditions and to confirm their resistance to CBD and to Leaf Rust.

Table 3. Types of variants and their frequency within plants derived from four *C. arabica* hybrid genotypes

Genotype (Clone)	Number of plants	Thick leaf	Dwarf	Dwarf/ Pea berries	Total frequency
1	199	1	2	0	0,5
2	136	0	1	0	0,7
3	161	8	1	1	6,2
4	148	0	0	0	0
Total	644	9	4	1	2,1

Photo f.

CONCLUSION AND PERSPECTIVES

One should notice that this technique, elaborated thanks to partnership between Cirad, Central America and Tanzania, might be applied to both cultivated coffee species, *C. arabica* and *C. canephora*, and to various genotypes. The results of the two pilot projects presented here confirm the feasibility and the industrial potential of this process. Indeed 100,000 germinated embryos could be produced in quite different conditions, Central America versus East Africa/France.

Software was developed in order to calculate production costs. It will allow the identification and optimisation of the bottleneck phases of the process.

Assessment of somaclonal variation goes on in Central America and should start soon in Tanzania before and after the plants are established in the field.

The next steps towards the complete validation of the technique for its use at industrial stage are:

- perfect control of somaclonal variation (less than 5%);
- development of routine industrial production;
- commercial valorisation of the hybrids in the middle term.

Photo g.

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