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STUDIES ON GENETIC TRANSFORMATION OF COFFEE BY USING ELECTROPORATION AND THE BIOLISTIC METHOD

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op gezag van de rector magnificus,
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Abstract

The present study aimed simultaneously at an improvement of coffee regeneration systems and at a definition of factors influencing the efficiency of direct gene transfer methods. The development of an improved regeneration system, based on high frequency somatic embryogenesis from leaf explants, passing through multiplication of embryogenic callus in liquid medium, is described. This method can contribute to the obtaining of high protoplast yields and offers perspectives for use in genetic transformation systems of coffee. Several factors affecting protoplast isolation, electroporation and regeneration were studied. This system appeared to be appropriate for transient expression studies but, due to difficulties with protoplast regeneration, less promising for achieving stable expression. Further expression studies were performed using particle gun bombardment on different tissues of several coffee genotypes. Best results were obtained using *in vitro* cultured leaves of *Coffea arabica* and plasmids carrying the EF1α-A1 promoter of *Arabidopsis thaliana*. The effect of tungsten particles on callus induction and the fate of GUS-expressing cells after bombardement on leaves has been described, and the consequences for their use are discussed. Studies on five selective agents showed best prospects of the herbicide glufosinate for detection of stably transformed coffee tissue. It was concluded that avoidance of polyphenolic oxidation, caused by tissue wounding, is of great importance for the development of a reliable genetic transformation method for coffee.

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Prologue

This thesis is the result of research carried out between May 1990 and January 1994 in the BIOTROP laboratory, which is part of the "Centre de Coopération Internationale de Recherche Agronomique pour le Développement" (CIRAD) in Montpellier, France. During three years this work was financed by the CIRAD department "Cultures Pérennes" (CP). Further 6-months financial support was provided by the French ministery of Foreign Affairs. I am grateful to the association "Landbouw Export Bureau 1916-1918" for their financial support which amongst others, allowed the publication of this thesis. Besides my appreciation for these financial contributions, I would like to thank all personal contributions of people who where essential for the progress of this work and for my 4½ year living in Montpellier.

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List of abbreviations

BA	6-benzylaminopurine
Bt	*Bacillus thuringiensis*
pCaMV-E35S	cauliflower mosaic virus enhanced 35S promoter
CBD	coffee berry disease
CIRAD	Centre de coopération internationale en recherche agronomique pour le développement
EDTA	ethylenediaminetetraacetic acid
EF1α	elongation factor 1α
FDA	fluorecein diacetate
GGP	greenhouse grown plants
GUS	β-glucuronidase
HFSE	high frequency somatic embryogenesis
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IRCC	Institut de recherches du café, du cacao et autres plantes stimulantes
KIN	kinetin
LFSE	low frequency somatic embryogenesis
MES	2-(N-morpholino)-ethanesulfonic acid
MR	multiplication rate
MS	Murashige & Skoog
MUG	4-methylumbelliferyl β-D-glucuronide
NBB	naphthol blue black (protein specific coloration)
OP	open pollinated progeny
PAS	periodic acid - Schiff (polysaccharid specific coloration)
PCR	poly chain reaction
PCV	packed cell volume
PEG	polyethylene glycol
PVP	polyvinylpyrrolidone
rpm	rotations per minute
SCSE	self controlled somatic embryogenesis
SE	somatic embryogenesis (in general)
SEM	somatic embryo-derived microcuttings
TEM	transmission electron microscopy
UBQ	ubiquitine
X-Gluc	5-bromo-3-chloro-3-indolyl-β-D-glucuronic acid
2,4-D	2,4-dichlorophenoxyacetic acid
2-iP	6-(γ,γ-dimetylallylamino)-purine

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STELLINGEN

1. Op basis van de waarneming dat de EF1α-A1 promoter goed tot expressie komt in de koffieplant, zou een selectiegen ontwikkeld kunnen worden dat optimaal functioneert in deze plant.

Dit proefschrift.

2. Zowel regeneratie via somatische embryogenese, als transiente expressie van GUS-genen, geïntroduceerd m.b.v. een "particle gun", zijn bij koffie genotype-afhankelijk

Dit proefschrift.

3. Polyfenolische afweerstoffen in de koffieplant zijn niet alleen hinderlijk voor pathogenen, maar ook voor ingrepen door de mens die tot genetische modificatie moeten leiden.

Dit proefschrift.

4. Voor de genetische modificatie van koffie moet uitgekeken worden naar een alternatief voor het detectiegen β-glucuronidase.

Dit proefschrift.

5. Het verdient aanbeveling om een resistentiegen, gebaseerd op Bt-toxine en gericht tegen bladvretende insekten, niet tot expressie te laten komen in de koffieboon. Dit is mogelijk door het kiezen van een weefsel-specifieke promoter.

6. De moeite die besteed wordt aan het produceren van goede koffiebonen staat vaak in geen verhouding met de kwaliteit van de ervan bereide koffie.

7. De kwaliteit van het gemiddelde Franse fietspad doet vermoeden dat de aanleggers ervan zich per auto naar hun werk begeven.

8. De Franse genegenheid voor Nederlanders is omgekeerd evenredig aan de hoeveelheid meegebrachte conservenblikken in hun caravan.

9. Hondenpoep houdt mensen lenig.

10. Het aannemen van de prijs van een" BigMac" als norm voor de levenstandaard van een land, leidt tot normvervaging.

Het Parool, 12 september 1994.

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Table 2: Somatic embryogenesis procedures developed for coffee

Author(s)	species	starting material	SE procedure	medium	light cond.	growth regulators ($mg.l^{-1}$)	obtained morphology	time (weeks)	total time to plantlet	remarks
Staritsky 1970	*C.canephora*	young internodes	direct	1.solid	dark light	KIN 0.1 + 2,4-D 0.1	embryos plantlets	n.s. n.s.	n.s.	first successful SE experiments
Sharp *et al.* 1973	*C.arabica* cv M.N. + B.V.	orthotrop. shoots	indirect	1.solid 2.solid	dark n.s.	KIN 0.1 + 2,4-D 0.1 KIN 0.1	callus plantlets	n.s. n.s.	n.s.	also reaction of leaves and anthers
Herman & Haas 1975	*C.arabica*	leaves	direct	1.solid 2.solid	dark light	KIN 0.1 + 2,4-D 0.1 NAA 0.1	organoïds plantlets plants	9 17 13	26	first plant regeneration
Söndahl & Sharp 1977	*C.arabica* var. Bourbon	leaves	LFSE1	1.solid 2.solid 3.solid	dark low low	KIN 4 + 2,4-D 1 idem KIN 0.5 + NAA 0.05	pro-embryos embryos plantlets	6-10 4 6-12	21	low, but rapid embryo production
			LFSE2	1.solid 2.solid	dark low	KIN 2 + NAA 8 KIN 0.5 + NAA 0.1	pro-embryos plantlets	14 5	19	risk of adventitious root formation
			HFSE	1.solid 2.solid 3.solid 4.solid	dark low low light	KIN 4 + 2,4-D 1 idem KIN 0.5 + NAA 0.05 hormone-free	prim.callus sec.callus pro-embryos plantlets	6-10 4 13-16 10	35	friable embryogenic callus, leading to high embryo production
Crocomo *et al.* 1979	*C.arabica* cv Catuaí	internodes	direct	1.solid 2.solid	dark low	KIN 0.05 + 2,4-D 0.075 KIN 0.5	pro-embryos shoots	7 4	n.s.	relation callus morphol.-embryo formation
Staritsky & van Hasselt 1980	*C.canephora*	internodes	indirect	1.solid 2.liquid 3.solid	dark dark light	BA 1 + IBA 5 BA 5 + IBA 1 hormone-free	callus embryos plantlets	6-8 8-10 n.s.	n.s.	indirect, but LFSE; attempts in liquid medium
Dublin 1980	Arabusta	internodes	indirect	1.solid 2.solid 3.solid	light light light	IAA 0.5 BA 1 KIN 0.1 + IAA 0.5	callus embryos plantlets	4-6 8-10 10	24	
Lanaud 1981	*C.canephora*	ovules + antheres	indirect	1.solid 2.solid 3.solid 4.solid	dark light light light	2,4 D 0.2 + NAA 1 idem hormone-free BA 0.1 + IAA 0.5	pro-embryos plantlets plants	1 18 13 22	32	haploid production not achieved
Dublin 1981	Arabusta	leaves	direct	1.solid 2.solid	light light	BA 1-10 KIN 0.1 + IAA 0.5	embryos plantlets	13 8	21	
			LFSE	1.solid 2.solid 3.solid 4.solid 5.solid	dark light light light light	2,4-D 0.01-1 BA 1-10 KIN 0.1 + IAA 0.5 KIN 1 + IAA 1.5 + IBA 0.5 KIN 1	pr.callus sec.callus embryos plantlets plants	4-6 8-10 10 n.s. 8-10	32	
Pierson *et al.* 1983	*C.canephora*	leaves	LFSE	1.solid 2.solid	dark n.s.	2-iP 1 + IBA 5 n.s.	pr.callus sec.callus	7 15	n.o.	histolog. study LFSE-callus formation
de Pena 1983	*C.arabica* cv Mundo Novo	leaves	indirect	1.solid 2.liquid 3.solid	dark dark light	KIN 8 + 2,4-D 1 NAA 0.8 idem	callus embryos plantlets	13 11 4	28	plantlet production in liquid medium
				1.solid	dark	KIN 1-2 + 2,4-D 0.1-0.5	callus	4-6	n.s.	no HFSE-callus

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Dublin 1984	*C.arabica* + Arabusta	internodes leaves ovules	indirect	1.solid 2.solid 3.solid 4.solid	dark low light light	BA 1-3 BA 1-5 KIN 1 + IAA 1.5 + IBA 0.5	embryos plantlets plants	13 n.s. n.s.		obtained
Yasuda *et al.* 1985	*C.arabica*	leaves	direct	1.solid 2.solid	light light	BA 1.125 hormone-free	pro-embryos embryos plantlets	16 4 n.s.	n.s.	no auxin used
de Garcia & Menendez 1987	*C.arabica* cv Catimor	leaves	direct	1.solid	light	2-iP 1 + IBA 5	plantlets	n.s.	n.s.	
			indirect	1.solid 2.solid	dark low	BA 8 + 2,4-D 1 NAA 0.3	embryos plantlets	14 n.s.	n.s.	
Hatanaka *et al.* 1991	*C.canephora*	leaves	direct	1.solid 2.solid	light light	2-iP 1 n.s.	pro-embryos plantlets	9 n.s.	n.s.	no auxin used; high germination level
Zamarripa *et al.* 1991	*C.canephora* *C.arabica* cv Catuai + Caturra Arabusta	leaves	indirect	1.solid 2.solid 3.liquid 4.liquid 5.solid 6.solid	dark low low low light light	KIN 1.5 + 2,4-D 0.3 BA 1 idem BA 5 BA 0.225 hormone-free	pr.callus sec.callus embryog.mass embryos shoots plantlets	9 13 35 8 4 7	70	callus not of HFSE-type; long multipl. phase in liq. medium; very high embryo production in bio-reactor
Neuenschwander & Baumann 1992	*C.arabica*	leaves	HFSE	1.solid 2.liquid 3.liquid 4.liquid 5.solid	dark dark dark low light	KIN 4 + 2,4-D 1 idem KIN 0.5 + NAA 0.05 ABA 2.6 KIN 0.05	pr.callus callus pro-embryos embryos plantlets	7 4 12 5 5	33	improvements of maturation of HFSE procedure and conversion rate
			SCSE	1.solid 2.liquid 3.solid	dark dark light	KIN 4 + 2,4-D 1 KIN 0.5 + NAA 0.05 KIN 0.05	callus embryos plantlets	7 18 5	30	exact mechanism of embryo formation unknown
Marques 1993	*C.eugenioïdes*	leaves	direct	1.solid 2.solid	dark dark	KIN 4.3 + 2,4-D 1.1 KIN 1 + NAA 0.1	callus embryos	6 13	n.o.	
Noriega & Söndahl 1993	*C.arabica* cv Red Catuai	leaves	HFSE	1.solid 2.solid 3.liquid 4.liquid 5.liquid 6.solid	dark dark dark dark n.s. n.s.	KIN 4 + 2,4-D 1 KIN 1 + NAA 0.1 idem KIN 5-15 + ABA 0.3-3 ABA 0.1-0.8 Z 0.1-1 + ABA 0.01-0.1	prim.callus sec.callus embryog.mass embryos shoots plantlets	6-8 22 10-12 9 5 10	63	high embryo production in bio-reactor; moderate conversion rate
Aponte Acuna 1993	*C.arabica* cv Caturra x H.T.	leaves	direct	1.solid 2.n.s.	dark light	2-iP 1 n.s.	embryos plantlets	9 n.s.	n.s.	
Ramos *et al.* 1993	*C.canephora*	leaves	direct	1.solid	light	KIN 10.8 + Z 2.2	callus embryos	4 9	n.o.	
This work	*C.arabica* cv Catuai *C.canephora* Arabusta Congusta	leaves	HFSE	1.solid 2.solid 3.liquid 4.liquid 5.solid 6.solid	dark low low low light light	2-iP 2 + IBA 1 + 2,4-D 0.5 BA 4 + 2,4-D 1 KIN 1 + 2,4-D 1 BA 1-5 BA 0.25 + IAA 0.45 BA 0.3	prim.callus sec.callus embryog.mass embryos plantlets plants	4 8-14 8 8-10 6-8 9	36	high production of HFSE-callus is genotype dependent

LFSE: Low Frequency Somatic Embryogenesis
HFSE: High Frequency Somatic Embryogenesis
SCSE: Self-Controlled Somatic Embryogenesis
SE: Somatic Embryogenesis

B.V.: Bourbon Vermelho
H.T.: Hybrid de Timor
M.N.: Mundo Novo

KIN: kinetin
2,4-D: 2,4-dichlorophenoxyacetic acid
BA: 6-benzylaminopurine
NAA: naphtalene-acetic acid
2-iP: 2-isopentenyladenin
Z: zeatin
IAA: indole-3-acetic acid
IBA: indole-3-butyric acid

n.s.: not specified
n.o.: not obtained

CHAPTER 1

General introduction

1. Coffee culture

More than 70 coffee species are known, belonging to the genus *Coffea* which is part of the large family of Rubiaceae. Commercially, *C. arabica* and *C. canephora* are the main species (Coste 1989). The beverage with stimulating effect is prepared from the roasted beans. Arabica coffee is more aromatic, sometimes acid and with less caffeine, while *C. canephora* (commercially called Robusta) gives a stronger and less aromated drink, often bitter and caffeine rich (Leroy 1993). Coffee is one of the most important products in the international world trade. Thus, coffee growing is a very important agricultural occupation, upon which more than 50 countries are more or less dependent for their economy, especially for foreign exchange (Kushalappa & Eskes 1989). Arabica coffee, mainly produced in South and Central America, is responsible for 75% of the worlds coffee consumption and Robusta, producted in Brazil, Africa and Asia, for about 25% (Coste 1989). Actually, the principal coffee producers are Brazil with about 1,500,000 tonnes of green coffee produced annually (of which 20% is Robusta), Colombia with 980,000 tonnes (Arabica), Indonesia with 480,000 tonnes (mainly Robusta), Mexico with 310,000 tonnes (Arabica) and Côte d'Ivoire with 240,000 tonnes (Robusta) (Leroy 1993).

2. Coffee breeding

In spite of the economic importance of coffee, relatively few research centers are working on coffee genetics and breeding. The main coffee breeding projects are being conducted in Brazil, Colombia, India, Kenya and Central America. Two French research institutes are involved. The Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) is engaged in more applied research, by supporting breeding projects in different countries. The Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM) is responsible for collections of genetic resources and carries out work concerning the systematics and evolution of *Coffea* (Carvalho 1985). With increasing consciousness since the 1960s of the risks of genetic erosion, both institutes have given attention to the establishment of germplasm collections. The main field collections are in Madagascar (local *Coffea* species), Côte d'Ivoire (mainly diploïd *Coffea* species) and Ethiopia (*C. arabica*). The coffee research centers have developed high-yielding coffee cultivars, which are being extensively cultivated. Being a perennial crop, the importance of the development of well adapted coffee cultivars with durable resistance to major pests and diseases, is even more obvious than in the case of annual crops (Carvalho 1985).

2.1 *C. arabica*

Origin. *C. arabica* L., which center of origin is Ethiopia, is a predominantly self-pollinating allotetraploïd (2n=4x=44). Some cross-pollination due to transport by wind and insects is responsible for maintenance of heterozygozity. Average cross-pollination in plantations has been assessed to be about 10%. Arabica's allotetraploid origin implies a relatively narrow gene base in descendant lines of the cultivated varieties. Most of the highly productive cultivars as Mundo Novo, Caturra and Catuai, selected in Brazil and Central America, originate from a small genetic basis (mainly from some trees introduced from Yemen in the 17th century).

Breeding criteria. The majority of commercial cultivars is susceptible to the main coffee pests and diseases, which limits production in many regions. Therefore, most breeding efforts are actually directed to resistance. Coffee leaf rust (caused by *Hemileia vastatrix*), CBD, coffee berry disease (*Colletotrichum coffeanum*) and nematodes (*Meloidogyne incognita* and others) are most destructive. With regard to insects, arabica is most seriously attacked by lepidopterous larvae of which leaf miners are the most widespread: *Perileucoptera coffeella* in Central and South America, *P. meyricki* in Africa (Mitchell 1985). Recently, some cultivars have been bred with resistance to rust or nematodes, but so far no variety with resistance to pests is available. Effective resistance to rust, CBD, nematodes and to the leaf miner is found in wild species and its transfer to arabica is possible (Carvalho 1985). However, taken in account the 5 year duration of one breeding cycle, this transfer takes at least 25-35 years when classical breeding methods are applied.

Because of the desired adaptation to manual and mechanical harvest, dwarfness is another important selection criterion in breeding programs. Compact stature varieties have been developed by crossing dwarf mutants with cultivars. Some other selection criteria of potential interest are herbicide resistance, drought tolerance, cold tolerance, short maturation cycle and varying levels of caffeine content.

The awareness that intraspecific arabica F1 hybrids can be superiour in vigour and yield has stimulated breeding programs for this goal. High producing cultivars should be crossed with wild arabica species bearing multiple disease resistance (Eskes 1989). Production of intraspecific F1 hybrids has already resulted in cultivars with both resitance to CBD and rust, like Ruiru 11 in Kenya. Multiplication of F1 hybrids can be carried out vegetatively or by F1 seed production. Since arabica is a self-pollinator, programs aimed at F1 seed production are very laborious, as it demands large scale emasculation and controlled pollination: prevention of self-fertilization is a key requirement in F1 hybrid seed production. Hybrid seed production would be more easily achieved if an appropiate type of genetic male sterility would be available in mother trees of commercial arabica cultivars (Kushalappa & Eskes 1989). Male sterility as a natural phenomenon is occurring sporadically: some male sterile arabica plants have been found after screening of several thousands of plants in Brazil (Mazzafera *et*

al. 1989).

Breeding methods. In the traditional pedigree method of selection, elite mother trees are evaluated by replicated progeny tests. Successfull progenies should maintain a high yield over 6-8 years. For the backcross method of selection, high yielding F2 or F3 plants from artificial hybridization programmes are backcrossed with cultivars, in order to isolate new high-yielding lines with desired characteristics. Natural haploids might be used to develop pure lines after doubling of chromosome number and subsequent use for artificial crosses between selected parent types. This method is not yet applied to arabica as very few natural haploids are found; anther culture is actually tried as alternative. Doubled forms of canephora have been crossed with arabica to create interspecific hybrids, called Arabusta (Capot 1972).

2.2 *C. canephora*

Origin. *C. canephora* PIERRE is a self-incompatible diploïd species (2n=2x=22). Cross-pollination has resulted in polymorphic populations of highly heterozygous individuals. Two genetic groups of wild canephora genotypes can be distinguished: the Congolese type, originating from Central Africa, and the Guinean type from West Africa. F1 hybrids between these types have shown to be generally more vigorous and productive, than hybrids between clones of the same group (Leroy *et al.* 1993).

Breeding criteria. Most important selection criteria in canephora breeding programs are yield, moderate vigour, quality and resistance to pests and diseases. Major pests of canephora are the coffee berry borer (*Hypothenemus hampei*), several species of twig and stem borers and the tailed caterpillar (*Epicampoptera* sp.) (Snoeck 1985). So far practically no breeding efforts for insect resistance have been made (Charrier & Berthaud 1985). Canephora is used in interspecific breeding programs for introgression into arabica of genes confering resistance to coffee leaf rust, nematodes and CBD.

Breeding methods. Mass selection in populations, combined with vegetative propagation, is mostly applied for obtaining superiour clones. Clonal selection is made among plantations and collections, and the best performing trees are subsequently chosen in comparitive trials. Production of synthetic varieties is performed by planting together several plants possessing good general combining ability. Hybrid varieties are created in biparental fields consisting of parents with good specific combining ability. Reciprocal recurrent selection is applied to intergroup crosses between the Guinean and Congolese gene pools. Long-term progress can be achieved by this method by increasing the frequency of favorable alleles in the base populations. Since the first report by Dublin & Parvais (1975) of spontaneous development of haploid embryos in canephora, a method of doubled haploid (DH) production has been developed (Lashermes *et al.* 1993). Whether or not hybridization of these homozygous DH with arabica may result in homogenous hybrids, requires additional research.

2.3 Interspecific crosses

With the objective to combine the qualities of each species, crosses were made between arabica (cup quality) and canephora (disease resistance). Commercial testing has been performed with F1 tetraploïd hybrids and a few derivatives of backcross populations, have resulted in tetraploïd or hexaploïd Arabusta (Capot 1972), and the backcross populations Icatu and Catimor. Selected Arabusta hybrids are multiplied vegetatively, whereas several generations of selfing have been applied to the backcross populations in order to obtain homogeneous lines.

The transfer of resistance genes, present in wild coffee species, to selected arabica cultivars constitutes one of the main objectives in all current coffee breeding programs.

2.4 Conclusions

It might be concluded that conventional coffee breeding is a long term effort, taking 25-35 years for new variety production. Efforts to introduce genes by interspecific crosses are partially hindered by sexual barriers (hybrid sterility due to ploïdy level) and require many cycles of selfing and backcrossing to eliminate deleterious genes and gene combinations (Söndahl 1985b). The long cycle of traditional breeding and important advances acquired during the last 25 years in *in vitro* culture techniques of coffee, make the crop suitable for other approaches, like genetic transformation.

3. Genetic transformation: history and methods

The application of biotechnology has created rapid developments in agriculture. Biotechnology uses the technical approaches of molecular biology and plant cell culture systems to develop commercial products. With the introduction of genetic engineering techniques, based on knowledge of gene structure and function, plant breeding methodologies and objectives can be broadened (Kung 1993).

It was only in 1983 that scientists inserted the first foreign genes into plants by use of *A. tumefaciens* vectors (Herrera-Estrella *et al.* 1983; Fraley *et al.* 1983; Bevan *et al.* 1983). In the early experiments protoplasts were used as recipient, later on Horsch *et al.* (1985) developed a method based on explants, such as leaf and stem explants, in which high transformation capacity was combined with good regeneration capacity. Since this breakthrough, several important traits in crops might be considered as targets for application (like insect, virus and herbicide resistance, and modification of seed storage products).

Studies for DNA transfer by fusion of protoplasts had resulted in the development of the so-called somatic hybridization technique. Many attempts were

made to establish fusions between protoplasts of phylogenetically unrelated species (interfamiliar), but only a restricted number of attempts succeeded (Dudits *et al.* 1987; Kisaka & Kameya 1994). Ramulu *et al.* (1994) reported gene transfer by means of fusion of potato microprotoplasts, containing only one chromosome, with tobacco protoplasts.

Direct gene transfer concerns the methods of gene introduction without involving any vector. It avoids the need of introduction of cloned DNA into the T-DNA of the *A. tumefaciens*-Ti plasmid or its equivalent, before insertion into the plant. Especially the development of uptake of naked DNA by electroporation (since 1985) and by particle gun bombardment (since 1987) have seen a large progress during the last years. In Table 1a and 1b, the main results obtained with several methods of direct gene transfer into protoplasts, cells and whole tissue are summarized.

The methods based on the use of protoplasts, which have resulted in the obtainment of stably transformed plants are PEG/high pH, liposomes fusion and electroporation. These methods may have an advantage for species which are not infected by *Agrobacterium*, like some monocots. Since application of the methods influences negatively protoplast viability, high protoplast regeneration capacity is an absolute requirement for successfull transformation.

Micro-injection, particle gun bombardment and tissue electroporation are the methods based on the use of intact tissue, having resulted in stably transformed plants. These methods may have an advantage over the former, in case of non-susceptibility to *Agrobacterium* or absence of critical protoplast regeneration capacity.

4. Coffee biotechnology

4.1 *In vitro* culture techniques

Nowadays, *in vitro* culture techniques are being applied to many agricultural crops. Main objective is usually mass multiplication of heterozygous genotypes by using tissue culture methods as axillary and adventitious budding, or somatic embryogenesis from leaf or stem explants, passing by callus or cell suspension cultures. These methods permit the production of uniform plants on a mass scale, in a far shorter period than is possible by the conventional multiplication methods. At the same time, a perfect regeneration method is a first requisite for application of genetic transformation procedures, either for indirect or direct transfer of isolated valuable genes into cultivated plants. Some *in vitro* culture techniques developed with coffee will be described.

Table 1: Methods of direct gene transfer to cultivated plants and pioneer results obtained

a: gene transfer into protoplasts

method	species	TE[a]	TP[b]	TD[c]	author(s)
PEG/high pH	*N. tabacum*	x	x		Paszkowski *et al.* 1984
	O. sativa	x	x	x	Zhang & Wu 1988
liposomes fusion	*N. tabacum*	x			Deshayes *et al.* 1985
	N. tabacum	x	x		Bellini *et al.* 1989
	N. tabacum	x	x	x	Spörlein & Koop 1991
electroporation	*Z. mays*	x			Fromm *et al.* 1985
	D. carota	x	x		Langridge *et al.* 1985
	O. sativa	x	x	x	Shimamoto *et al.* 1989
electroporation/PEG	*N. tabacum*	x			Shillito *et al.* 1985
	D. glomerata	x	x		Horn *et al.* 1988
micro-injection	*P. hybrida*	x			Griesbach 1983
	N. tabacum	x	x		Crossway *et al.* 1986
sonication	*B. vulgaris*	x			Joersbo & Brunstedt 1990
	N. tabacum	x	x		Zhang *et al.* 1991

b: gene transfer into cells and tissues

method	species	TE[a]	TP[b]	TD[c]	author(s)
macro-injection	*G. hirsutum*	x			Zhou *et al.* 1983
	S. cereale	x	x		de la Pena *et al.* 1987
micro-injection	*B. napus*	x	x		Neuhaus *et al.* 1987
	N. tabacum	x	x	x	Potrykus *et al.* 1990
DNA/pollen mixture	*Z. mays*	x			Ohta 1986
pollen tube pathway	*O. sativa*	x			Duan & Chen 1985
	O. sativa	x	x		Luo & Wu 1988
embryo imbibition	*T. aestivum*	x			Töpfer *et al.* 1989
silicon carbide fibers	*Z. mays*	x			Kaeppler *et al.* 1990
electroporation	*B. vulgaris*	x			Lindsey & Jones 1987
	O. sativa	x	x		Dekeyser *et al.* 1990
	Z. mays	x	x	x	D'Halluin *et al.* 1992
particle gun	*A. cepa*	x			Klein *et al.* 1987
	G. max	x	x		McCabe *et al.* 1988
	Z. mays	x	x	x	Gordon-Kamm *et al.* 1990

[a] TE, transient expression
[b] TP, transgenic plants
[c] TD, transgenic descendants

4.1.1 Microcuttings

Micropropagation by culture of nodal explants, axillary buds or apical meristems of arabica, Arabusta and interspecific hybrids was reported by several authors (Dublin1980a, 1981; Custers 1980; Kartha *et al.* 1981; Söndahl 1982; Berthouly *et al.* 1987). These techniques are useful for establishing clonal nurseries or in vitro collections, but less for large-scale multiplication. However, recent application of microcutting culture in a temporary immersion system (Berthouly *et al.* 1994) has demonstrated a 6-fold increase in multiplication rate, in relation to microcuttings multiplied on solid medium.

4.1.2 Somatic embryogenesis

Procedures for somatic embryogenesis in coffee are summarized in Table 2. Coffee tissue culture was first carried out by Staritsky (1970), who induced somatic embryos with successive plantlet regeneration, from soft green internodes taken at the top of orthotropic shoots of *C. canephora*. Colonna (1972) demonstrated the *in vitro* germination and subsequent culture of disinfected coffee beans of *C. canephora*. Keller *et al.* (1972) reported production and release of caffeine from primary callus cultures derived from endosperm and pericarp of *C. arabica*. Townsley (1974) grew viable callus suspension cultures from stem sections of *C. arabica*, for the production of coffee aroma compounds. Sharp *et al.* (1973) grew callus cultures from seeds, shoots, leaves, and anthers of *C. arabica*. Shoots and primary roots were obtained, but no further development occurred. Rooting and transfer to soil after somatic embryogenesis from leaf explants of *C. arabica* was reported for the first time by Herman & Haas (1975). Until then, in all procedures described, somatic embryos arose from small sized and compact nodular callus, formed after a relatively short period on callus induction medium. Because of the short duration and little callus formation, this process was called "direct somatic embryogenesis". The process was further improved by Dublin (1981), Pierson *et al.* (1983), Yasuda *et al.* (1985) and Hatanaka *et al.* (1991). Although rapid (6 months from leaf explant to soil transfer), this method results in relatively small amounts of somatic embryos and is subject to genotype effects (Bieysse *et al.* 1993; Ramos *et al.* 1993).

Söndahl & Sharp (1977) distinguished two types of somatic embryogenesis from leaf sections of *C. arabica* var Bourbon. They obtained embryogenic callus which yielded two waves of somatic embryo production named Low-Frequency Somatic Embryogenesis (LFSE) and High-Frequency Somatic Embryogenesis (HFSE). Following a primary culture on callus induction medium, LFSE developed after 70 days of secondary culture, whereas HFSE appeared only after 90-120 days. Because of its highly embryogenic character, resulting in abundant embryo formation, and its friability, which facilitates manipulation, the advantages of HFSE-callus production were recognized. The disadvantage of the HFSE procedure is its duration, taking at

least 10 months from leaf explant to soil transfer.

Other two-step somatic embryogenesis procedures, not always resulting in HFSE-callus, were described by Dublin (1980b) and Pierson *et al.* (1983). Neuenschwander & Baumann (1992) described a process, called Self-Controlled Somatic Embryogenesis (SCSE), which yields numerous, well-formed embryos with a high developmental synchronism. SCSE in contrary to the HFSE-way does not require a step on a maturation medium.

Multiplication of embryogenic suspensions followed by production of somatic embryos in liquid medium, was described by Staritsky & van Hasselt (1980), Dublin (1980b), de Pena (1983), Söndahl *et al.* (1985a), Neuenschwander & Baumann (1992) and Zamarripa *et al.* (1991). The work of Ducos *et al.* (1993) and Noriega & Söndahl (1993) extended and improved somatic embryogenesis of coffee for use in industrial bioreactors.

4.1.3 Androgenesis

Attempts have been made for obtaining haploid plants by androgenesis from culture of intact anthers (Lanaud 1981; Carneiro 1993) or isolated microspores (Carneiro 1993; Neuenschwander *et al.* 1993), but although haploid callus was obtained, complete regeneration was not reported. Recently, Ascanio & Asdrubal Arcia (1994) described regeneration of dihaploid *C. arabica* plants from cold shock stimulated anther culture. They obtained completely homozygous tetraploid genotypes after chromosome doubling by colchicine treatment.

4.1.4 Protoplasts

C. arabica is the only tetraploid species in the genus *Coffea*. This makes the transfer of genetic traits from wild diploid species of the genus to the cultivated *C. arabica* more difficult, although not impossible. In the early 1980s emphasize was laid on studies on coffee protoplasts, aiming at their possible use for interspecific somatic hybridization between dihaploid *C. arabica* protoplasts, obtained after androgenesis, and somatic protoplasts of diploid wild species (Söndahl *et al* 1981). Regeneration would be possible by following the procedures for regeneration of callus-derived cell suspensions, which were known for several species by that time.

Söndahl *et al.* (1980) pioneered in coffee protoplast studies; by using friable embryogenic callus as the source, they were able to isolate *C. arabica* protoplasts and regenerate them up to callus proliferation. Orozco & Schieder (1984) used young greenhouse grown leaves of hybrids of *C. arabica* and *C. canephora* as the source for protoplast isolation. The protoplasts survived until cell wall formation and some cell divisions. Schöpke *et al.* (1987) used somatic embryos of *C. canephora* as protoplast source; they reported regeneration of protoplasts into plantlets. However, efficiency was yet low, anomalities occurred and plantlets died after transfer to the greenhouse.

1991, Spiral & Pétiard reported plant regeneration after protoplast isolation from embryogenic callus suspensions of *C. arabica, C. canephora* and Arabusta. Regeneration into plantlets was achieved 12 months after isolation. Several well-matured protoplast-derived embryos could be successfully transfered to soil. Protoplast regeneration was improved by Acuna & de Pena (1991). Embryogenic callus suspension-derived protoplasts of *C. arabica* could be regenerated into plantlets within 9-10 months after isolation. Fifty six percent of the regenerated embryos developed into plantlets, which were transfered succesfully to greenhouse conditions.

4.1.5 Conclusions

It might be concluded that several aspects of the *in vitro* culture of coffee are actually well developed. Studies on regeneration from protoplasts or by androgenesis are still in progress but some results have already been obtained. Microcuttings and somatic embryogenesis are actually commonly used for multiplication purposes. "Direct" somatic embryogenesis appears to be dependent on genotype-effects (Bieysse *et al.* 1993; Ramos *et al.* 1993), whereas these effects are not yet clear with LFSE and HFSE (Söndahl *et al.* 1981). However, *in vitro* culture steps demand to be fully understood before being efficiently applied in a genetic transformation system. For example, somatic embryogenesis on solid or in liquid medium, used for such aim, must be considered as routine work, not asking for special attention or provoking inconvenient obstacles. In short, the advancements in *in vitro* culture of coffee has supported the way for its genetic transformation.

4.2 Genetic transformation

The first reports on genetic transformation of coffee tissue appeared in 1991. Barton *et al.* (1991) described the use of electroporation to integrate foreign DNA into *C. arabica* protoplasts and their subsequent regeneration into plantlets under kanamycin selection. Southern blot analysis was used to detect the introduced traits after transformation in one regenerated plantlet, which subsequently died. The same year, Ocampo & Manzanera (1991) showed that *C. arabica* tissues could be infected by wild *Agrobacterium tumefaciens* strains. Analysis of opines, showed the bacterial character of tumors developed on the young *in vitro* hypocotyls, which however, are not the best tissue for regeneration into complete plants. Other starting material, such as cell suspension cultures (if susceptible for *Agrobacterium*-mediated infection) would be more interesting.

The most recent report on genetic transformation appeared in 1993. Spiral & Pétiard used *A. rhizogenes* strains to infect somatic embryos of *C. canephora.* They used GUS expression and root formation as selection criteria for the transgenic character of tissue. A number of copies of the integrated exogenous DNA could be

detected in the plantlets regenerated from secondary somatic embryos. It is not yet clear if the genes responsible for hairy root phenotypes obtained by this method, can be genetically seperated from the integrated desired genes by a sexual step.

Until now, a reliable selective growth system for detection of transformed coffee tissue could not yet be described. Commonly used selective agents as kanamycin, geneticin and hygromycin are thought to be unreliable with regard to different coffee genotypes and tissues (Spiral & Pétiard 1993).

5. Aim and outline of the study

The study focusses on the development of a reliable genetic transformation method for coffee. At the start, in 1990, CIRAD had some experience with genetic transformation of cotton and was soon after involved in transformation studies with other tropical crops, considering that important applications can be forseen. For the coffee breeding program the possible applications are the integration of genes coding for important agronomic or quality traits:

1) Integration of genes, which are coding for insect resistance by the synthesis of *Bacillus thuringiensis* (Bt) toxins. Since 1991, studies were being carried out in parallel to this thesis, to demonstrate activity of Bt-toxins with regard to the most important coffee pest, the coffee leaf miner (*Perileucoptera coffeella*). Evaluation through immunocytochemical and biological assays revealed that the crystal proteins CryIA(c) and CryIB can be considered as active. The corresponding genes may be used for genetic transformation of coffee (Guerreiro *et al.* 1993). Actually, CIRAD is also testing Bt-genes for action against the coffee berry borer. Recently, in Colombia a large research program started for achieving transgenic resistance to the berry borer, as other methods to control this pest are of low efficiency and no resistance can be found in *C. arabica* or in diploid species.

2) Another potential application is the introduction of genes encoding for male sterility, of interest for F1 hybrid seed production. As noticed before, the occurrence of natural male sterile coffee plants, is rare. For example, the Belgium PGS group has developed a dominant gene for male sterility (Mariani *et al.* 1990) and a gene for restoration of male fertility (Mariani *et al.* 1992). The first gene is encoding for a ribonuclease, which, because of the selected promoter, is characterized by its extreme specificity for tapetal cell layers surrounding the pollen sac. Expression of the ribonuclease leads to the precocious degeneration of the tapetum cells, the arrest of microspore development, and male sterility. This male sterility gene has already been successfully introduced in oilseed rape, cauliflower, chicory, lettuce, tomato, cotton and corn (Leemans 1993).

3) Identification of enzymes interfering in caffeine synthesis, has been claimed

by private companies in the USA. The genes encoding for these enzymes are of potential importance for production of caffeine-free or high caffeine-content coffee seeds, which is of commercially interest (Lee 1990).

As mentioned before, genetic transformation can be efficiently applied only if *in vitro* culture methods are well established. Although CIRAD has long experience in this research field, some more recent techniques, like use of embryogenic callus suspensions had to be further investigated in the course of this thesis. Results are presented in chapter 2.

With regard to genetic transformation of coffee, CIRAD firstly investigated the virulence of six *A. tumefaciens* strains on leaf explants, hypocotyledons, cotyledons and stem fragments, without positive results (Barré 1990). Because of this result and because of information on protoplast-mediated transformation (Lee 1990), it was proposed that the present thesis would be directed to the improvement of protoplast isolation and, possibly, regeneration, aiming at electroporation studies later on.

Schöpke *et al.* (1987) had been able to regenerate some plantlets from isolated *C. canephora* protoplasts. However, this procedure needed improvement and, more importantly, regeneration of *C. arabica* protoplasts had not yet been achieved. Protoplast work in the framework of this thesis was realized between May 1990 until July 1992 and is described in chapter 3 (protoplast isolation and attempted regeneration) and chapter 4 (electroporation).

During the course of the thesis the difficulties and the long time needed for protoplast regeneration became evident; the same difficulties were also considered in other laboratories. At the same time, preliminary studies on the use of the biolistic system for transformation of coffee tissue realized at CIRAD and elsewhere, had demonstrated promising results. The development of a regeneration and mass multiplication procedure by somatic embryogenesis (Zamarripa *et al.* 1991; Berthouly & Michaux-Ferrière, submitted; chapter 2 of this work) supported further development of this system. It was therefore decided to stop further work with protoplasts and to develop studies on the biolistic method from September 1992 onward. Experiments were realized on different coffee tissues, from different genotypes, using four different promoters. Improved transient expression of introduced exogenous DNA is described in chapter 5.

In order to explore the possibilities of obtaining transgenic coffee tissue from somatic embryogenesis after biolistic treatment of leaves, as a first approach a histological study was carried out to characterize the effect and early events originating from the treatment. In chapter 6 the use of light and electron microscopy for this purpose is described.

Chapter 7 shows results from a study on a selective culture system for obtaining transgenic coffee tissue. The action of some antibiotics and herbicides was tested on

the induction of leaf callus and on growth of embryogenic suspension cultures.

In chapter 8 results are linked to each other, evaluated and discussed, and the implications of this study on coffee breeding programs and genetic transformation, is discussed as well as perspectives for further studies.

6. Published work

Results of the present thesis have been or will be published in:

van Boxtel J, Dufour M, Eskes A. 1991. Callus formation from isolated coffee protoplasts (*Coffea arabica* and *C. canephora*). *Physiologia Plantarum* **82**, A14.

van Boxtel J, Berthouly M, Carasco C, Eskes A. 1993. Transient expression of β-glucuronidase following biolistic delivery of foreign DNA into coffee tissues. In: ***15ème Colloque Scientifique International sur le Café*** (Montpellier). Paris: ASIC, 757-759.

van Boxtel J, Berthouly M. High frequency somatic embryogenesis from coffee leaves: factors influencing callogenesis, and subsequent multiplication and regeneration in liquid medium. *Plant Cell Tisssue & Organ Culture*, submitted.

van Boxtel J, Berthouly M, Carasco C, Dufour M, Eskes A. Transient expression of β-glucuronidase following biolistic delivery of foreign DNA into coffee tissues. *Plant Cell Reports*, submitted.

van Boxtel J, Berthouly M, Besson A, Pujol R, Michaux-Ferrière N. Fate of GUS-expressing cells during biolistically-induced callus formation on *Coffea* sp. leaves: a histological study. *Journal of Experimental Botany*, submitted.

van Boxtel J, Berthouly M, Eskes A. Inhibitory effect of selective agents on callus development for selection of genetically transformed coffee tissues. *HortScience*, submitted.

CHAPTER 2

High frequency somatic embryogenesis from coffee leaves: factors influencing callus induction, and subsequent multiplication and regeneration in liquid medium

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Abstract

An improved procedure for the induction, multiplication and regeneration of embryogenic high frequency callus from coffee leaf explants was developed for different genotypes. The used sequence of media for callus and embryo induction, yielded embryogenic callus more rapidly and abundantly with *Coffea canephora*, Arabusta and Congusta, in relation to other procedures. However, *C. arabica* genotypes proved to be less rapidly reacting to the procedure. Recalcitrance for somatic embryogenesis of certain genotypes will require adaptation of growth regulator composition in the media sequence for achievement of such regeneration method.

Highest multiplication rate of embryogenic callus in liquid culture, while avoiding occurrence of embryos, was obtained by an initial density of 10 g callus.l^{-1} in medium containing 4.5 µM 2,4-D, under 3 µmol.m^{-2}.s^{-1} illumination, and subcultured each 7-10 days. Best long-term maintenance of embryogenic strains was obtained by culture of aggregates, sized between 250 µm and 1000 µm, at a density of 5 g.l^{-1}, with medium renewal each 3-4 weeks. This way, embryogenic potential of *C. canephora* callus was maintained over two years. Analysis of nutrient absorption by callus cultures demonstrated that application of MS macro- and micro-minerals at half strength did not lead to their depletion during at least 3 weeks of sustained culture.

Best regeneration efficiency of embryogenic callus required omission of auxin and lowering of culturing density to 1 g.l^{-1}. Thus, one gram *C. canephora* or Arabusta callus produced respectively 1.2 and 0.9 x 10^5 somatic embryos after 8-10 weeks in liquid regeneration medium. After transfer of embryos to germination medium and medium for plantlet development, roots were formed and the plantlets could be adapted to greenhouse conditions. The duration of the complete procedure (from leaf explants to plantlet regeneration passing through callus multiplication in liquid medium) was about 9 months, which is 4-6 months shorter compared to other procedures described. A histological study confirmed the unicellular origin of the high frequency somatic embryos.

Introduction

Various procedures for somatic embryogenesis (SE) have been described in coffee species since it was firstly reported by Staritsky (1970) from *Coffea canephora* internodes. High frequency somatic embryogenesis (HFSE) was firstly reported by Söndahl & Sharp (1977) on leaves of *C. arabica* 'Bourbon'. They obtained HFSE-callus after a two-step procedure, consisting of a primary culture on auxin-containing medium for callus induction, followed by a second culture on auxin-free medium for embryo induction. The advantages of HFSE-callus are being found in its friability, its abundant appearance and its highly embryogenic character. These advantages permit the use of HFSE-callus in liquid culture systems, as previously described by Söndahl *et al.* (1985) and Zamarripa *et al.* (1991). HFSE in liquid medium has potential

applications in coffee breeding programs.

Firstly, a fast multiplication method which allows the availability of large amounts of uniform plant material is of great value in speeding up and facilitate classical coffee breeding methods, e.g. the rapid multiplication of intraspecific hybrid varieties of *C. arabica*, combining multiple disease resistance and hybrid vigour. The recent application of bioreactors, providing large scale multiplication of coffee plants, has advanced these developments (Ducos *et al.* 1993; Noriega & Söndahl 1993). Secondly, somaclonal variation induced as a result of SE, may be used in obtaining mutant forms of high yielding varieties (Söndahl & Bragin 1991). Thirdly, a well-controlled SE procedure is fundamental for genetic transformation purposes. *Agrobacterium*- and PEG-mediated transformation, electroporation or the biolistic method can be applied on tissues which undergo subsequent SE: directly on intact plant tissue, on suspension cultures initiated from embryogenic callus, or on protoplasts isolated from embryogenic suspensions.

In general, two approaches for liquid culture conditions can be distinguished: a) rapid growth of embryogenic callus mass, or b) slow growth for long-term maintenance of embryogenic strains. The first approach is of importance when a large quantity of embryogenic callus is required, e.g. for genetic transformation experiments. The second prefers a slow growth combined to a minimum of culture handling. The priorities for both approaches are the same: maintenance of embryogenic potential and suspension quality, without the occurrence of embryo formation. Similar systems have been developed in carrot (Halperin 1966), maize (Vasil & Vasil 1986), grape (Gray & Mortensen 1987), eucalyptus (Muralidharan *et al.* 1989), celery (Nadel *et al.*, 1989), sweet potato (Chee & Cantliffe 1989) and citrus (Gavish *et al.* 1991).

In the present study, main objectives are a) to improve the yield of the present SE techniques for coffee, by combining accelerated regeneration to a considerable increase in number of plantlets regenerated, and b) to control culture steps necessary in genetic transformation systems. The two-step procedure on solid medium for the obtainment of HFSE-callus from leaf explants as described by Berthouly & Michaux-Ferrière (submitted) was applied. The efficiency of this procedure was tested on a range of coffee genotypes belonging to different species. Secondly, a continuation is described for the multiplication and regeneration of HFSE-callus in liquid medium. The obtainment of a self-maintained embryogenic callus suspension is desired which can, when required, be directed towards embryo formation. Elimination of drawbacks of SE in solid medium, like asynchronism and the need to regularly use new explants, should thus be achieved. Finally, a histological study was carried out for characterization of developmental events of HFSE in liquid medium.

Materials and methods

Plant material

Induction of embryogenic callus

Tested genotypes belonged to different *Coffea* species: *C. arabica* varieties (Mundo Novo, Caturra and Catuai) and semi-wild accessions from Ethiopia (KF2.1, AR15); *C. canephora* clones from Togo (107, 181, 182, 197, 202), Costa Rica (T3561-2.1 and T3561-2.3), and open pollinated progenies (OP) from Côte d'Ivoire (OP461, OP597); Arabusta clones from French Guiana (1307, 1312) and one Congusta clone from Madagascar (HA). Healthy greenhouse grown plants were selected as source for leaf explants. Sterilized young leaves from orthotropic nodes were cut into pieces of one cm^2 and placed, upper surface down, on medium **C** for callus induction (Table 1). The explants were cultured in dark at 27°C in plates (OPTILUX, Falcon). After one month, primary callus and explants were transferred to 100 ml glass jars, containing 25 ml of medium **E** for embryo induction (Table 1), and cultured at low light intensity (2 $\mu mol.m^{-2}.s^{-1}$; 12 h/d).

Table 1. Composition of used media in $mg.l^{-1}$

	C[1]	E[1]	CP	R	EG[2]	DEV[3]
macrominerals	MS[4]/2	MS/2	MS/2	MS/2	MS/2	MS
microminerals	MS/2	MS/2	MS/2	MS/2	MS/2	MS
$FeSO_4.7H_20$	13.9	13.9	13.9	13.9	13.9	27.8
Na_2EDTA	18.65	18.65	18.65	18.65	18.65	37.3
thiamine-HCl	10	20	5	10	8	1
pyridoxine-HCl	1	-	0.5	1	3.2	1
nicotinic acid	1	-	0.5	1	-	1
calcium pantothenate	-	-	-	-	-	1
biotine	-	-	-	-	-	0.01
glycine	1	20	-	2	-	-
L-cystein	-	40	10	-	-	-
myo-inositol	100	200	50	200	100	100
adenine sulfate	-	60	-	40	-	-
casein hydrolysate	100	200	100	400	-	-
malt extract	400	800	200	400	-	-
2,4-D	0.5	1	1	-	-	-
IBA	1	-	-	-	-	-
IAA	-	-	-	-	0.45	-
2-iP	2	-	-	-	-	-
KIN	-	-	1	-	-	-
BA	-	4	-	4	0.25	0.3
sucrose	30,000	30,000	15,000	40,000	20,000	30,000
phytagel	2,000	2,000	-	-	2,500	2,500
pH	5.6	5.6	5.6	5.6	5.6	5.6

[1] Berthouly & Michaux-Ferrière (submitted)

[2] Dufour & Carasco (unpublished)

[3] Dublin (1984)

[4] Murashige & Skoog (1962)

Supensions with embryogenic potential

Within two to 4 months (depending on genotype), friable embryogenic callus, developed on explants on E-medium, was transferred to liquid medium **CP** for callus proliferation (Table 1), in 150 ml erlenmeyers. The suspensions were cultured at 27°C on a gyratory shaker at 100 rpm. Stable embryogenic callus suspensions were obtained 2-3 months after initiation in liquid medium. Stationary growth of callus mass was then about 3 to 4 fold between each subculture, which was carried out in 250 ml erlenmeyers. For subculturing, only the fragments of size below 1 mm were collected, by using a narrow mouth pipet (Falcon). The stable embryogenic suspensions of several coffee genotypes obtained by this procedure were used for further testing of liquid culture parameters. Tested parameters were 2,4-D concentration in **CP**-medium (4.5 or 9 µM), light intensity (3 or 30 $\mu mol.m^{-2}.s^{-1}$), subculture intervals (3, 6 or 14 days), culture density (0.5, 2, 5, 10 or 20 g $callus.l^{-1}$) and density readjustment (to 10, 15 or 20 g $callus.l^{-1}$) during weekly subculturing. Measurement of callus fresh weight was carried out for quantification of effects. Multiplication rate (MR) was defined as:

$$MR = \frac{\text{fresh weight at week n+1}}{\text{fresh weight at week n}}$$

Regeneration of somatic embryos

Callus suspensions were directed to embryogenesis by transfer to liquid medium **R** (Table 1), containing 4.4 or 17.8 µM BA. They were cultured at low density (1, 3 or 5 g $callus.l^{-1}$) in 250 ml erlenmeyers, under indirect light (5 $\mu mol.m^{-2}.s^{-1}$; 12 h/day) at 100 rpm, and subcultured once a month. Eight to 10 weeks later mature embryos were placed, upper side down, in solid medium **EG** for embryo germination (Table 1).

Development into plantlets

About 6-8 weeks later, when planlets had developed a first pair of real leaves, cotyledons and roots were cut off, and the plantlets were transferred to medium **DEV** for plantlet development (Table 1). By the time that the plantlets had achieved 3-4 cm and possessed 4 leaf pairs, the *in vitro* developed root was again cut off and plants were immersed in a IBA/IAA-containing solution during one night, for rooting enhancement. Hardening of the plants in the greenhouse was achieved by an initial period of 3 weeks, individually planted in soil-mixture and covered under plastic.

Absorption of nutrients

Concentration of minerals in **CP**-medium during culture was determined after dilution in lanthane oxid, by atomic absorption spectrometry (PERKIN-ELMER 2380) as described in "Standard methods for the examination of water and wastewater" (1992). Analysis of nitrogen sources was performed by the Kjeldahl method.

Histological observation

Different types of *in vitro* grown tissues were used for a histological characterization. From initiation of liquid culture to obtaining of a stable embryogenic suspension, and during the first month in **R**-medium, samples were taken once a week, and prepared for histological observation. Fixation, dehydratation, embedding and colouration procedures are described in detail by Berthouly & Michaux-Ferrière (submitted).

Data analysis

For all data, analyses of variance using Type III sums of squares generated from the General Linear Model procedure in SAS were performed. Differences among treatment means were analyzed by Newman-Keuls test. Differences significant at 5% probability level were considered meaningful.

Results and discussion

Induction of embryogenic high frequency callus from leaves on solid culture medium

Media sequence **C** + **E** was initially developed at CATIE (Costa Rica) for obtaining of HFSE-callus with *C. canephora*. Here, the sequence was tested on a range of genotypes belonging to different species (Table 2). Development of a primary nodular callus seemed to be required for subsequent HFSE-callus formation: *C. canephora* clones 107 and 197 showed few nodular calli and practically no HFSE-callus formation. Other genotypes like *C. arabica* cv Mundo Novo and AR15 showed an exclusive formation of low frequency somatic embryogenesis (LFSE), i.e. somatic embryos developed rapidly from compact nodular callus. The histological characterization of such a process was described by Söndahl *et al.* (1979) and Michaux-Ferrière *et al.* (1989). In our study, first friable embryogenic callus on leaves of *C. arabica* cv Catuai and KF2.1 appeared only after some 3 to 4 months on **E**-medium (data not shown). Therefore, the procedure as described by Söndahl & Sharp (1977) might be preferable for HFSE on *C. arabica* species.

Although nodular callus had developed on the explants, *C. canephora* clone 182 and Arabusta clone 1307 showed an almost complete blockage of either LFSE or HFSE (Table 2). Best reacting genotypes for the HFSE procedure were *C. canephora* (181, 202, T3561-2.3 and OP461), Arabusta (1312) and Congusta (HA).

HFSE-callus formation seemed not always to be hindered by severe polyphenolic oxidation of tissue (clone 202), but in general better HFSE-response was obtained in combination with late occurrence of polyphenolic oxidation.

A large variability in HFSE-response is observed among *C. canephora* genotypes, compared to *C. arabica*. Due to the self-incompatible character of this species, a high level of heterozygozity is found in *C. canephora* germplasm, which might be a reason for the in general higher regeneration capacity of its tissue (Söndahl *et al.* 1981). Regarding at the phylogenetic background of the *C. canephora* clones (Congolese or Guinean types), no relation seems present with the observed differences in tissue reactivity (Table 2). We presume that rather different endogenous hormone balances would be responsible for specific reactions to the induction of HFSE-callus on growth regulator-containing culture media.

These results indicate a genotype **x** medium interaction and supports the awareness that in fact species-, or even genotype-adapted growth regulator treatments

Table 2. LFSE and HFSE-callus formation on leaf explants, expressed in % of reactive explants, of various *Coffea* genotypes after one month on **C**-medium and 75 days subsequent culture on **E**-medium.

Genotypes	total number of explants	% of explants with nodular callus at day 60	at day 105		
			polyphenolic oxidation and nodular callus degeneration[1]	% of explants with embryo formation of LFSE-origin	% of explants with friable HFSE-callus formation
C. arabica cv Mundo Novo	32	90.6	+	5.1	0.0
„ cv Catuai	33	100	+	8.0	5.7
„ KF 2.1	55	89.1	++	10.2	10.0
„ AR 15	20	90.0	+	2.0	0.0
C. canephora clone 107 (CxG)[2]	46	17.4	+	0	1.0
„ „ 181(CxG)	33	90.9	+	7.6	90.9
„ „ 182 (C)	82	61.0	++	0	0.5
„ „ 197 (G)	47	0.0	+	0	0.0
„ „ 202	56	100	+++	0	66.1
„ OP[3] 461 (CxG)	60	95.0	+	0	82.0
„ „ 597 (C)	20	100	+	0	25.0
„ clone T3561-2.1 (C)	59	83.1	++	3.4	35.6
„ „ T3561-2.3 (C)	97	100	+	9.3	96.9
Arabusta clone 1307	40	100	+++	0	0.0
„ 1312	53	100	+	0	66.0
Congusta clone HA	20	100	++	0	60.0

[1] +++ overall, ++ moderate, + slight.
[2] C: Congolese type; G: Guinean type; CxG: F_1 Hybrid
[3] OP, open pollinated progeny

are required for assuring a satisfying response to SE in coffee. Söndahl *et al.* (1981) and Bieysse *et al.* (1993) described also genotype x medium interactions for callus induction by HFSE and LFSE, respectively, with several coffee species and genotypes using different growth regulator treatments.

Multiplication of embryogenic callus in liquid culture medium

HFSE-callus obtained after application of media sequence **C** + **E**, was used to test several culture conditions in liquid medium. The basal medium is **CP**-medium (Table 1), which was developed for the proliferation of coffee callus in suspension cultures.

If not otherwise stated, the genotype used was *C. canephora* clone T3561-2.3, and initial culture density 6 g callus.l^{-1}.

Effect of 2,4-D concentration

Presuming that both cytokinins and auxins are required in a cell multiplication medium (Söndahl *et al.* 1981), two combinations were tested: KIN 4.6 μM (1 mg.l^{-1}) + 2,4-D 4.5 μM (1 mg.l^{-1}), and KIN 4.6 μM + 2,4-D 9 μM (2 mg.l^{-1}). Callus mass was subcultured each 2 weeks by a complete renewal of the culture medium. Cultures were grown under indirect light (3 μmol.m^{-2}.s^{-1}). Although 4.5 μM 2,4-D showed a slightly higher growth (Fig. 1a), no significant effect could be noticed between the two treatments. In order to reduce presumed somaclonal variation from 2,4-D application (Larkin & Scowcroft 1981), 4.5 μM 2,4-D was chosen for further testing of liquid culture conditions.

Effect of light intensity

The influence of light intensity on the multiplication of embryogenic callus mass was determined during a 10 week growing period, without subculturing. Fig. 1b demonstrates an inhibition of callus growth under direct light compared to the indirect light condition. Higher light intensity seems to retard growth and may therefore be used for long-term maintenance. However, polyphenolic oxidation in light limits the use of too high intensities (Monaco *et al.* 1977). Growth under indirect light is slowing down after 9 weeks of culture (Fig. 2b). This is probably due to a spatial saturation of the culture and a lack of vital nutrients. Higher callus densities than 400 gram of fresh weight per liter culture medium were never obtained without the occurrence of cell death or loss of embryogenic potential.

Effect of different subculture intervals

Less callus growth was observed when subculturing each 3 days (Fig. 1c). No difference occurred between the 6 and 14 days subculture treatments. Apparently *C. canephora* callus suspensions are better adapted to a low subculture frequency and are stressed by too rapidly succeeding subcultures. Similar results were obtained in citrus (Gavish *et al.* 1991) and oil palm (de Touchet 1991), but in rubber on the other hand, a 3 to 5 day subculture regime was prefered (Montoro *et al.* 1994). To minimize culture handling without negatively influencing suspension growth, a 14 day subculture frequency appears therefore acceptable when rapid growth of callus mass is required.

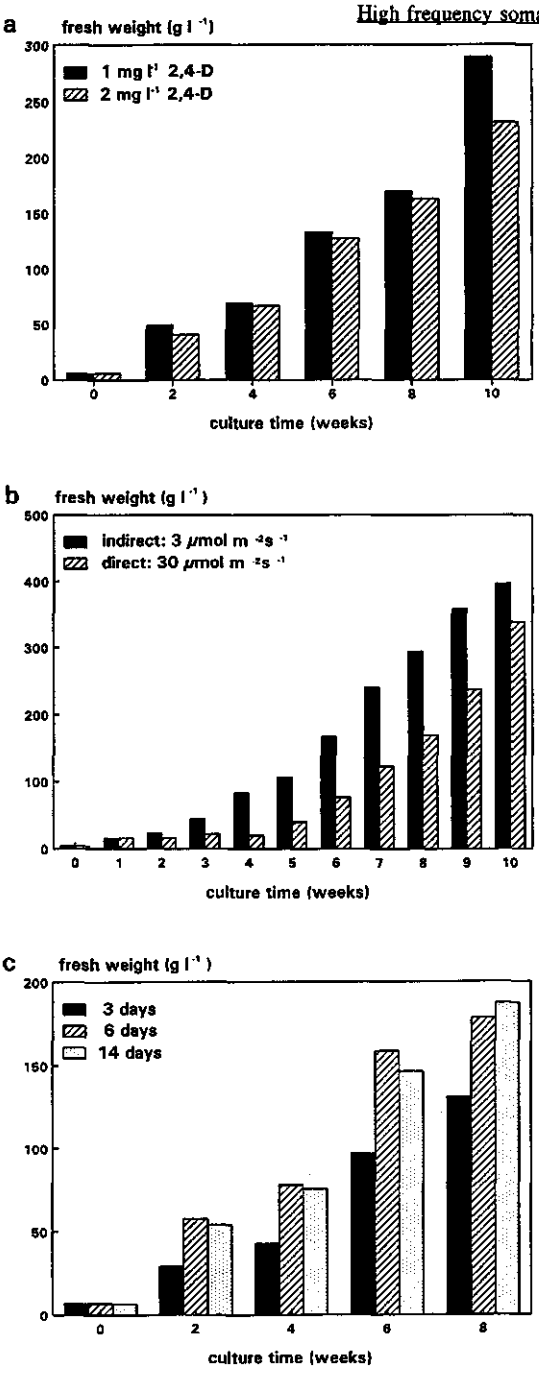

Fig. 1. Effect of a) 2,4-D concentration, b) light intensity and c) subculture frequency, on growth, expressed in fresh weight of embryogenic callus cultures of *C. canephora* clone 3561(2.3) in CP-medium.

Effect of culture density

Optimal culture density was studied with two objectives: a) for rapid growth of embryogenic callus mass, and b) for slow growth in long-term maintenance of embryogenic strains. In both cases embryogenic potential and suspension quality should be maintained, without the occurrence of embryo formation. Fig. 2 shows the growth curves of *C. canephora* OP597 suspensions followed during 8 weeks of culture. Initial densities of 0.5, 2, 5, 10 and 20 $g.l^{-1}$ were not adjusted during the 2-weekly renewal of medium. Growth of 20 $g.l^{-1}$ suspensions, and less so of 10 $g.l^{-1}$ suspensions, is slowing down between week 6 and 8. In 0.5 $g.l^{-1}$ cultures, and less so in 2 $g.l^{-1}$ cultures, mass formation of embryos occurred. Increase of weight in these cultures was partially due to embryo growth. Embryo formation was not observed in 5, 10 and 20 $g.l^{-1}$ cultures, showing the inhibitory effect of high inoculation densities. A minimal culture density of 5 $g.l^{-1}$ seems thus to be necessary for avoiding embryo formation. Calculation of multiplication rates (MR) demonstrated for each tested culture density an exponential growth during the first week of culture. MR of 0.5, 2, 5, 10 and 20 $g.l^{-1}$ cultures was respectively 16.6, 5.2, 2.9, 2.8 and 2.5. During the second week, MR of all cultures decreased to a level between 1.0 and 1.8. The 2 and 5 $g.l^{-1}$ cultures maintained the highest MR through the whole test period of 8 weeks, during which density was not readjusted.

In parallel, density of other cultures was weekly readjusted during medium renewal, to their initial densities of 10, 15 or 20 $g.l^{-1}$. MR of cultures determined during 4 weeks were respectively 2.01 ± 0.57, 1.81 ± 0.08 and 1.53 ± 0.36, being MR of 10 $g.l^{-1}$ cultures significantly different from MR of 20 $g.l^{-1}$ cultures. Thus, weekly readjustment of culture density to 10 g callus.l^{-1}, can be used for speeding up growth of callus suspensions.

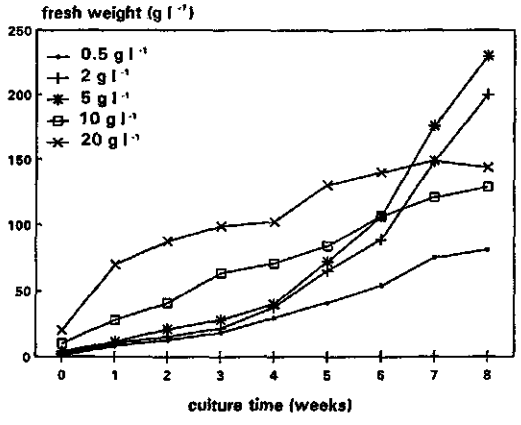

Fig. 2. Effect of culture density on growth, expressed in fresh weight of embryogenic callus cultures of *C. canephora* OP-597 in CP-medium.

In conclusion, a relatively low MR without embryo formation can be maintained by using an initial density of 5 to 10 $g.l^{-1}$ and decreasing subculture frequency to once each 3 to 4 weeks. These were optimal conditions for long-term maintenance of embryogenic strains. On the other hand, rapid production of embryogenic callus can be achieved using culture density of 10-15 $g.l^{-1}$, thus combining high MR to low quantity of culture units, which saves space in culture chambers. However, for continuous exponential growth of callus mass, a 7-10 days subculture interval is then required.

Absorption of nutrients by embryogenic callus

Absorption of some macro- and micro-nutrients by callus suspensions was followed during 3 weeks, without renewal of the culture medium. The amount of remaining nutrients in the culture medium was analyzed each 2 days. After 12 days of culture, 60 to 70% of the initial concentration is still present (fig 3a-c). After 3 weeks none of the analyzed macro-nutrients seems yet to be limiting in the culture medium. Macro-nutrients and nitrogen sources have decreased to 40-50%, wheras magnesium concentration is lowest (30%). Thus, supply of MS macro- and micro-nutients at half strength, as is the case for **CP**-medium, did not lead to their depletion in embryogenic coffee callus culture, for at least three weeks.

Compared to **CP**-medium, Zamarripa (1993) used a two times higher concentration of carbonic source in his culture medium for multiplication of coffee callus. He showed that after three weeks of culture 60% of carbonic source was still present in the medium. This means that if our suspensions had been cultured at the same density as Zamarripa's, which is 10 g $callus.l^{-1}$, carbon source would have become limiting in **CP**-medium after some four weeks.

Trying to optimize long-term maintenance of embryogenic strains, instead of subculturing in complete new medium, each two weeks a part of the callus mass was discarded and minerals in concentrated form were added to the conditioned culture. However, in all cases inhibited growth was observed after two months. A subculture by a complete or partial renewal of the culture medium could thus not be replaced by a simple addition of nutrients in concentrated form. Therefore, medium renewal remains necessary at regular time intervals, in order to eliminate substances excreted by the aggregates, having an inhibitory influence on embryogenic potential of the culture (Zamarripa, 1993). The nature of toxic substances can only be speculated, but they may be related to presence of polyphenols which oxidize by forming toxic quinones (Monaco *et al.* 1977).

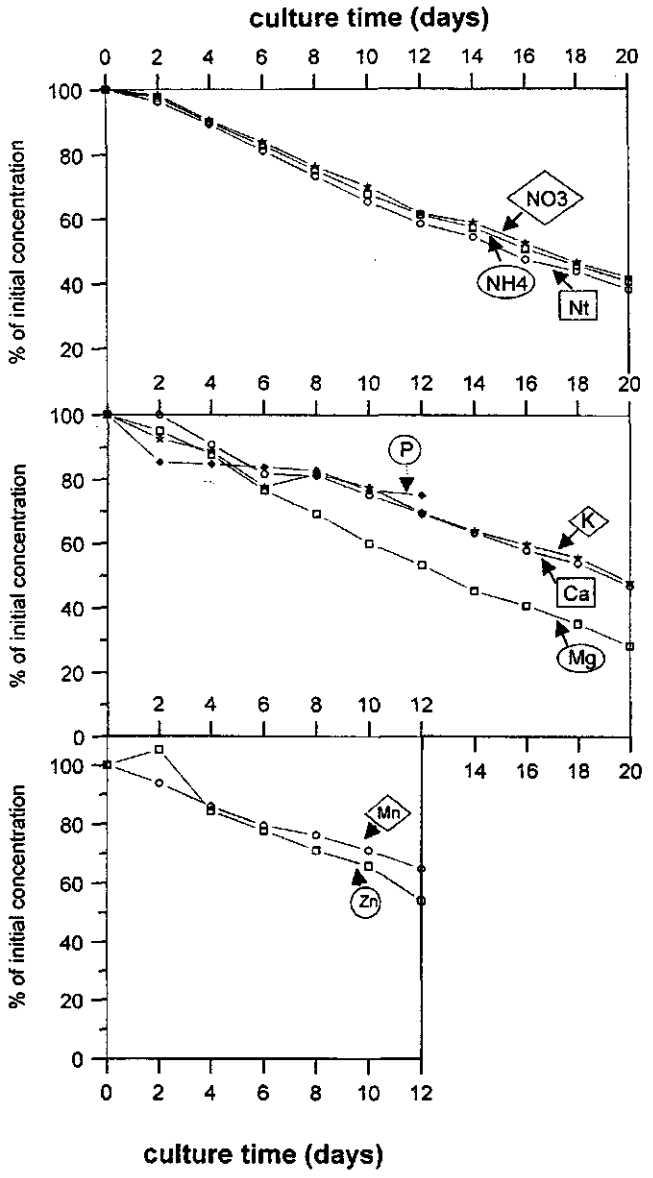

Fig. 3. Absorption of nutrients by embryogenic callus cultures of *C. canephora* clone 3561(2.3) in CP-medium. Concentrations of remaining nutrients were analyzed by Kjeldahl method or atomic absorption spectrometry, and data are given in percentages of initial concentrations in liquid medium. a) nitrogen sources: Nt, total nitrogen; NO_3, nitrate; NH_4, ammonium. b) macronutrients: Ca, calcium; K, potassium; Mg, magnesium; P, phospor. c) micronutrients: Mn, manganese; Zn, zinc.

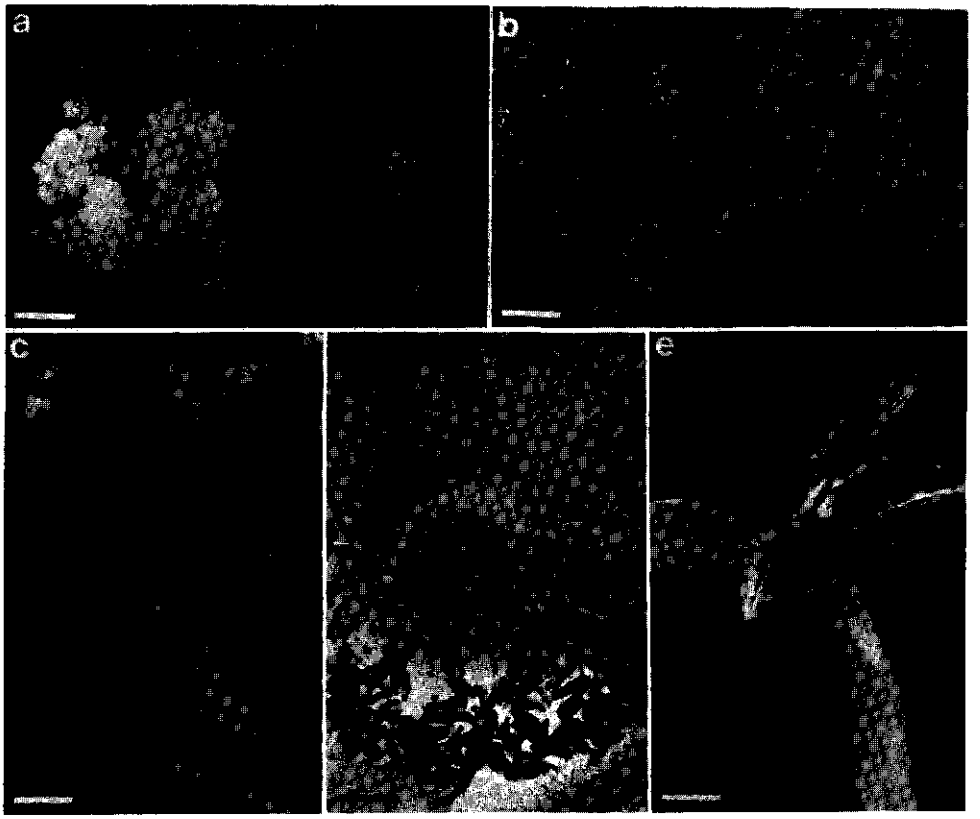

Plate 1. High frequency somatic embryogenesis in *C. canephora* genotypes. a) HF-callus on OP597 leaf explant after 15 weeks on **E**-medium (bar = 5 mm); b) detail of embryogenic callus of clone 181 after 3 months in **CP**-medium (bar = 0.5 mm); c) regenerating embryogenic callus of clone 3561(2.3) after 10 weeks in **R**-medium (bar = 2 mm); d) maturation of somatic embryos of clone 197 after 10 weeks in **R**-medium (bar = 8 mm); e) fully developed plantlet of *C. arabica* cv. Catuai after 2 months on **EG**-medium (bar = 3 mm).

Maintenance of embryogenic capacity

Embryogenic potential of suspension cultures was followed with different genotypes by regularly culturing small callus amounts in liquid regeneration medium (**R**). *C. arabica* callus was in general difficult to maintain in "undifferentiated" state. The exception were Catuai and Caturra, which lost their embryogenic potential only after 16-18 months of culture in **CP**-medium. Aggregates had gradually changed in size (< 250 μm), colour (white) and texture (spongy), and were constituted of very large, vacuolized cells. With Mundo Novo, KF2.1 and AR15, a shift towards somatic embryo

formation after 4-6 months of culture in CP-medium, was not possible to avoid. This might be due to the type of callus of these 3 genotypes used for suspension initiation, which was not friable, but rather a compact nodular callus formed after a short callus phase.

Also the highly embryogenic callus of clone 197 (induced on auxin-free callus induction medium) could not be maintained in undifferentiated state in CP-medium; after 7 months all aggregates had developed into embryos. Cultures of clones T3561-2.1 and T3561-2.3 preserved embryogenic capacity respectively during 13 and 16 months of cultivation. Callus suspensions of OP597 are actually still embryogenic after 24 month of culture.

The aggregate size which was most appropiate for multiplication has found to be between 250 and 1000 µm. The callus fraction below 250 µm contains small aggregates with less embryogenic cells and may therefore loose its embryogenic character during sustained culture. Experiments carried out with fractions below 100 µm (1-10 cells per aggregate), showed a rapid degeneration of cells or formation of non-embryogenic free cells. Chee & Cantliffe (1989) demonstrated similar results in sweet potato.

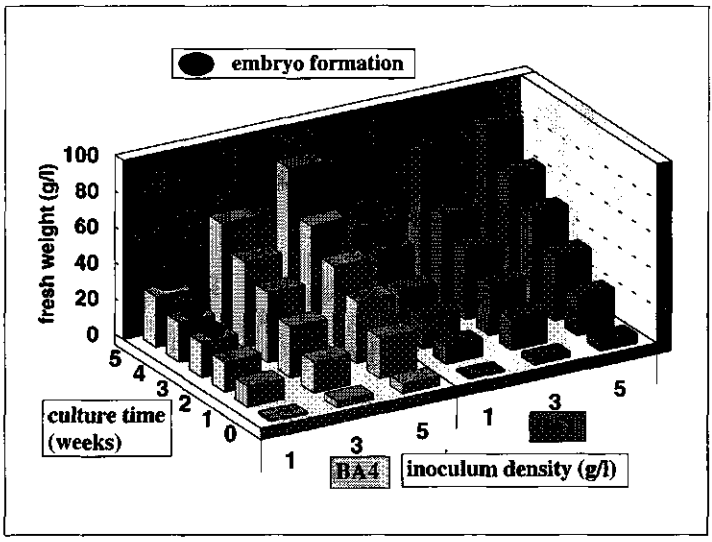

Fig. 4. Effect of BA concentration and culture density on growth, expressed in fresh weight, and embryo formation of embryogenic callus cultures of *C. canephora* clone T3561(2.3) in **R**-medium.

Regeneration of embryogenic callus in liquid culture medium

Effect of BA and inoculation density

The effect of benzyladenine (BA) concentration and inoculum density on the occurrence of globular somatic embryos and increase of fresh weight in **R**-medium-cultured embryogenic callus of *C. canephora* clone T3561-2.3, is summarized in Fig. 4. Increasing inoculum density was inversely related to the occurrence of globular embryos, and combination of high inoculum density (from 3 $g.l^{-1}$) with high BA concentration (4.4 µM = 4 $mg.l^{-1}$) suppressed embryo formation. Most early and abundant somatic embryo formation was observed with inoculum density of 1 $g.l^{-1}$ combined to BA concentration of 4.4 µM. These results are more or less in agreement with those reported by Dublin (1984) and Zamarripa *et al.* (1991) who demonstrated maximum somatic embryo formation in regeneration medium supplemented with 4.4-22.2 µM (1-5 $mg.l^{-1}$) BA.

Conversion rates

The presented SE procedure for mass propagation takes about 7-8 months for *C. canephora* and Arabusta, and 9-10 months for *C. arabica*, counted from leaf explants to regenerated plantlets (Plate 1a-e). Efficiency of SE procedures in liquid medium is summarized in Table 3. *C. arabica* is more recalcitrant to SE procedures since the level of somatic embryo production is far below that of *C. canephora* and Arabusta. Zamarripa (1993) obtained relative high rates of 200.000-500.000 somatic embryos from one gram of embryogenic *C. canephora* aggregates, using erlenmeyer flasks. The 8 month period that he needed for the establishment of suspension cultures could be diminished to 2-3 months in our procedure. Somatic embryo production on the other hand, also diminished about 4-fold in our procedure (Table 3).

Histological characterization of multiplication and regeneration of embryogenic callus in liquid medium

The multiplication phase

The induction and proliferation of embryogenic callus from leaf explants on solid medium following our procedure has been histologically described by Berthouly & Michaux-Ferrière (submitted). Seventy days after transfer to **E**-medium, friable callus, consisting of both oblong and embryogenic cells, was used for initiation of suspension cultures and continued histological observation.

Table 3. Efficiency of coffee micropropagation systems by somatic embryogenesis in liquid culture.

	Species	Culture method	multiplication rate (growth/week)	number of som. embryos/gram inoculum	% of som. embryos developed into greenhouse plants
Staritsky & van Hasselt (1980)	*C.canephora*	erlenmeyer	1.19	500-1000	40-60
Neuenschwander & Baumann (1992)	*C.arabica*	erlenmeyer	1.48	609	--
Zamarripa (1993)	*C.canephora*	erlenmeyer	1.36-1.52	200.000-500.000	48
	Arabusta	„	„	„	44
Noriega & Söndahl (1993)	*C.arabica*	bioreactor	--	12.500	--
Ducos *et al.* (1993)	*C.canephora*	bioreactor	--	600.000	47
	Arabusta	„	--	„	35
present paper	*C.canephora*	erlenmeyer	1.81	120.000	--
	Arabusta	„	--	92.300	--
	C.arabica	„	1.54	12.300	--

-- not evaluated

In liquid medium isolated oblong cells were coexisting with small embryogenic cells in aggregates. About two months after initiation (period of culture establishment) all oblong cells appeared to have degenerated and were eliminated during medium renewal. Due to culture agitation, smaller agregates continuously detached from the "mother"-aggregate and individually proceeded the same process (Plate 2a). Nodules observed in the centre of the aggregates were composed of differentiated, vacuolated cells, which degenerate. Nodules at the periphery can be considered as proembryogenic masses (PEM's; Halperin 1966). Following this process, multiplication in CP-medium of embryogenic aggregates, is controlled without a start of embryo formation.

The histological observations also clarified why medium sized aggregates (between 250-1000 μm) are best suited for suspension cultures. Cells at the interior of too large aggregates show necrosis, thus leading to liberation of oxidized polyphenols in the liquid medium. Embryogenic cells in too small aggregates loose their reserves, become vacuolized and differentiate (Plate 2b) .

The regeneration phase

After transfer to R-medium, embryogenic cells in the peripheric aggregate nodules divided, giving rise to embryonic cells. These cells contained no starch, a large diffuse nucleus in a dense cytoplasm and were rich in soluble proteins. The embryonic cells formed an area of meristematic cells, closely grouped together within an epidermal structure (Plate 2b). Such an independently functioning unit is a pro-embryo and is of

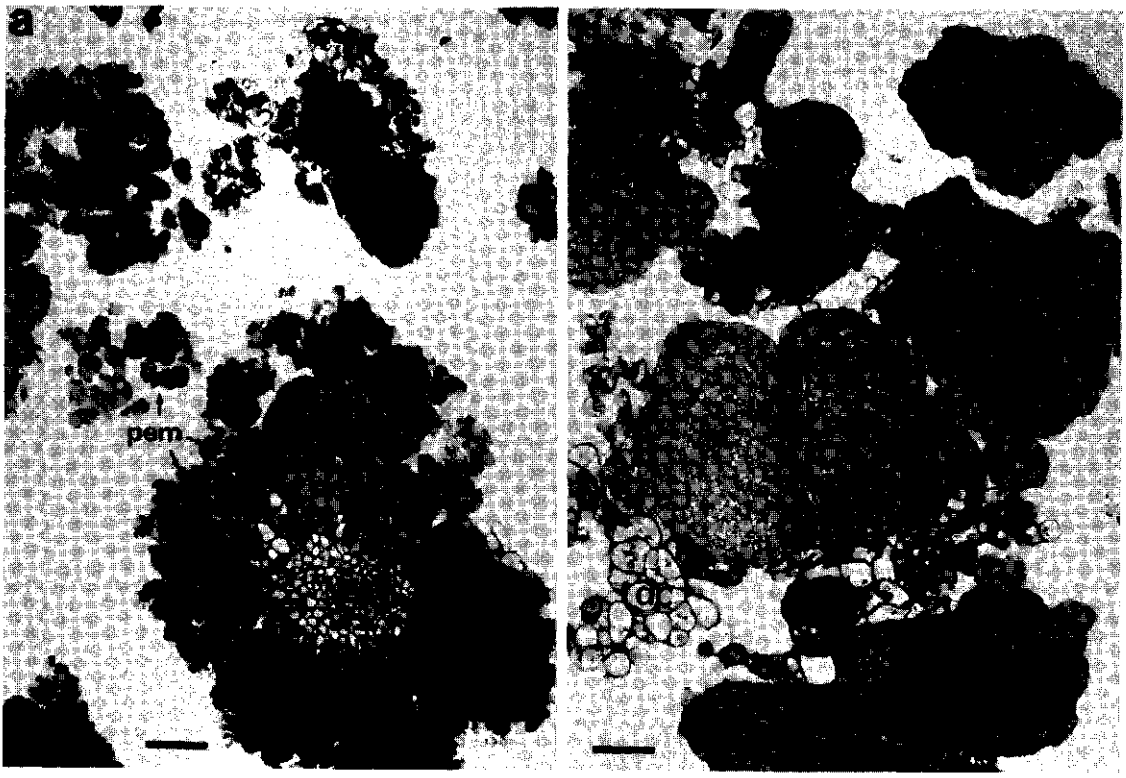

Plate 2. Histological aspects of high frequency somatic embryogenesis in *C. canephora* callus, a) during culture in CP-medium (multiplication phase); pem proembryogenic mass; cn central nodule; (bar = 150 µm); b) during culture in R-medium (regeneration phase); ec embryonic cells; pe proembryo; ge globular embryo; dc differentiated cells; ◂ epidermal structure; (bar = 75 µm).

unicellular origin, as already noted by Michaux-Ferrière *et al.* (1987). During further development, pro-embryos gradually isolated from the "mother"-aggregate (Plate 2b). A completely isolated pro-embryo can be considered as a globular embryo. Subsequent somatic embryo development and maturation into heart, oblong and torpedo stages took place in the same medium, and is analogous to zygotic embryo development (Carasco *et al.* 1994). The described regeneration process in liquid medium took about 6-10 weeks.

Conclusions

Somatic embryogenesis of coffee has been obtained by single-step and double-step procedures. For particular reasons the single-step procedure on auxin-free medium may be prefered (Dublin 1981; Yasuda *et al.* 1985; Hatanaka *et al.* 1991; Aponte Acuna 1993). It possesses the advantage of rapid embryo formation, high embryo germination

rate, and may function on genotypes recalcitrant for HFSE. The interest of HFSE arising from the double-step procedure, however, is clear with regard to large scale production of somatic embryos and the use of embryogenic suspensions for genetic transformation purposes. For some genotypes the HFSE process may be the sole procedure for obtaining of somatic embryogenesis, because of their recalcitrancy to "direct" SE (Bieysse *et al.* 1993).

In this report we have described a HFSE procedure, applied successfully on leaves of genotypes of *C. canephora*, Arabusta, Congusta and *C. arabica*. It appeared that response to the HFSE procedure is determined by factors like genotype, medium composition, occurrence of polyphenolic oxidation and endogenous hormone levels.

HFSE-response differed between species, being *C. arabica* less reactive, and also within species, as demonstrated by the HFSE variability among clones within the *C. canephora* species and within Arabusta. The composition of media in the HFSE procedure described by Söndahl & Sharp (1977), seems to be better adapted for HFSE-callus formation on *C. arabica* genotypes. Lower levels of polyphenolic oxidation of tissue were in general favorable for subsequent HFSE-callus formation.

Strong indications were found that the reaction of a genotype to a culture medium is due to the combination of exogenously applied growth regulators and endogenous hormone metabolism. A blockage in embryogenic callus formation of recalcitrant genotypes may be a result of a relatively high auxin/cytokinin ratio after one month of culture on **C**-medium. This presumption was supported by the results of preliminary experiments carried out in our laboratory. Determination by high pressure liquid chromatography (HPLC) of endogenous hormone levels in callus developed on **C**-medium, indicated that the auxin/cytokinin ratio at day 30 in callus of a recalcitrant genotype was 3-fold higher than the ratio in callus of a relatively well-reacting genotype. Controlled lowering of the ratio at this particular moment by transfer of explants to **E**-medium, permitted development of HFSE-callus on the latter genotype. It is known that in order to obtain optimal SE-response in a successive medium procedure, the medium must be changed when the callus contains a great number of typically embryogenic cells (Michaux-Ferrière & Schwendiman 1992). An increase of cytokinins or reduction of auxins in **C**-medium, combined to early transfer to **E**-medium, may be a support for stimulating embryogenic capacity of recalcitrant genotypes.

Two objectives for callus suspension cultures in liquid medium were described, leading to different culture procedures. Firstly, we have shown that for a long-term maintenance of embryogenic strains an initial density of 5-10 gram fresh weight per liter, with a monthly renewal of medium, was optimal. Depending on the quality and character of culture, part of the conditioned medium may be retained. Light intensity during culture seemed to be of less importance, but in order to avoid oxidation of polyphenols, tempered light is recommended. In this way, embryogenic potential of *C.*

canephora suspensions could be maintained for at least 24 months. Secondly, when a large quantity of callus mass is needed, e.g. for propagation or for genetic transformation, rapid suspension growth is desired. An initial density of 10-15 $g.l^{-1}$ was optimal, with a 10 days complete renewal of medium during which culture density must be readjusted to 10-15 $g.l^{-1}$. Light intensity during culture must be kept relatively low (less than 5 $\mu mol.m^{-2}.s^{-1}$). For both objectives aggregate sizes of 250-1000 µm appeared to be optimal for nutrient absorption and maintenance of embryogenic potential.

Embryogenic supensions could be directed to embryo formation and subsequent ontogenesis, by omission of auxin and increase of cytokinin dosis in the medium, combined with a lowering of culture density (1 $g.l^{-1}$) and increase of light intensity (5-10 $\mu mol.m^{-2}.s^{-1}$). A histological study confirmed the unicellular origin of the produced HFSE-way embryos, which is an important reason for use of the procedure in genetic transformation systems.

The presented SE procedure for mass propagation takes about 7-8 months for *C. canephora* and Arabusta, and 9-10 months for *C. arabica*, counted from leaf explants to regenerated plantlets. The multiplication phase in liquid medium should be prolonged if higher production of embryogenic aggregates is required. But it must be taken into consideration that the more the undifferentiated cell state is maintained for a long period, the higher the risk of somaclonal variation seems to be (Smith & Street 1974). The gain in time (6 months) between this procedure and the SE procedure as described by Zamarripa *et al.* (1991) is due to the difference in time needed for culture establishment in liquid medium. Callus in their procedure originates from secondary culture on embryo induction medium (Yasuda *et al.* 1985), and is less abundant and less friable than the HFSE-callus described in our procedure. For this reason, optimal suspension quality could already be achieved 6 to 8 weeks after culture initiation in **CP**-medium.

Embryo maturation and conversion into plantlets are factors that might still be improved in the HFSE procedure. Michaux-Ferrière & Schwendiman (1992) reported that too rapid somatic embryo formation could induce abnormalities (low protein reserves, absence of shoot apices) due to embryo immaturity. Somatic embryo conversion rates should be optimized by a reconsideration of embryo maturation, especially the dessication and dormancy process. Actually we are trying to adapt our SE procedure to the SIT-system (Système en Immersion Temporaire) as described by Alvard *et al.* (1993). This system may permit HFSE from callus induction to plantlet regeneration in one and the same culture vessel, in which optimal conditions for embryo maturation can be well-controlled.

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CHAPTER 3

Isolation of viable coffee protoplasts and preliminary outgrowth studies

Jos van Boxtel

Abstract

For the development of a simple and reliable method of isolation of viable coffee protoplasts, different tissues were used as source and treated under various conditions. Use of non-embryogenic suspensions of *Coffea arabica* resulted in highest protoplast yield (36.8 x 10^6 $P.g^{-1}$ fresh weight) and viability (90%). Use of such suspensions is adequate as protoplast source, when subsequently experiments on transient expression of introduced reporter genes are carried out. Best performing isolation method tested was as according to Spiral & Pétiard (1991). Preliminary studies on outgrowth of isolated protoplasts showed best results when using embryogenic suspensions of *C. arabica* or *C. canephora* as starting material. Subsequent protoplast culture in liquid medium demonstrated best results. Thus, protoplasts restored cell walls and divided up to stage of protocolony formation, two months after isolation, but no further outgrowth was obtained.

Introduction

Since Vardi *et al.* (1975) described the regeneration of citrus protoplasts, several other protoplast regeneration procedures with woody species have been published (for a recent overview see Roest & Gilissen 1993). Sources used for protoplast isolation are leaves, callus or embryogenic suspension cultures, and in 90% of the reports, protoplast culture of woody species was carried out in liquid media.

Many studies on protoplasts of tropical tree species have been carried out, but only recently some resulted in complete regeneration (Manders *et al.* 1991).

First studies on coffee protoplasts were realized by Söndahl *et al.* (1980) and Orozco & Schieder (1984); they achieved regeneration up to callus stage, but no regeneration into plantlets. Reports of Yasuda *et al.* (1986) and Acuna & de Pena (1987) did not give detailed descriptions of used procedures. Schöpke (1989) carried out a thorough study with several isolation and regeneration conditions. Among different plant materials tested as protoplast source, the use of somatic embryos of *Coffea canephora* permitted the regeneration into plantlets. However, the regenerated plantlets suffered from anomalities and transfer to greenhouse conditions was not possible.

During development of the present study, three other procedures, describing the regeneration of coffee protoplasts came available. Spiral & Pétiard (1991) reported plant regeneration of embryogenic suspension-derived protoplasts of *C. arabica, C. canephora* and Arabusta. It took 11 months from protoplast to plantlet stage. Acuna & de Pena (1991) reported a procedure in which 10 months were needed for regeneration and subsequent transfer to soil of plants obtained from embryogenic suspension-derived *C. arabica* protoplasts. Barton *et al.* (1991) reported protoplast isolation, electroporation and regeneration of two *C. arabica* plantlets, which

subsequently died.

Very recently Grèzes *et al.* (1994) described a protoplast isolation procedure from non-embryogenic *C. arabica* suspensions and Tahara *et al.* (1994) reported regeneration of fertile plants from embryogenic *C. arabica* callus-derived protoplasts, using BA as the sole growth regulator.

The present study tried to establish a reliable protoplast isolation procedure for subsequent use in electroporation experiments. Moreover, studies on conditions required for protoplast regeneration were performed. Several conditions were tested: different plant sources, isolation procedures (pretreatments, enzyme combinations, protoplast purification media) and culture procedures (media, densities and methods).

Materials and methods

Preparation of protoplast sources

Young leaves from greenhouse grown plants or *in vitro* microcuttings were cut in fine ribbons in CPW-salts (Frearson *et al.* 1973) with 0.4 M sucrose. After rinsing in similar solution, slices were transferred to enzyme solution.

The obtaining of friable HFSE-callus from leaf explants was according to the method described by Berthouly & Michaux-Ferrière (submitted). HFSE-callus was cultured on solid embryo expression medium, **E**-medium, and used for incubation in enzyme solution, without any pretreatment.

Initiation, maintenance and regeneration of embryogenic suspension cultures from HFSE-callus was done following the standard method described by Van Boxtel & Berthouly (submitted). Suspension cultures were maintained in medium for proliferation of embryogenic callus, **CP**-medium, and subcultured each 20 days. Callus suspensions, at least 3 months old and 7-12 days after last subculturing, were sieved over 500 µm and the fine callus fraction was incubated in enzyme solution.

Immature somatic embryos regenerated from embryogenic suspensions in regeneration medium, **R**-medium, according to Dublin (1984), were sliced after a 100 min preplasmolysis procedure in modified Gamborg *et al.* (1968) medium, B5-1 (Schöpke *et al.* 1987). After rinsing in similar solution, slices were transferred to enzyme solution.

Standard protoplast isolation procedure

One gram of prepared plant material (see above) was incubated in 15 ml enzyme mixture solution during 16 hours on a rotary shaker (40 rpm), at 27°C in obscurity. Enzyme mixture consisted of 1% Cellulase R10, 0.5% Macerozyme R10 and 0.4 M sucrose in CPW-salts. Released protoplasts were sieved over a 50 µm stainless steel mesh and washed three times by spinning them down in CPW-salts + 0.4 M sucrose. Centrifugation was performed once during 10 min at 1000 rpm and twice 5 min at 600 rpm. All protoplast preparation media were filter sterilized.

Variables tested in protoplast isolation procedure

- Premaceration treatments:
  - *addition of 100 mg.l^{-1} L-cystein to leaf maceration medium or preplasmolysis medium, and subsequent washing in order to inhibit polyphenolic oxidation (Butt 1985),
  - * mechanical grinding of leaf tissue.

- Enzyme mixtures (for 16 h incubation):
  * 0.25 % Cellulase R10 + 0.25% Driselase + 0.05% Pectinase in CPW-salts,
  * 0.5% Cellulase R10 + 0.2% Macerozyme R10 + 0.05% Pectolyase Y23 in salts (Spiral & Pétiard 1991).
- Short incubation period of 5-6 hours (only for callus), with enzyme mixture four-fold concentrated.
- Osmotic pressure:
  * mannitol 0.4 M, 0.5 M or 0.6 M.
- Sieving over 38 µm stainless steel mesh.
- Washing procedure:
  * W5 salt-sugar solution (Menczel *et al.* 1981)
  * CPW-salts with high sucrose gradient of 0.6 M or 0.7 M,
  * modified Blaydes (1966) medium, BL, with 0.51 M glucose + 0.06 M sucrose (Spiral & Pétiard 1991).
- Centrifugation procedure:
  * 8 min at 800 rpm + 2 x 5 min at 600 rpm,
  * 8 min at 1000 rpm + 2 x 5 min at 600 rpm,
  * 10 min at 12000 rpm + 2 x 5 min at 750 rpm,
  * 12 min at 900 rpm + 2 x 5 min at 750 rpm,
  * 3 x 5 min at 750 rpm,
  * 3 x 5 min at 600 rpm,
  * 3 x 7 min at 500 rpm.

Standard protoplast culture procedure

Purified protoplasts (1.5 ml) in modified Gamborg (1968) medium, B5-2, containing 0.51 M glucose (Schöpke *et al.* 1987), were cultured in ⊖ 3 cm plates, at a density of 5 $x10^5$ $P.ml^{-1}$, 27°C in obscurity. Each week 0.5 ml B5-2 medium, containing 0.46 M glucose was added. Cultures were transferred to ⊖ 5.5 cm plates after one month and kept further at dim light (2-3 $\mu mol.m^{-2}.s^{-1}$). Hereafter, weekly dillution was performed by adding 0.5 ml B5-2 medium with 0.36 M glucose. All protoplast culture media were filter sterilized.

Variables tested in protoplast culture procedure

- Plating density:
  * 5 x 10^4 $P.ml^{-1}$
  * 1 x 10^5 $P.ml^{-1}$
  * 2 x 10^5 $P.ml^{-1}$
  * 4 x 10^5 $P.ml^{-1}$
  * 5 x 10^5 $P.ml^{-1}$
- Medium type:
  * liquid,
  * semi-solid (agarose 0.6 or 0.8 %), applied directly or 2 days after isolation.
- Culture conditions:
  * in dim light (3-4 $\mu mol.m^{-2}.s^{-1}$),
  * under agitation (30 rpm), in order to avoid protoplast agglomeration in centre of plates,
  * protoplasts on membrane superposed on semi-solid medium layer,
  * protoplasts on membrane floating on liquid medium.
- Culture media:
  * TM_2G medium, 0.51 M glucose + 0.08 M sugars (Shahin 1985),
  * BL medium, 0.51 M glucose + 0.06 M sucrose.
- Medium renewal with:
  * TM_2G medium, 0.46 M or 0.36 M glucose + 0.08 M sugars,
  * BL medium, 0.3 M or 0.2 M glucose + 0.06 M sucrose.

- Subculture conditions:
    * moment of medium renewal (weekly, 2-weekly or monthly),
    * moment of transfer to light (after 2 weeks, 1 month or 2 months).
- Other conditions:
    * separation of dead cell remainders in cultures by centrifugation at day 10.

Observations realized

Protoplast counting with a light microscope was performed by using a haemocytometer and fluorescein diacetate (FDA) staining (Widholm 1972). Staining with phenosafranine was performed for evaluation of cell mortality. Cell wall formation was visualized by Calcofluor White staining (Constabel 1975). Cell survival was expressed in the number of weeks until viable cells could be detected in culture.

Results and discussion

Factors influencing protoplast yield and viability

Protoplast yields obtained from suspension cultures, HFSE-callus, somatic embryos and leaves are shown in Table 1. Yields with HFSE-callus and embryogenic suspensions were superiour to the yields reported by Söndahl *et al.* (1980) and Acuna & de Pena (1991), which were respectively 8.7 x 10^5 P.g^{-1} by using HFSE-callus and 7.5 x 10^5 P.g^{-1} with embryogenic suspensions. Schöpke *et al.* (1987) obtained 4.5 x 10^6 P.g^{-1} fresh weight from somatic embryos of *C. canephora*. Because of large quantities of regenerable protoplasts being required, embryogenic suspension cultures of *C. arabica* and *C. canephora* appear best suited as source for regeneration studies. On the other hand, the high yields obtained from suspensions of non-embryogenic character, could make this tissue an excellent source for transient expression studies, as regeneration is not required.

Table 1. Maximum yields obtained with protoplast isolation procedures from different coffee plant tissue sources.

Protoplast source	Protoplast yield (x 10^6 P. g^{-1} fresh weight)
C. arabica greenhouse grown leaves	0.08
in vitro leaves	2.0
embryogenic callus suspensions	22.0
non-embryogenic callus suspensions	36.8
somatic embryos	4.6
C. canephora friable HFSE-callus	26.5
embryogenic callus suspensions	35.0

Viability of leaf protoplasts was enhanced by addition of L-cystein to the maceration solution and subsequent tissue washing. According to Butt (1985) protoplasts of deciduous tree species could only be released if chopped leaf tissue was thoroughly washed prior to addition of digestive enzymes. It is thought that polyphenolic compounds released by chopping inhibit cell-wall digestion.

The enzyme mixture consisting of only one pectinase (Macerozyme R10) and one cellulase (Cellulase R10) did not result in a satisfying protoplast release. Many undigested tissue structures remained after incubation. The combination of two pectinases (Macerozyme R10 and Pectolyase Y-23) with one cellulase (Cellulase R10) performed better than the combination of one pectinase (Pectinase) with two cellulases (Driselase and Cellulase R10). Replacement of Cellulase R10 by Cellulase RS further enhanced yield and viability. In accordance with Tahara *et al.* (1994), mannitol in concentration of 0.5 M served best as osmotic regulator during incubation. When callus tissue was used as protoplast source, a short incubation period (5-6 hours) in a four-fold concentrated enzyme mixture solution was sufficient. Since enzymes may become toxic for protoplasts when remaining in prolonged contact, long-term incubation has to be avoided for maintenance of viability.

CPW osmoticum (Frearson *et al.* 1973), W5 washing solution (Menczel *et al.* 1981) and TM_2G culture medium (Shahin 1985) is a media-sequence developed for isolation and regeneration of *Solanacea* protoplasts (tobacco, tomato, petunia and potato). Application of this media-sequence to the coffee protoplast system did not improve results, in relation to sequences based on modified B5-medium (Gamborg *et al.* 1968) or BL-medium (Blaydes 1966). B5-medium is rich in sugars and vitamins, BL-medium is rich in macro- and micro-elements. Both these media were developed for soybean tissue culture.

For washing by a centrifugation/precipitation procedure, mannitol at 0.5-0.6 M was used as osmoticum. But protoplasts were damaged while spunn down in pellet in the centrifugation tube, and firmly performed resuspension was often required. Lowering of centrifugation speed to 750 rpm showed a slight improvement for protoplast viability. Using 0.6 M sucrose in the washing medium for achieving protoplast floatation by high gradient centrifugation did not result in a proper purification, while 0.7 M sucrose provoked plasmolysis. Due to their small and dense character, floatation of coffee protoplasts seemed to require centrifugation in a high density solution. Orozco & Schieder (1984) used therefore 70% Percoll dissolved in 0.6 M mannitol as washing solution. Acuna & de Pena (1991) achieved floatation of protoplasts in a 40% Percoll solution in 0.51 M glucose.

An important improvement appeared to be the sieving over a 38 µm mesh instead of a 50 µm. The homogenous population of small dense cells obtained in this way showed higher viability and more rapid divisions than larger cells. Cell survival, however, was not prolonged.

Optimal plating density was dependent on the protoplast viability and applied culture method. A density of 2 x 10^5 P.ml^{-1} in liquid medium was preferable for high cell survival. This is in agreement with other reports on coffee protoplasts (Schöpke *et al.* 1987; Spiral & Pétiard 1991; Acuna & de Pena 1991). Too rapid dilution and reduction of plating density (10 days after isolation) showed not to be favorable for protocolony formation.

The composition of the culture medium seemed to be of less importance, since results obtained with the different media applied did not vary substantially. On the other hand, choise of culture method was important in improving outgrowth of protoplasts. Culture in liquid medium always resulted in an extreme agglomeration of protoplasts in the centre of plates. This agglomeration of protoplasts resulted in formation of floating aggregates or chains, constituted of dead cells. A slight agitation was applied to prevent this phenomenon; however increased mortality was then obtained. It was tried to eliminate dead aggregates from 10 days-old cultures by centrifugation and precipitation. But since this led to cell damage without satisfying purification, also here a centrifugation on Percoll gradient might have been more appropriate. Embedding in agarose Sea-plaque, either directly after purification or later, led to increased necrosis compared to liquid culture. Protoplasts spread out over a membrane, superposed on a semi-solid medium layer, were immerged in a thin film of liquid medium. Some divisions were observed but formation of protocolonies did not occur. A similar result was obtained when the membrane with protoplasts on it, was placed floating on liquid culture medium. Following this method Russel & McCown (1986) had been able to regenerate mesophyll-derived *Populus* protoplasts. As a side step, experiments were carried out for evaluating the possibilities of nurse culture on a layer of embedded cells. Therefore non-embryogenic cell suspensions of *C. arabica* cv Caturra were included in 0.8% agarose in different densities (40, 80 and 120 ml of packed cell volume per liter culture medium). However, embedded cells necrosed rapidly and thus did not seem appropriate for a nurse culture feeder layer.

No clear results were obtained on the optimal moment and amount of osmolality reduction during subculturing. In general, a too rapid decrease (10 days after isolation) was not favorable.

It was clearly shown that initial culture of protoplasts has to be carried out in obscurity for at least one month. Transfer of cultures to light during the first month led always to rapid necrosis. It was not determined which developmental stage was best for transfer of cultures to light conditions.

By coincidence, a stimulatory effect of an antibiotic treatment on protoplast viability was observed. HFSE-callus of *C. canephora* obtained on medium **C** for callus induction (Berthouly & Michaux-Ferrière, submitted), supplemented with cefotaxime, was used for protoplast isolation, under optimized conditions as mentioned below. Fig. 1 shows that highest yield of viable protoplasts was obtained when HFSE-callus was

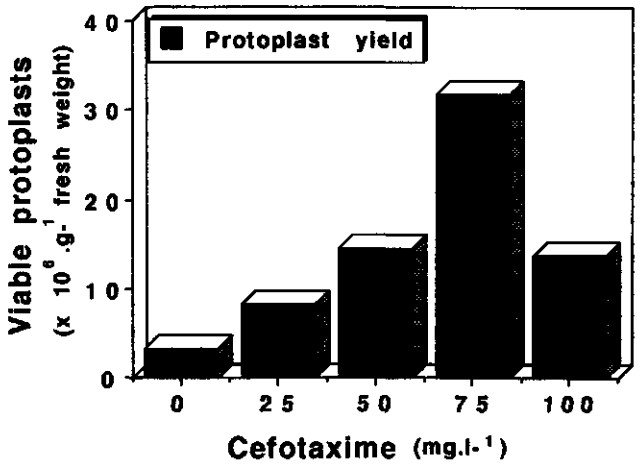

Fig. 1. Effect of cefotaxime-induced HFSE-callus on subsequent isolation of viable protoplast yield of *C. canephora*. HFSE-callus was obtained from leaf explants cultured during one month on cefotaxime-containing callus induction medium and 2 months on cefotaxime-free embryo induction medium.

induced on 75 mg.l^{-1} cefotaxime-containing C-medium. Cefotaxime-induced callus formation for protoplast isolation, can be considered as a viability-enhancing pretreatment. But since HFSE-callus formation takes 3-4 months, this pretreatment has low practical value. Whether or not an enhancing effect of cefotaxime application also exists during protoplast culture, needs further investigations. d'Utra Vaz *et al.* (1993) showed an enhancing effect of 50-250 mg.l^{-1} cefotaxime on cell divisions in passionfruit protoplast culture.

Optimal isolation and culture conditions

For analyzing differences between tested conditions, the parameter "duration of cell survival" was used as criterion. A description of conditions resulting in longest cell survival is given below.

A one year-old embryogenic suspension culture of *C. arabica* cv Caturra, was sieved over 500 µm and incubated in enzyme mixture (2% Cellulase RS, 1% Macerozyme R10 and 0.2% Pectolyase Y-23) in 0.5 mM MES, 25 mM $CaCl_2.2H_2O$, 0.5 M mannitol and pH 5.5 (Spiral & Pétiard 1991). Incubation lasted 6 hours at 27°C in obscurity under 40 rpm agitation. Protoplasts were sieved over 38 µm and washed in BL-medium by 3 x centrifugation of 7 min at 500 rpm. Protoplast yield was 2.2 x 10^7 P.g^{-1} fresh weight, with a viability of 52%. They were resuspended in

BL-medium at a density of 5 x 10^5 viable $P.ml^{-1}$, and 1 ml of it was cultured in a ∅ 3 cm plate, at 27°C in obscurity. After one day, first cell wall formations were observed and after four days first cell divisions. One week after isolation viability had decreased to 34%. Hereafter, protoplast viability was difficult to determine, due to agglomeration. Four weeks after isolation, 1 ml BL-medium (0.3 M glucose) was added to cultures, and again 4 weeks later 1 ml of culture medium was replaced by 1 ml fresh BL-medium (0.2 M glucose). At that moment, some cultures showed regenerative development and clumps of 20-30 cells had been formed, which can be considered as protocolonies. Two weeks later, however, all protocolonies consisted of dead cells only. Thus, longest cell survival achieved was 9 weeks.

The better isolation conditions were also those which resulted in highest protoplast yield and viability. With a 16 month-old non-embryogenic suspension of *C. arabica* cv Caturra, 36.8 x 10^6 $P.g^{-1}$ fresh weight were obtained with a 90% viability.

Conclusions

The present studies were successful in obtaining high yields of viable protoplasts. However, the best result on outgrowth studies was the obtaining and maintenance of viable protocolonies only up to two months of culturing. Despite the many different parameters tested, complete regeneration was not achieved. As explained in the scope of the study (chapter 1.5), it was decided for different reasons to orientate the study to biolistic methods. Therefore, the research on protoplast outgrowth should be considered as incomplete.

For obtaining good quality protoplasts, the importance of adequate starting material is evident. Our results showed that suspension cultures of highly embryogenic character in exponential growth stage are most appropiate. For maceration, an enzyme mixture consisting of two macerases and one cellulase in a 1:2 proportion performed best. Sieving over 38 µm resulted in populations of small dense and more viable protoplasts. Since purification by centrifugation/precipitation resulted in protoplast damage, a floatation procedure in a high density gradient is preferable. For this goal, use of Percoll dissolved in culture medium, should be considered. This type of improvement of purification procedure might also contribute to a better regeneration capacity.

The physical way of culturing is crucial for a potential outgrowth of protoplasts. Embedding in agarose, directly after isolation led to cell wall restoration and some cell divisions. With culture in liquid medium, agglomeration occurred in our experiments, although this method may result in protocolony formation (Spiral & Pétiard 1991). Since subsequent further outgrowth in liquid medium was not observed in our studies, growth enhancement might be obtained by embedding of the

protoplasts in agarose at a certain stage. Tahara *et al.* (1994) reported success with embedding in gelrite directly after isolation, while Schöpke *et al.* (1987) and Acuna & de Pena (1991) used addition of agarose after 5 and 20 days of culture, respectively.

Because of the described difficulties and long duration for regeneration of coffee protoplasts, their use for achievement of genetic transformation, appears less recommendable. The most successfull procedures mentioned regeneration in 9-12 months at low efficiencies (Spiral & Pétiard 1991; Acuna & de Pena 1991; Tahara *et al.* 1994). Application of electroporation on protoplasts further reduces regeneration capacity (Barton *et al.* 1991; chapter 4). Hidano & Niizeki (1988) described in a review article regeneration of deciduous fruit tree protoplasts and their use for genetic transfer of traits. In their point of view, protoplasts offer more perspectives for somatic hybridization than for direct gene transfer.

Nevertheless, in all coffee studies the isolation of large quantities of viable protoplasts has been reported. Use of non-embryogenic suspensions even resulted in higher protoplast yields compared to embryogenic suspensions (Grèzes *et al.* 1994; chapter 4 of this work). Since outgrowth is not required, the use of coffee protoplasts for transient expression studies may be of some practical value. If the physical conditions (field strength and capacitor charge) of protoplast electroporation for optimal transient expression of an introduced reporter gene are known, the system can then be applied for studies of promoter strengths.

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CHAPTER 4

Transient expression studies of β-glucuronidase in electroporated coffee protoplasts

Jos van Boxtel

Abstract

This report describes the use of coffee protoplasts, derived from embryogenic or non-embryogenic callus suspensions, for transient expression studies of genes introduced by electroporation. Physical electroporation conditions (electric field strength and capacitor charge) were optimized using fluorometrically assayed transient expression of the introduced GUS gene. Because of the high yield (3.3 x 10^7 $P.g^{-1}$ fresh weight) and viability (90.2%), non-embryogenic suspensions were a preferable source for protoplast isolation with subsequent transient expression studies. GUS activity reached a maximum level 6-9 days after electroporation, which is long in comparison to other plant species. Discrimination between observed "false" GUS activity and introduced plasmid GUS activity was, therefore, not reliable until six days after electroporation. A capacitor discharge of 250 μF by three exponential decaying pulses under 250-350 $V.cm^{-1}$ field strength was optimal for highest transient GUS expression. An increase of these variables was not related to a decrease in protoplast viability after electroporation. Viability in embryogenic suspension-derived protoplasts was enhanced by treatment under 150 $V.cm^{-1}$ field strength, compared to lower or higher values. With this optimization of physical electroporation conditions, coffee protoplasts can now be used for studies on promoters.

Introduction

Protoplasts can be isolated in large quantities from different plant tissues and organs. They have shown the ability to synthesize new cell walls and to regenerate complete plants when cultured in the appropriate media. Therefore, protoplasts are regarded as totipotent cells, which are well suited for genetic manipulation studies (Roest & Gilissen, 1993). Especially their use for the analysis of promoter structures and function, by measuring temporary expression of chimeric gene constructs, so-called transient expression, has been proven a powerful tool (see Chang *et al.* 1992, for a recent overview). Transient expression does not require the regeneration of treated protoplasts. A short culture time (± 2 days) has appeared sufficient for transient expression to be detected in most species (Van der Steege 1991). Under some conditions, however, exogenous DNA expression in protoplasts was not detectable as early as 4 days after uptake (Huttly & Baulcombe 1989). Stable transformation on the other hand requires the potency of protoplasts to regenerate, in addition to the ability to integrate the introduced DNA into the plant genome. For this purpose, protoplasts should thus be competent to both processes.

Electroporation was initially developed to achieve DNA transfer into animal cells (Neumann *et al.* 1982), and was later successfully applied to plant protoplasts (Fromm *et al.* 1985; Shillito *et al.* 1985) or enzymatically wounded plant tissues

plant tissues (Dekeyser *et al.* 1990; D'Halluin *et al.* 1992). In this system, optimal transfer conditions seem to be protoplast type- and apparatus-dependent and have to be established for specific purposes (Van der Steege & Tempelaar 1991). The efficiency of conditions leading to DNA transfer can be measured in terms of transient expression levels, which may not only vary between species, but also between different genotypes of particular species (Hobbs *et al.* 1990).

Procedures for the isolation and regeneration of coffee (*Coffea* sp.) protoplasts were described by Schöpke *et al.* (1987), Spiral & Pétiard (1991) and Acuna & de Pena (1991). Barton *et al.* (1991) reported *C. arabica* protoplast isolation, electroporation and transformation. Southern blot analysis confirmed the stable transformation character in one plantlet, which was unable to survive. These four procedures mentioned for coffee were relatively inefficient with regard to regeneration capacity, compared to other plant species (see for review Roest & Gilissen 1993), being suspension quality-dependance and the long regeneration time (10 to 15 months) some of the major reasons. However, the ease of coffee protoplast isolation may make this tissue appropriate for determination of promoter strength and function in transient expression studies.

The objective of the present study was optimization of electroporation parameters (physical and physiological), which subsequently can be used in a promoter analysis. Transient expression of the β-glucuronidase reporter gene in *C. arabica* protoplasts was measured at various time intervals after electroporation, using different protoplast sources (embryogenic and non-embryogenic suspension cultures) and variable capacitor discharges and electric field strengths.

Materials and Methods

Plasmid isolation

Plasmid pCH1 was provided by Laboratoire Biologie Cellulaire, INRA/Versailles, France. pCH1 contains the *uidA* gene, coding for β-glucuronidase, under control of the CaMV 35S promoter with doubled enhancer part (Kay *et al.* 1987). Plasmids were propagated in Luria-Broth cultured *E. coli*, isolated and purified according to Birnboim, as described in Sambrook *et al.* (1989).

Callus suspensions and protoplast preparation

Initiation of suspension cultures from HFSE-callus was realized as described by Van Boxtel & Berthouly (submitted). A 13 months-old embryogenic and a 16 months-old non-embryogenic callus suspension of *C. arabica* cv Caturra were used as sources for protoplast isolation. Suspension cultures were maintained in liquid medium **CP** for callus proliferation and subcultured each two weeks. After sieving over a 500 µm mesh, one gram of the fine callus aggregates was incubated in 15 ml enzyme mixture (Spiral & Pétiard 1991) during 5 hours on a rotary shaker (40 rpm), at 27°C in obscurity. Released protoplasts were sieved over a 38 µm stainless steel mesh and washed once with 25 ml Blaydes medium (1966), modified for coffee protoplast culture by Spiral & Pétiard (1991). After

centrifugation during 5 min at 500 rpm, protoplast pellet was resuspended in electroporation buffer (EB) at a final concentration of $2x10^6$ P.ml^{-1}. EB consisted of 5 mM MES, 10 mM $MgCl_2$ and 0.6 M mannitol, at pH 6.0.

Determination of protoplast viability
Protoplast counting with a light microscope was performed by using a heamocytometer and viability was determined using fluorescein diacetate (FDA) staining (Widholm 1972).

Electroporation conditions
Electroporation device was constructed at Laboratoire Biologie Cellulaire, INRA/Versailles, France and was described by Guerche (1988). Power supply was regulated by a SEBIA GD251E generator (Paris, France). One ml of protoplast suspension was mixed with 35 µg of plasmid DNA (supercoiled pCH1) and 40 µg of Calf-thymus DNA in the 1 x 1 x 1 cm electroporation cuvette. Different capacitor charges were discharged into the cuvette by performing three exponentially decaying pulses (of about 100 ms) at 5 sec intervals (parameters determined in pilot studies) under variable field strenghts. Controls were not electroporated. All treatments were carried out twice. After electroporation treatment, protoplasts were transferred to ∅ 55 mm plates and 10 min later, modified Blaydes medium was added upto a final concentration of $5x10^5$ P.ml^{-1}. Plates were sealed and kept in dark at 27°C. Viability of protoplast cultures was measured six days after electroporation by FDA staining.

Fluorimetric GUS assay
Protein extraction and quantification, and fluorimetric measurement of GUS activity were carried out 3, 6, 9, 12 and 16 days after electroporation. Therefore, protoplasts were washed twice with washing buffer, containing 0.3 M KCl and 5 mM $CaCl_2$, at pH 5.6, and pellet was resuspended in 200 µl GUS extraction buffer as described by Jefferson (1987). After transfer to microtubes, proteins were extracted by moderate sonication during 30 sec, and supernatant was recovered after 5 min centrifugation at 10,000g. After addition of 1 mM 4-MUG-containing GUS assay buffer (Jefferson 1987) in extract volumes, containing 200 µg proteins, samples were incubated at 37°C. At regular intervals (0, 60 and 120 min) reactions were stopped by adding 2 ml 0.2 M Na_2CO_3. GUS activity was quantified in a fluorimeter TKO100 (Hoefer, San Francisco, USA) at 365 nm excitation/456 nm emission. Protein concentration in extracts was measured by bicinchoninic acid assay (SIGMA) according to Smith *et al.* (1985) on a spectrophotometer Shimadzu UV-1200 (Kyoto, Japan).

Results and Discussion

Protoplast yields

Average yield from embryogenic callus suspensions was $1.55x10^7$ protoplasts per gram fresh weight, with viability of 31.2%. This differs from results obtained by Acuna & de Pena (1991) using embryogenic suspensions of the same genotype: $7.5x10^5$ P.g^{-1} with 80% viability. The lower viability obtained in our experiments may be due to the fact that part of the embryogenic suspensions used, apparently consisted of cells, less competent for viable protoplast release. The low quality protoplasts resulting from it, appeared not sufficiently removed during the subsequent protoplast purification

Table 1. Variation in GUS activity of non-embryogenic callus suspension-derived *C. arabica* protoplasts at various time intervals after electroporation with a GUS-containing plasmid. Data are average results of 20 electroporations with 50-250 μF capacitor discharge under 100-350 $V.cm^{-1}$ field strength. Controls were not electroporated.

Days after electroporation	GUS activity (nmol 4-MU.min^{-1}.mg^{-1} protein)	
	Electroporated	Controls
3	1	0.5
6	3-10	1
9	5-15	1-2
12	4-7	1
16	3-9	1

procedure. Embryogenic coffee calli cultured in liquid medium are characterized by hard crumbly and relatively large aggregates. In studies of Zamarripa (1993) and Van Boxtel & Berthouly (submitted), size of *C. arabica* calli appeared to be between 100 and 1500 μm, with most viable aggregates being found between 200 and 1000 μm. In order to allow correct penetration of enzymes, isolation of protoplasts as described here was carried out with the fraction below 500 μm, which might implicate that a large portion consisted of less viable cells. Protoplast isolation from embryogenic callus suspensions of *C. arabica* may therefore be optimized by 1) using aggregate size between 200 and 500 μm, 2) performing a preplasmolysis for maceration of aggregates, and 3) improving purification procedure.

Non-embryogenic suspensions yielded in average $3.3x10^7$ protoplasts per gram fresh weight with 90.2% viability, which is respectively 2- and 3-fold higher than that of protoplasts obtained from embryogenic suspensions of this genotype. Recently Grèzes *et al.* (1994) reported protoplast yield of $5.1x10^6$ per gram fresh weight with 90% viability, using non-embryogenic suspensions of *C. arabica.* In our study, the larger portion of the soft non-embryogenic calli in liquid culture medium, had an aggregate size below 500 μm, and consisted each of 1-50 cells. Compared to embryogenic suspensions, the fraction below 500 μm of non-embryogenic suspensions is of higher viability, and protoplast isolation from this tissue is more efficient.

GUS activity at different time intervals after electroporation

Analysis of GUS activity in non-embryogenic suspension-derived protoplasts, at various time points after electroporation, showed highest level at day nine (Table 1). As already noticed in previous experiments with coffee (data not shown), also here the

level of GUS expression 2 or 3 days after electroporation was low and did not differ significantly from unelectroporated control treatments. Better discrimination between GUS activity levels was observed at day 6 or 9. It may be that, due to low metabolic activity of *C. arabica* protoplasts, combined with the persistent nature of the synthesized β-glucuronidase enzyme, GUS acitivity was highest only 6 to 9 days after DNA uptake. But on the other hand, it has been reported that once DNA has entered the cells, it is subject to rapid degradation, due to the action of nucleic acid degrading factors (Pröls *et al.* 1988).

Treatment of protoplasts from embryogenic suspensions

Results of measurements (viability at day 6 and GUS activity at day 9) are shown in Table 2. At day 6, viability had decreased under all electroporation conditions. But also control treatments showed a considerable decrease in viablity, an indication for moderate quality of suspensions used as protoplast source. It is not clear why viability in 150 $V.cm^{-1}$ treatments was higher than in control treatments. A relation with reported enhancement of division of cultured protoplasts by electroporation treatment (Rech *et al.* 1987), may exist.

Fungal contaminated treatments (150 $V.cm^{-1}$; 150 μF) showed no viability and GUS activity was low in relation to other treatments. GUS activity in protoplasts was highest when electroporated with 200-250 μF capacitor discharge under 200 $V.cm^{-1}$ field strength. Although differences between treatments were not significant, a tendency seemed to exist for higher transient GUS expression levels under more severe

Table 2. GUS activity of embryogenic callus supension-derived *C. arabica* protoplasts, 9 days after electroporation with a GUS-containing plasmid. Viability before electroporation was 31.2 %. Controls were not electroporated.

Electroporation conditions	Protoplast viability (%)[a] at day 6	GUS activity[b] at day 9 (pmol 4-MU.min^{-1}.mg^{-1} protein)
150 $V.cm^{-1}$, 50 μF	25	1185.0
, 150 μF	0[c]	150.0[c]
, 200 μF	15	881.5
, 250 μF	10	931.1
200 $V.cm^{-1}$, 100 μF	5	574.7
, 150 μF	<5	634.8
, 200 μF	<5	1443.8
, 250 μF	<5	1366.9
Controls	5	11.3

[a] estimation by two FDA staining assays.

[b] average of two fluorometric assays, background fluorescence substracted.

[c] contaminated cultures.

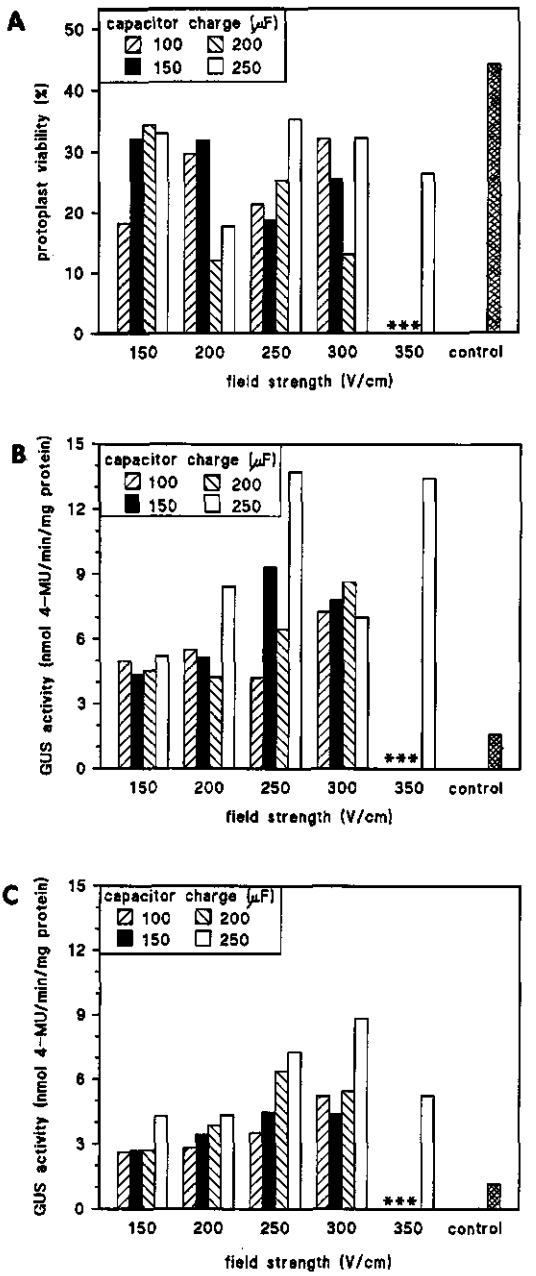

Fig 1. Effect of capacitor discharge and electric field strength on A) viability at day 6, B) GUS activity at day 9 and C) GUS activity at day 16 after electroporation of non-embryogenic suspension-derived *C. arabica* protoplasts with a GUS-containing plasmid. Protoplast viability before electroporation was 90.2% (* not executed).

electroporation conditions. These conditions on the other hand, were negatively related to viability after electroporation. Van der Steege & Tempelaar (1991) showed that in spite of the dramatic loss of viable protoplasts, transient expression experiments are better served with fairly severe electrical conditions.

Treatment of protoplasts from non-embryogenic suspensions

In Figure 1a, b and c results of viability measurements and quantifications of GUS activities are shown. Firstly, comparison of GUS activity levels at day 9 of electroporated protoplasts from embryogenic (Table 2) to non-embryogenic suspensions (Fig 1b), revealed up to a 10-fold higher level of the latter. Probably differences in suspension quality was a major cause for this difference in GUS activity.

No clear relationship between discharged condensor capacity or applied field strength on one hand, and protoplast viability after six days on the other hand, was observed (Fig. 1a). Viability reduction was about 50% compared to non-electroporated control treatments. GUS activity after 9 days was highest when protoplasts had been electroporated with 250 μF capacitor discharge under 250-350 $V.cm^{-1}$ field strength (Fig. 1b). GUS activity after 16 days (Fig. 1c) was reduced by about 40% in comparison to that after 9 days. Highest transient expression however, was also here obtained by 250 μF capacitor discharge under 250-350 $V.cm^{-1}$ field strength. Due to high variability in results, differences between treatments were not significant.

Conclusions

Use of a direct gene transfer method as electroporation, allows rapid evaluation of biological activity of genes in plasmid constructs, without being restricted by host-range limitations of *Agrobacterium*-mediated transfer methods. The purpose of this study was to determine if electroporation was applicable for use with coffee and whether it was possible to optimize transient GUS expression. This was achieved by variation of protoplast source and electroporation parameters, and by assessment of GUS activity at various time intervals after electroporation.

It was shown that quality of suspension cultures is of major importance for isolating protoplast populations of good viability. When used for subsequent transient expression experiments, protoplasts derived from non-embryogenic suspensions may serve better than protoplasts derived from embryogenic suspensions. Besides their higher multiplication rate, non-embryogenic suspensions yield protoplasts in higher quantity and of higher viability.

In protoplasts derived from embryogenic suspensions, viability was enhanced by electroporation treatments using a 150 $V.cm^{-1}$ field strength, compared to untreated

protoplasts. Further studies are required to clarify whether or not this observation may have similarities to the effect of enhanced division and regeneration of protoplasts by electro-pulses, as described by Rech *et al.* (1987) and Ochatt *et al.* (1988).

The nature of undesired GUS expression in non-electroporated protoplasts observed in all experiments, could not be identified. This "false" GUS activity may be attributed to presence of endogenous bacteria, an often encountered problem in *in vitro* cultured tropical woody species (Tör *et al.* 1992; Van Boxtel *et al.* submitted), to intrinsic GUS-like activity, described in a range of plant species (Hu *et al.* 1990), or to contaminating GUS activity from the cell wall-digesting enzymes used in protoplast preparation (Jefferson 1987). Discrimination between "false" GUS activity and introduced plasmid related GUS activity was not reliable until six days after electroporation of non-embryogenic suspension-derived protoplasts. While GUS activity in untreated protoplasts remained more or less at the same level, in treated protoplasts it increased upto a maximum nine days after electroporation. Total GUS activity in treated protoplasts may therefore be considered as the sum of "false" and plasmid related GUS activity.

Highest GUS activity was observed nine days after electroporation, when protoplasts had been electroporated with 250 μF capacitor discharge under 250-350 $V.cm^{-1}$ electric field strength. Similar optimal field strenghts for transient expression were described by Guerche *et al.* (1987) in tobacco, by Tautorus *et al.* (1989) in jack pine and by Hobbs *et al.* (1990) in pea. Capacitor discharges on the other hand, are in general applied at higher levels for obtaining optimal transient expression (Chang *et al.* 1992). In further studies with coffee protoplasts, capacitor discharge can therefore be extended to higher values.

Although reports have described a rapid degradation of exogenous DNA once entered into the cells (Bates *et al.* 1990), we found highest GUS activity only 6 to 9 days after electroporation. It is known that the enzyme β-glucuronidase is quite stable once present in cells and can be detected a relatively long time, even in dead cells (Gaudin 1992). On the other hand, the period of transcriptional activity of transiently expressed foreign DNA may be promoter or plant species dependent. This is on its turn related to specific metabolic activity, which seems to be low in coffee protoplasts. With coffee callus suspensions and leaves, a delay of two days between biolistic treatment and histochemical GUS-assay has shown to be sufficient for discriminating between plasmid-induced GUS expression and controls (chapter 5). One week after bombardment, the level of transient expression was reduced by half in leaves (chapter 6). However, it must be taken in account that the histochemical GUS assay is less hindered by quantitative background activities. In other species, transcriptional activity has shown to vary between 30 min (PEG-treated tobacco protoplasts using CaMV 35S; Pröls *et al.* 1988), to 4 days (oat protoplasts using a wheat α-amylase promoter; Huttly & Baulcombe 1989).

In order to obtain stable transformation after electroporation of protoplasts, the ability to integrate DNA should be combined with the potency to regenerate. Therefore viability of protoplasts must remain at a reasonable level after electroporation treatment. In general, electroporation parameters aimed at stable transformation have to be chosen less severe, than those leading to optimal transient expression, in order to maintain protoplast viability at an acceptable level.

In this study optimal electroporation parameters for transient expression in *C. arabica* protoplasts have been explored. A rapid evaluation of strength of plasmid constructions, bearing promoters other than the enhanced CaMV 35S promoter tested here, is now more accessible. These results may be used for standardizing a system for the genetic transformation of coffee.

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CHAPTER 5

Transient expression of ß-glucuronidase following biolistic delivery of foreign DNA into coffee tissues

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Abstract

The availability of an efficient callus induction and regeneration method for *Coffea* spp., permits application of the biolistic method for obtaining transgenic coffee plants. Biolistic experiments were applied to different types of tissue (leaves, somatic embryos and suspension cultures) of genotypes of *C. arabica*, *C. canephora* and Arabusta, using four different promoter sequences. Tobacco leaves were used as a comparison. In general, similar large variation and mean values of transient GUS expression were observed between coffee and tobacco leaves. With regard to the coffee tissue effect, transient expression was best detectable and most frequently observed with bombarded leaves of microcuttings. Disturbing endogenous light blue staining was found with control treatments of somatic embryos. For the three coffee species tested, the most effective promoter was the EF1α-A1 promoter of *Arabidopsis thaliana*.

Introduction

Coffee (*Coffea* sp.) is one of the most important international trade products. World coffee production is ensured by the species *C. arabica* (75%) and *C. canephora* (25%). Since conventional breeding programs take 25 to 30 years for new variety production, genetic modification would be a valuable support. Possible applications are the use of genes for insect resistance (e.g. *Bacillus thuringiensis* genes), use of male sterility for production of F1 hybrids and gene modification for caffeine-free coffee seeds. Techniques such as *Agrobacterium*-mediated transformation and electroporation have been studied with variable success. The use of protoplasts and subsequent electroporation is hampered by difficulties in protoplast regeneration (Barton *et al.* 1991; Spiral & Pétiard 1991; Acuna & de Pena 1991; Van Boxtel *et al.* 1991). Regeneration frequency is strongly dependent on the genotype and the quality of its source, usually embryogenic suspension cultures. *A. tumefaciens*-mediated transformation seems to be hindered by an extremely low infection rate of coffee plant material. Studies of Barré (1990) and Ocampo & Manzanera (1991) have not resulted in a satisfying transformation procedure. Spiral & Pétiard (1993) recently demonstrated stable transformation of coffee after infection of *C. canephora* somatic embryos with a binary *A. rhizogenes* vector. It is not yet clear whether the genes responsible for the hairy root phenotypes obtained by this method can be genetically separated from the desirable genes.

The rapid development of genetic manipulation through microprojectile-delivered DNA, has been the reason for us to study this transformation method with coffee. The availability of efficient callus induction methods and regeneration from embryogenic callus, cultured on solid or in liquid media (Dublin 1984; Söndahl *et al.* 1985; Zamarripa *et al.* 1991; Neuenschwander & Baumann 1992; chapter 2), would

permit the application of the biolistic method to different types of tissue.

The present study investigates optimal conditions for biolistic treatment of coffee tissues by observations on transient expression of the ß-glucuronidase (GUS) gene (Jefferson *et al.* 1987), using different plant tissues and promoter sequences.

As far as known, this report describes the first demonstration of transient expression of the GUS marker gene following biolistic delivery of foreign DNA into coffee tissue.

Materials and Methods

Plant Tissue Preparation

Suspension cultures. Plants of the CIRAD greenhouse collection were grown under daylight conditions, at 25°C and 70% relative humidity. Sterilized young leaves from orthotropic nodes of these plants were cut into pieces of one cm^2 and placed with upper surface down on medium **C** for callus induction (Table 1). The explants were cultured in dark at 27°C in ∅ 10 cm plates (OPTILUX, Falcon). After one month, a primary callus was formed, explants were transferred to medium **E** for embryo induction (Table 1), and cultured at low light intensity (2 $\mu mol.m^{-2}.s^{-1}$; 12 h/d). Three months later, friable HFSE-callus, developed on the explants, was transferred to liquid medium **CP** for callus proliferation (Table 1), in 250 ml erlenmeyers, one gram for 60 ml of medium. The suspensions were cultured at 27°C (indirect light, 5 $\mu mol.m^{-2}.s^{-1}$; 12 h/d) on a gyratory shaker at 100 rpm, until a stable suspension was obtained after about 2 months of initiation, and subcultured each 12 days. Following this procedure embryogenic and non-embryogenic suspension cultures were obtained. Both were used for biolistic treatment. Non-embryogenic suspensions were used directly by spreading them out over a filter paper using a Buchner funnel, and placing them one day before biolistic treatment on a modified Murashige & Skoog (1962) medium ($\mathbf{MS_{pg}}$, Table 1) with sucrose concentration increased to 100 $g.l^{-1}$. This osmotic pretreatment has shown to be stimulatory for transient expression of biolistically introduced genes into several plants (Ye *et al.* 1990; Russel *et al.* 1992b; Perl *et al.* 1992; Vain *et al.* 1993; Van Boxtel *et al.* 1993). Embryogenic suspensions were first cultured during one week in liquid regeneration medium, **R** (Table 1), before exposing them to the same osmotic pretreatment.

Somatic embryos. Embryogenic coffee suspensions from HFSE-callus can regenerate large amounts of somatic embryos. They can be obtained two months after transfer of embryogenic suspensions into liquid **R**-medium containing, 5 $mg.l^{-1}$ BA. The embryos of different developmental stages were transferred to liquid $\mathbf{MS_{pg}}$-medium and agitated at 100 rpm, five hours before biolistic treatment.

Leaves of somatic embryo-derived microcuttings. Following maturation in liquid **R**-medium the somatic embryos were transferred to culture boxes (Magenta, Chicago IL, USA) containing medium **EG** for embryo germination (Table 1), and cultured at 27°C and 30 $\mu mol.m^{-2}.s^{-1}$ (12 h/d). After development of a first pair of real leaves, the plantlets were transferred to medium **DEV** for plantlet

development (Table 1), in 250 ml glass yars, and kept at 27°C and 50 $\mu mol.m^{-2}.s^{-1}$ (12 h/d). Fully developed leaves of these somatic embryo-derived microcuttings (somaplants) were exposed to biolistic treatments by placing them one day before bombardment with upper surface down on MS_{pg}-medium.

Table 1. Composition of used media in $mg.l^{-1}$

components	C[a]	E[a]	CP[b]	R[c]	EG[d]	DEV[c]	MS_{pg}[e]
macro minerals	MS/2	MS/2	MS/2	MS	MS/2	MS	MS
micro minerals	MS/2	MS/2	MS/2	MS	MS/2	MS	MS
$FeSO_4.7H_2O$	13.9	13.9	13.9	27.8	13.9	27.8	27.8
Na_2EDTA	18.65	18.65	18.65	37.3	18.65	37.3	37.3
thiamine-HCl	10	20	5	1	8	1	1
pyridoxine-HCl	1	-	0.5	1	3.2	1	1
nicotinic acid	1	-	0.5	1	-	1	1
calcium pantothenate	-	-	-	1	-	1	1
biotine	-	-	-	0.01	-	0.01	0.01
glycine	1	20	-	-	-	-	-
L-cystein	-	40	10	-	-	-	-
myo-inositol	100	200	50	100	100	100	100
adenine sulfate	-	60	-	40	-	-	-
casein hydrolysate	100	200	100	-	-	-	-
malt extract	400	800	200	400	-	-	-
2,4-D	0.5	1	1	-	-	-	-
IBA	1	-	-	-	-	-	-
IAA	-	-	-	-	0.45	-	-
2-iP	2	-	-	-	-	-	-
kinetine	-	-	1	-	-	-	-
BA	-	4	-	5	0.25	0.3	-
sucrose	30,000	30,000	15,000	30,000	20,000	30,000	100,000
phytagel	2,000	2,000	-	-	2,500	2,500	4,000
pH	5.6	5.6	5.6	5.6	5.6	5.6	5.6

[a] Berthouly & Michaux-Ferrière (submitted)
[b] Van Boxtel & Berthouly (submitted)
[c] Dublin (1984)
[d] Dufour & Carasco (unpublished)
[e] Murashige & Skoog (1962); modified for particle gun use by van Boxtel *et al.* (1993)

Tobacco plants. Seeds of *Nicotiana tabacum* var. W38 were surface sterilized, germinated and cultured on **MS10** medium at 20°C. Transgenic tobacco microcuttings bearing the GUS gene, kindly provided by Laboratoire de Biologie Cellulaire INRA/Versailles France, were maintained on the same medium and subcultured monthly. Leaves of transgenic and non-transgenic tobacco were used as positive and negative controls in the experiments.

Plasmids

Plasmids pCH1 and pBMCV102120k were obtained from L. Jouanin, Laboratoire de Biologie Cellulaire, INRA/Versailles, France. Plasmid p1932 was provided by J. Callis, Dept. of Biochemistry and Biophysics, Davis/California, USA. Plasmids pP1G and pP1GK were a gift from B. Lescure, Laboratoire de Biologie Moleculaire des Relations Plantes-Microorganismes, CNRS-

INRA/Toulouse,France. Plasmid pΔ1932 was kindly provided by C. Franche, Laboratoire de Biotechnologie des Symbioses Forestières Tropicales, Nogent s/Marne, France. Details of plasmid contents are shown in Table 2. All plasmids were purified by passing them over plasmid purification collumns (Qiagen, Chatsworth CA, USA), after alcalin-denaturation extraction (Sambrook *et al.* 1989). Plasmid pCH1 was used for optimization purposes of the biolistic apparatus.

Table 2. Schematic diagram of the chimeric gene constructs used in particle gun experiments. pCH1 contains the GUS gene under control of the E35S promoter of CaMV. This promoter bears a duplication of the strong transcriptional enhancer part, which can give 10-fold higher gene expression in dicot cells (Kay *et al.* 1987). In pBMCV, the GUS gene is under control of the Tnt1 transposable element of tobacco. Tnt1 is known to contain two long terminal repeats (LTRs) which show high level in transient expression assays (Pouteau *et al.* 1991). Both p1932 and pΔ1932 contain ubiquitin extension protein genes of *A. thaliana* which direct the expression of GUS (Callis *et al.* 1990). The plasmids pP1GK and pP1G contain the A1 gene promoter of the *A. thaliana* translation elongation factor EF1α. In *A. thaliana* this promoter has shown an increase of transient expression of about twofold higher than using the CaMV 35S promoter (Axelos *et al.* 1989).

Expression vector	Gene construct	Cloning plasmid	Size (bp)	Source
pCH1	p19s:nptII:t35s/pE35s:gus:tnos	pBS-SK$^-$	8500	Horlow (unpubl.)
pBMCV102120k	pnos:nptII:tnos/pLTR:gus:tnos	pBS-SK$^-$	9200	Pouteau *et al.* (1991)
p1932	pnos:nptII:tnos/pUBQ1:gus:tnos	pBIN19	16740	Callis *et al.* (1990)
pΔ1932	pUBQ1:gus:tnos	pUC118	6870	Lappartient (1993)
pP1GK	pnos:nptII:tnos/t35s:gus:pEF1α-A1	pBI101	14800	Curie *et al.* (1993)
pP1G	t35s:gus:pEF1α-A1	pUC19	6600	Curie *et al.* (1993)

Particle gun device

The device used for our experiments was a powder driven gun (Zumbrunn *et al.* 1989), modified by F. Quetier and co-workers, Laboratoire de Biologie Moleculaire Végétale, Univerité Paris XI/Orsay, France. In pilot studies adaptation of device functioning was carried out. HC100 tungsten particles, with a mean diameter of approximately 1.0 μm (METABAP, Paris), were coated with plasmid DNA using calcium/spermidine precipitation (Daniell *et al.* 1990). About 2 μl of the DNA/particle suspension was loaded onto the nylon macroprojectiles, thus theoretically containing 10 μg DNA and 2.5 mg tungsten particles per shot. The target cells were placed 180 mm below the macroprojectile stopping plate and bombarded with a single shot under partial vacuum of 40 mbar.

Postbombardment handling

After bombardment, the petri dishes containing coffee suspensions, somatic embryos or leaves were placed for two days at 27°C with indirect light (2 $\mu mol.m^{-2}.s^{-1}$, 12 h/d). Plant material for histochemical GUS assays was stained with 1 $mg.ml^{-1}$ 5-bromo-3-chloro-3-indolyl-ß-D-glucuronic acid solution (Biosynth AG, Staad, Switzerland) following an overnight incubation at 37°C, and leaves

were discoloured with alcohol 95°. The GUS assay according to Jefferson *et al.* (1987) was modified with regard to the composition of the incubation solution: 50 mM $NaHPO_4$, 10 mM Na_2EDTA, 0.1% Triton-X100 and antioxidants (sodium metabisulfite, PVP-10 or caffeine).

Data analysis

The number of blue spots on coffee leaves was expressed as a relative number of the mean number of spots obtained on three tobacco leaves, when using pCH1 construct. This was used as standard in each experiment and considered as 100, thus making it possible to analyse data of four experiments together. The mean absolute number of blue spots per plate with pCH1 on tobacco, over four experiments, was 30.7. Number of repetitions for coffee treatments varied from 3-13 with an average of 6.5. Results shown in Table 3 and 4, and in Fig. 1 were obtained in four experiments. For all data, except those in Fig. 1, analyses of variance using Type III sums of squares generated from the General Linear Model procedure in SAS were performed. Significance of differences among treatment means was analyzed by Newman-Keuls test. For analysis of results shown in Fig. 1 the non-parametric test of Kruskal-Wallis was used. For both analyses, differences significant at 5% probability level were considered meaningful.

Results and Discussion

Suspension cultures

First, non-embryogenic suspensions of *C. arabica* were tested. Increase of transient expression of GUS gene, under control of pE35S promoter, was previously obtained by an osmotic treatment of suspensions on 0.35 M sucrose, before bombardment (Van Boxtel *et al.* 1993). However, the absolute level of expression remained low, up to 50 blue spots per plate. As described by Iida *et al.* (1991), the efficiency of biolistic transformation is strongly influenced by growth stage of cultured cells. Cell cultures used for our experiments were in exponential growth phase (5-10 days after subculture), but their mitotic index was not identified.

Variable results were obtained with embryogenic suspensions. For *C. canephora* the number of blue spots per plate varied between 0 and 10, and for *C. arabica* between 0 and 50. Continuation of culture in liquid medium directly after bombardment, enhanced transient expression, in comparison to culture on solid medium. The number of spots per plate was 9 to 48 for *C. canephora* and 0 to 375 for *C. arabica* by using this method (Plate 1A).

The large variation in number of spots is partly due to the morphology of embryogenic callus aggregates, which are difficult to immobilize on filter paper. The impact of bombardment provoked often ejection of callus outside the petri plate. Adaptations tested to solve this problem include decrease of powder charge, use of a

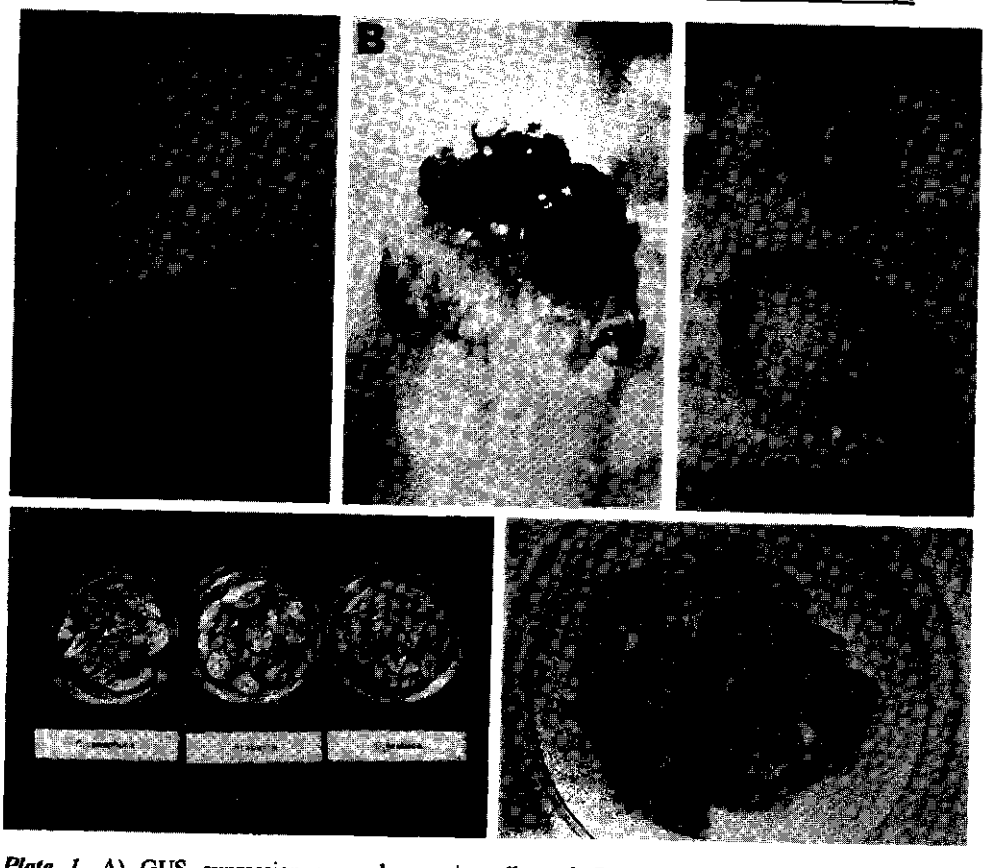

Plate 1. A) GUS expression on embryogenic callus of *C. arabica* cv. Catuai, 2 days after bombardment with plasmid pP1G, and being contineously cultured in liquid medium; B) Blue stained secondary somatic embryo at root apex of mature embryo of *C. arabica* cv. Catuai, 10 days after bombardment with plasmid pCH1; C) Light bluish stained immature embryos of *C. arabica* cv. Catuai, in control treatment; D) Effect of biolistic treatment on leaf necrosis of *Coffea* somaplants, 2 days after bombardment; E) GUS expression on leaves of *C. arabica* KF2.1 somaplants, 2 days after bombardment with plasmid pP1GK.

screen 2 cm under the stopping plate in order to improve particle dispersion, or use of a protective screen (iron, nylon or cotton) on top of the callus layer. These adaptations reduced the impact, but at the same time the transient expression level.

Regeneration on solid medium after biolistic treatment was not possible, due to general necrosis of callus aggregates. On the other hand, use of liquid medium allowedcultures of *C. arabica* and *C. canephora* to recover from biolistic handling and regain normal growth. Regeneration by somatic embryo formation in liquid R-medium took about 3 months for the two species, which is retarded in comparison to untreated suspensions (6 to 8 weeks).

The mechanical problem of ejection of aggregates and the lower availability of

embryogenic suspensions were connected with this source material. Due to the variable results obtained, this tissue was considered less appropriate for use in transient expression studies.

Somatic embryos

High intensity of GUS expression was observed 10 days after bombardment at the root apex of somatic embryos, in regions where fast growing secondary embryos are formed (Plate 1B). However, in contrast to its expression by distinct blue spots in callus and leaf tissue, GUS was expressed by vaste blue regions on somatic embryos. The problems mentioned above with the use of embryogenic suspensions related to the impact, were also encountered with somatic embryos.

Light bluish staining after X-gluc incubation has sometimes been observed with immature and mature somatic embryos in control treatments of regenerating embryogenic suspension cultures. This endogenous blue colouring in embryo structures, appears to be different from GUS expression, being lighter coloured and more diffused (Plate 1C). Such has never been observed in leaf explants or cell suspensions without embryo structures. The appearance of "false" GUS possitives may be due to the presence of endophytic bacteria, often encountered in cultured tissue of tropical woody species, and was previously reported by Tör *et al.* (1992) in yam (*Dioscorea* sp.). It appeared that a long X-gluc incubation time increased the risk of such staining and they proposed several antibiotics for inhibition of false staining. Since addition of chloramphenicol to our incubation solution could not suppress the pale blue staining in somatic embryos, may be attributed to intrinsic GUS-like activity, a phenomenon that has been observed also in other species (Hu *et al.* 1990).

Leaf explants

The wounding of coffee leaves due to biolistic treatment induces callus formation. The possibility exists that a touched cell, receiving foreign DNA, is at the base of induced callus. Theoretically, this could be the ideal situation for induction of transformed callus. The latter, combined to their easy availability, was the reason why coffee leaves were used for further transient expression studies.

Effect of antioxidants. None of the three tested antioxidants could consequently suppress tissue browning during X-gluc incubation. Addition of caffeine resulted in a low number of blue spots, which, moreover, were less distinct. No difference was observed between PVP-10 and sodium metabisulfite with regard to antioxidative effect or GUS staining intensity.

Effect of leaf origin. Three types of leaf origin of *C. canephora* clone 197 were compared for their response to GUS-expression: greenhouse grown plants, microcuttings derived from greenhouse plants and somaplants (=somatic embryo-

derived microcuttings). Table 3 shows large variation within and between treatments; leaves from microcuttings and somaplants reacted more favorable than those from greenhouse grown plants. This difference was significant at p = 0.08. Level of transient expression on tobacco leaves was lower or higher than that in coffee, depending on the used plasmid.

Table 3. Effect of plant origin on GUS-expression in leaves of *C. canephora* clone 197 and of tobacco W38. Data are average number of blue spots per plate in relation to tobacco leaves with plasmid pCH1 (30.7 spots = 100). Minimum and maximum relative values are marked in parentheses.

Leaf origin	Plasmid	
	pCH1	pP1GK
coffee:		
greenhouse plants	5.1 (0-14)	39.1 (2-110)
microcuttings	90.5 (16-180)	92.7 (46-172)
somaplants	61.7 (0-358)	193.0 (0-542)
tobacco: microcuttings	100 (63-157)	50.9 (0-142)

A possible explanation for the lower GUS expression of greenhouse grown leaves can be the higher tendency for polyphenolic oxidation. The sensitivity of these leaves to intrusion of tungsten particles provokes abundant death of cells. Polyphenols in stressed cells are rapidly oxidized and cause toxicity for neighbouring cells (Monaco *et al.* 1977). An introduced GUS-gene may for this reason not be able to express itself by forming the ß-glucuronidase enzyme. A more detailed histological study of this phenomenon is presented in chapter 6 of this work.

Furthermore, having passed through one or more cycles of callus inductions by using auxins, tissue from somaplants may have undergone some alterations in hormonal balance. The process of somatic embryogenesis, which is considered to cause rejuvenation (Bonga 1982), might be a stimulation for tissue reactivity. Such tissue may be more competent for genetic transformation by its physiological state (Sangwan *et al.* 1992). The juvenile coffee tissues appeared to react better with plasmid pP1GK, bearing promoter EF1α-A1. This could be an indication for an increased expression of this promoter in meristematic plant regions, as also reported by Ursin *et al.* (1991). Greenhouse coffee leaves are thus less competent for studying transient expression of introduced genes by the biolistic method, in relation to leaves from *in vitro* cultured plants. Further studies were carried out by using only leaves from somaplants.

Genotype effect. Results of different coffee genotypes are presented in Table 4 and in Fig. 1 and 2. Although variation between genotypes for GUS expression was observed, the differences were not significant according to analysis of variance and to the non-parametric test of Kruskal-Wallis. However, the results presented in Fig. 2 show a tendency for lower expression of *C. canephora* clone 197 in comparison with Arabusta clone 1312 and *C. arabica* KF2.1. Differences in expression level were inversely related to the intensity of necrosis after particle gun bombardment observed between these genotypes (Plate 1D), in this experiment as well as in others (chapter 6). Therefore, it might well be that genotypes which are more sensitive to wounding, induced by bombardment, are also less capable to express GUS activity.

Table 4. Effect of genotype on GUS-expression in leaves from somaplants of *Coffea* spp., when using plasmid pCH1. Data are average number of blue spots per plate in relation to tobacco leaves (30.7 spots = 100). Minimum and maximum relative values are marked in parentheses.

Species	Genotype	Relative number of blue spots	
C. arabica	KF 2.1	80.3	(5-142)
	Mundo Novo	27.7	(1-69)
C. canephora	clone 197	61.7	(0-358)
	clone 3561 (2.3)	44.5	(11-90)
Hybrids: Arabusta	clone 1312	107.3	(10-326)
,,	clone Nakety-2	56.6	(7-118)
control: tobacco	W38	100	(63-157)

Promoter effect. Results of experiments with four different promoter sequences in different plasmids are presented in Table 3, and in Fig. 1 and 2. These promoters are known to give high levels of constitutive gene expression in dicotyledonous species (see Table 2). In tobacco leaves, plasmid pCH1 showed higher GUS expression than plasmid pP1GK (Table 3). On the other hand, on two of the three different coffee tissues tested, pCH1, compared to pP1GK, seemed less favorable. The higher expression in coffee of pP1GK (bearing promoter EF1α-A1), was confirmed by results shown in Fig. 1. Analysis of variance showed a significance of plasmid effect, and Newman-Keuls test identified superiority of plasmid pP1GK in relation to plasmids pCH1 and p1932. Use of promoter EF1α-A1 increased by 2- to 5-fold GUS expression in coffee leaves compared to plasmid pCH1. Besides an increase in blue spots per bombarded plate (up to 1300; Plate 1E), also their staining intensity was improved. These results are a strong indication for the better expression of promoter EF1α-A1, despite the heterogeneity in sequence and size of used plasmids. The size of p1932 (16.7 kb) and pP1GK (14.8 kb) are not in the same order of magnitude as the two other plasmids, pCH1 (8.5 kb) and pBMCV (9.2 kb).

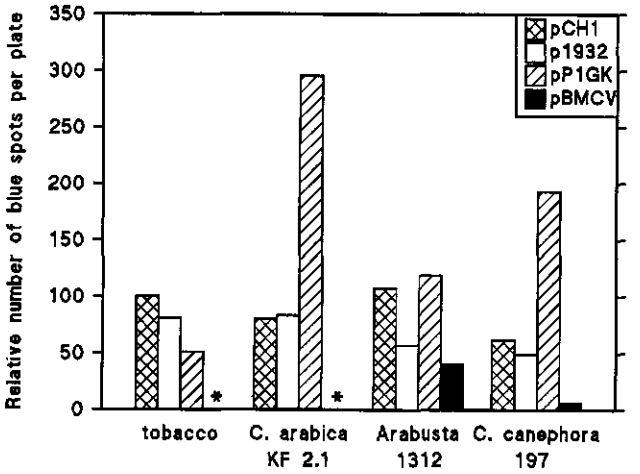

Fig. 1. Effect of plasmid/promoter on GUS-expression in leaves of somaplants of three selected coffee genotypes. Data are average number of blue spots per plate in relation to tobacco leaves with plasmid pCH1 (30.7 spots = 100). * = not executed

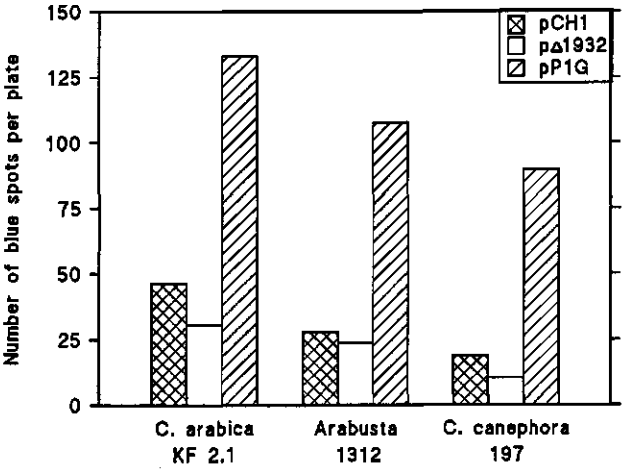

Fig. 2. Effect of plasmid/promoter on GUS-expression in leaves of somaplants of three coffee genotypes.

GUS expression was also compared using plasmids pCH1, pΔ1932 and pP1G, containing the same promoters as their homologues used in Fig. 1, but each of them being more or less of the same size (Table 2). Results shown in Fig. 2 confirmed superiority of plasmid pP1G, as statistically analyzed by Fisher's test ($p = 0.004$) and

Newman-Keuls test ($p<0.05$). Since effect of plasmid size was equal, the better expression of pP1G was most likely due to promoter EF1α-A1. It should be remarked that pP1G bears the 3' end of 35S, whereas the other plasmids were constructed with a 3' *nos* fragment. However, 3'-untranslated regions do not seem to influence the level of transiently expressed genes (Ingelbrecht *et al.* 1989; Franche *et al.* 1991).

Conclusions

This paper describes for the first time application of the biolistic system on coffee tissues.

Leaves of tobacco microcuttings were used for the improvement of experimental conditions of the device and as control in experiments on coffee tissues. General level of transient expression in coffee tissues was similar to that obtained on tobacco leaves. Therefore, coffee should not be considered as a recalcitrant species, and it is likely that high levels of transient expression can be obtained after further improvement of biolistic conditions.

The experiments carried out on different coffee tissues revealed that suspension cultures and somatic embryos were less appropiate for transient expression studies. Firstly, because of their morphology, which makes these tissues more difficult for bombardment handling, and secondly, because of the fact that sometimes endogenous blue staining was observed on somatic embryos of control treatments.

Because of their easy availability and handling, leaf explants were used for promoter studies. Leaves of greenhouse grown plants were more sensitive to biolistic treatment than leaves from *in vitro* microcuttings, due to their higher tendency for polyphenolic oxidation, visible as necrosis after bombardment. Histological observations on bombarded greenhouse grown coffee leaves were carried out in parallel to study the effect of bombardment (chapter 6). Tungsten particles could be localized in cells capable of forming callus, but when combined to GUS expression, these cells appeared rarely able to initiate callus. Thus, for both transient and stable expression, greenhouse grown leaves were less appropriate. The fact that highest transient expression levels were found in leaves of somaplants, might be related to the more juvenile character of this tissue.

The variation in GUS expression between tested genotypes was not significant. However, GUS expression was inversely related to polyphenolic oxidation and necrosis in leaves. More sensitive genotypes, like *C. canephora* clone 197, might require a lower amount of tungsten per shot. Compared to *C. arabica* KF2.1, this genotype appears less appropriate for transient expression studies.

Among four tested promoters controlling GUS expression, best results on coffee were obtained using the EF1α-A1 promoter from *A. thaliana*, in contrast to tobacco leaves, for which this promoter was less favorable. Besides an increase in

blue events per bombarded plate (up to 1300), also their intensity could be improved. This facilitates the recognition of blue spots on coffee leaves, often masked during X-gluc incubation as a result of polyphenol production. The fact that promoter EF1α-A1 showed higher expression in more juvenile coffee tissues and in tissues having passed through auxin-induced somatic embryogenesis, may indicate hormone sensitiveness of this promoter. Ursin *et al.* (1991) and Curie *et al.* (1993) reported constitutive expression of promoter EF1α-A1, with increased expression in regions of high protein synthesis.

In more recent experiments, selective growth agents were tested for inhibition of suspension growth and callus induction on leaves, in order to be able to detect transformed tissue after biolistic treatment. In both cases, glufosinate was most reliable as selective agent for coffee tissue (chapter 7).

Use of the EF1α-A1 promoter combined with the *bar* gene (glufosinate resistance) would offer best perspectives in obtaining and selecting for transformed coffee tissues. If adequate adaptations should be found for better handling of embryogenic suspensions, this tissue might be most prone for achieving this goal.

As a conclusion, the present report shows that the transient expression system using high-velocity microprojectiles provides valuable information concerning gene expression in coffee. The readily detectable expression of the GUS gene in coffee leaves supports the way for genetic engineering of this important crop.

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CHAPTER 6

Fate of GUS-expressing cells during biolistically-induced callus formation on *Coffea* spp. leaves: a histological study

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Abstract

This report describes the effect of biolistic treatment on callus development from greenhouse grown leaves of three coffee types (*Coffea arabica, C. canephora* and Arabusta), in order to evaluate their use in a biolistic transformation system. Furthermore, for exploring the possibilities of obtaining stably transformed tissue, the fate of GUS-expressing cells was followed during callus formation on callus induction medium. It appeared that the effect of bombardment easily induced formation of primary callus, but GUS-expressing cells were not found in callus tissue. If tungsten particles had entered cells, their presence in non-aggregated form was a requisite for cell survival. Despite of being less aggregated, use of gold did not increase GUS expression. Since induction of primary callus in coffee leaves is exclusively committed to perivascular cells or receptive parenchyma cells close to wounding, a plasmid should be introduced into these cells to increase the chance of transgenic tissue formation. On the other hand, proliferation of GUS-expressing cells was hindered by polyphenolic oxidation of surrounding necrosing tissue, wounded by particle intrusion. Polyphenolic oxidation was also responsible for masking of staining in histochemical GUS assay, and use of anti-oxidants showed no improvement of this effect. As a consequence, leaves of greenhouse or field grown coffee plants, which are highly sensitive to polyphenolic oxidation, provoked by wounding, appeared not to be a favorable type of tissue for biolistic transformation. Bombardment of primary leaf callus, whereon subsequently HFSE-callus is formed, seems to offer better perspectives for obtaining stably transformed coffee tissue.

Introduction

Since the first report of genetic transformation on plant material by using the so-called biolistic method (particle gun bombardment or high velocity microprojectile delivery) by Klein *et al.* (1987), this technique has been rapidly developed. Nowadays it has become a reliable method of direct gene transfer, of special interest for plant species in which *Agrobacterium*-mediated transformation seems to be difficult. Progress was achieved with regard to the technical and physical part: after the device described by Sanford and co-workers (1987), other systems using high velocity microprojectiles were demonstrated by Christou *et al.* (1988), Morikawa *et al.* (1989), Oard *et al.* (1989), Sautter *et al.* (1991) and Finer *et al.* (1992). But also the knowledge of the optimal physiological state of targetted plant material has increased (Sangwan *et al.*, 1992). Transient expression was obtained in a large number of species and stable expression achieved in several important agronomic crops as soybean (Christou *et al.* 1989), maize (Gordon-Kamm *et al.* 1990), cotton (Finer & McMullen 1990), rice (Christou *et al.* 1991) and wheat (Vasil *et al.* 1992).

Despite of its economical importance, coffee (*Coffea* spp.) is a less studied crop with regard to genetic manipulation. Transformed plantlets were obtained after regeneration of electroporated *C. arabica* protoplasts (Barton *et al.* 1991) and low *A.*

tumefaciens infection rate was observed by Ocampo & Manzanera (1991). Recently, *A. rhizogenes*-mediated infection of somatic embryos of *C. canephora* resulted in stably transformed plants (Spiral & Pétiard 1993), with "hairy-root" morphology.

Van Boxtel *et al.* (1993) demonstrated transient expression of ß-glucuronidase (GUS) in coffee tissues after biolistic treatment. Best results were obtained by using leaves of somatic embryo-derived microcuttings of *C. arabica*. GUS gene under control of the EF1α-A1 promoter of *Arabidopsis thaliana* (Axelos *et al.* 1989) showed highest level of transient expression. Regeneration and achievement of stable expression from bombarded leaves may be possible by induction of the well-controlled high frequency somatic embryogenesis (HFSE) process (Berthouly & Michaux-Ferrière, submitted). The advantage of application of a HFSE procedure is being found in its abundant friable callus formation, which can be conserved and multiplied in undifferentiated state, and the successive development of high amounts of somatic embryos of unicellular origin. Induced by the death of neighbour cells, the HFSE process arises from dedifferentiation and multiplication of perivascular and parenchymatic cells near veins, and leads to the formation of HFSE-callus after about 3-4 months of leaf explant culture on appropriate media. If cells, capable of callus formation, are touched by plasmid-carrying particles during bombardment, it might theoretically be possible to obtain transgenic callus arising from such events.

We have tried to explore the possibilities of using HFSE from coffee leaves, for obtaining stable transformants after bombardment with our gun device. In a first approach, this study is making appeal to light and electron microscopy in order to clarify the early events after bombardment. During one month, developments in biolistically treated leaves of greenhouse grown coffee plants were observed with a histological study. The GUS gene fusion marker which codes for β-glucuronidase of *Escherichia coli* (Jefferson *et al.* 1987) served as reporter for transformation events by its characteristic to form a blue indigo precipitate after hydrolysis and oxidation of glucuronides. In a similar study, Noël (1992) showed that particles and GUS-expressing cells could be traced upto the 14th cell layer in biolistically-treated cotyledons of courgette, close to perivascular cells, capable of callus formation.

Our study concerned the localization of tungsten particles and their impact on the induction of somatic embryogenesis. The relationship between presence of particles and presence of blue diX-indigo precipitates was studied, as well as the fate of GUS-expressing cells during callus development.

Materials and methods

Plant tissue preparation

The genotypes used for light microscopy experiments were *C. arabica* cv Catuai, two open-pollinated

progenies of *C. canephora*, OP-KB9 and OP-597, and Arabusta clone 4302. Healthy young leaves from orthotropic nodes from greenhouse plants of the CIRAD collection were used, growing at 25°C and 70% relative humidity. The sterilized leaves were cut in discs, completely filling up the surface of a ⌀ 55 mm plate. Plates contained 8 ml of modified Murashige & Skoog (1962) medium (MS_{pg}), modified for particle gun use (Van Boxtel *et al.* 1993). MS_{pg} pretreatment consisted of increased concentration of sugar (for enhancement of transient expression) and gelifying agent (for shock absorption), and was applied one day before bombardment. The leaves were cultured at 27°C in low light intensity (5 $\mu mol.m^{-2}.s^{-1}$; 12 h/d).

For electron microscopy study, leaves from microcuttings of *C. arabica* KF2.1 were used. Microcuttings were cultured on medium **DEV** for plantlet development (Dublin 1984), at 27°C under 50 $\mu mol.m^{-2}.s^{-1}$ light, 12 h/d. Pretreatment was similar to leaves used for light microscopy.

Plasmid

Plasmid pP1G (kindly provided by B. Lescure, INRA/CNRS, Toulouse, France) containing the EF1α-A1 promoter of *A. thaliana* (Axelos *et al.* 1989), was used for all experiments. Plasmid pP1G is a pUC19 derivative with promoter EF1α-A1 placed downstream of the GUS coding region and a CaMV 35S terminator upstream for poly A signal. After amplification in Luria Broth-cultured *Escherichia coli* and purification over columns (Qiagen, USA), plasmid concentration was adjusted to 5 $\mu g.\mu l^{-1}$.

Particle gun bombardment

The device used for our experiments is a powder driven gun as described by Zumbrunn *et al.* (1989), and modified by F. Quetier and co-workers, Laboratoire de Biologie Moleculaire Végétale, Université Paris XI, Orsay, France. HC100 tungsten particles, which have a mean diameter of approximately 1.0 μm (METABAP, Paris), were coated with plasmid DNA using calcium/spermidine precipitation (Daniell *et al.* 1990). Bombardment was carried out at conditions as described previously (Van Boxtel *et al.* 1993). For study of tungsten particle effect only, leaves of Arabusta clone 4302 and *C. arabica* cv Catuai were bombarded with a plasmid-free mix. Controls were not exposed to bombardment.

Postbombardment handling

Directly after bombardment, leaves for light microscopy were transferred to medium **C** for callus induction (Berthouly & Michaux-Ferrière, submitted) in ⌀ 10 cm plates (OPTILUX, Falcon). C-medium contains 0.5 $mg.l^{-1}$ 2,4-D, 1 $mg.l^{-1}$ IBA and 2 $mg.l^{-1}$ 2-iP. The plates were sealed and cultured at 27°C in dark. At regular days (1, 7, 14, 21 and 28) during callus formation, samples were taken for GUS analysis and subsequent histological study. Each sample consisted of 2 to 6 bombarded plates and one control per genotype.

Leaves for electron microscopy were transferred to fresh solid **MS30** medium after bombardment and kept for 2 days at 27°C in dark, until GUS assay.

GUS assay

Precautions were taken (prefixation, wounding avoidance) during the GUS assay in order to ensure tissue quality for subsequent microscope observations and to prevent bacterial contamination: solutions were filter sterilized before use and incubation was carried out under sterile laminar air flow conditions.

Tissue for light microscopy. A prefixation was applied by 30 min immersion in a 0.5% glutaraldehyde solution (SIGMA) in 25 mM monobasic/dibasic sodium phosphate buffer mixture at pH 7.0 (Merck), of which 10 min under vacuum filtration. Prefixation was followed by 5 x 3 min rinsing in 25 mM phosphate buffer. Incubation in 5 ml GUS assay solution was carried out during one night at 37°C in obscurity (Jefferson 1987). The composition of this solution was 50 mM phosphate buffer, 5 mM Na_2EDTA, 0.1% Triton X-100, 1% w/v PVP-10 (all SIGMA) and 0.7 $mg.ml^{-1}$ X-gluc (Biosynth AG, Switzerland). After the overnight incubation, explants were rinsed three times in 25 mM phosphate buffer. GUS expressing spots were counted with a binocular (Wild M3Z, Switzerland) at magnification 25x. Representative tissue parts were excised and fixed for histological observation.
Tissue for electron microscopy. All steps, except X-gluc incubation, were performed on crushed ice, to avoid extraction of cellular residues. Prefixation in 1% glutaraldehyde in 50mM phosphate buffer was carried out only during 10 min. After washing in 50 mM phosphate buffer explants were incubated in GUS-assay solution, consisting of 50 mM phosphate buffer, 10 mM Na_2EDTA, 0.1% Triton X-100, 1% w/v caffeine (SIGMA), 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide (both SIGMA). These oxidation catalysts were added to ensure rapid precipitation of diX-indigo, without artifacts in localization (De Block & Debrouwer 1992). Incubation at 37°C took 6 hours and was preceded by 1 min under vacuum. Three times rinsing in phosphate buffer was followed by excision of representative tissue parts, which measured about 5 mm^2.

Light microscopy

After the GUS assay, representative tissue parts were transferred to fixation solution and kept two days at 4°C, after having passed 10 min under vacuum. The fixation solution consisted of 100 mM sodium phosphate buffer (pH 7.2), 2% glutaraldehyde, 2% acroleine (Rectapur) and 1% w/v caffeine. The combination of glutaraldehyde and acroleine ensures a rapid fixation. Caffeine inhibits the diffusion of oxidized polyphenols in the cytoplasm, by their *in situ* precipitation (Mueller & Greenwood 1978). Fixation was followed by dehydration in a series of baths with progressively increasing ethanol concentrations, and 2 hours in a pre-impregnation bath (50% ethanol 95° + 50% impregnation medium). The composition of the impregnation medium was: 0.5% w/v benzoylperoxid, 3% PEG 400 (both Technovit7100, Kulzer, Germany), 0.5% glycol dimethacrylate ethylene (Aldrich) in liquid resin (Kulzer). Pre-impregnation was followed by one night in impregnation medium at 4°C, after having passed 5 min under vacuum. The excised explants were embedded in a mix of 7% hardener (Kulzer) in impregnation medium for polymerisation, and dried at 28°C during one night. Semi-fine sections (3 or 5 µm thick) were cut on a LKB Historange microtome and mounted on glass slides. Histological sections were stained by periodic acid-Schiff only (PAS: polysaccharide specific coloration; Feder & O'Brien 1968) or by PAS plus naphthol blue black (NBB: protein specific coloration; Fisher *et al.* 1968). Both stainings were preceded by a 5 min bath in DNPH solution in obscurity. DNPH solution consisted of 0.33% w/v 2,4 dinitrophenylhydrazine (SIGMA) in acidic acid at 15% (Merck), and is applied for blockage of aldehydes. After drying on a 60°C slide warming plate, slides were covered and fixed permanently with varnish. Observations and photographs were taken on a Leitz DMRB photomicroscope and recorded on 200 ASA Ektachrome Color film.

Electron microscopy

Fixation solution was the same as for light microscopy but fixation lasted only 2 hours including 1 min of vacuum filtration. Fixation was followed by 3 x rinsing with phosphate buffer and tissue parts were kept in the last bath during one night. Postfixation was carried out during 1 hour in 1% osmium

tetroxide in Millonig buffer (2,226% monobasic sodium phosphate + 2,52% sodium hydroxide; pH 7.3), followed by 3 x rinsing in Millonig buffer. After dehydration in an ethanol series, the samples were embedded in SPURR resin (TAAB, UK) and polymerized during 8 hours at 70°C. Ultrathin sections of 50-100 nm were cut with a diamond knife (DIATOME, Switzerland) on an ultramicrotome (Reichert-Jung, Austria) and mounted on copper grids. Unstained grids were examined on a high contrast TEM (Hitachi H-7100, Japan). An adjacent semi-thin section was taken prior to each grid of ultrathin sections. These semi-thin sections were examined with a Nomarski optic photo microscope (Zeiss, Germany).

Results and discussion

Macroscopic observations of callus development

A powdery tungsten layer was visible immediately after bombardment and the center of the impact often showed severe wounded leaf tissue. Amount of tungsten decreased gradually towards the periphery of the petri plate. Necrosis of damaged parts and of parts which have received more tungsten, started almost immediately after bombardment. Necrosis became more important during the days following bombardment and sometimes resulted in a complete browning of tissue. The less sensitive Arabusta leaves showed slight necrosis and stayed relatively green, even after one month, whereas *C. canephora* OP-597 turned completely brown.

Callus became visible after about one week on C-medium. The callus was of a white compact type and its formation was found at the site of the wounds from intrusion of tungsten particles. No callus was formed on severely wounded leaf parts. Sometimes callus formation in a circular form around the necrosed impact center was observed (Plate 1A). The reason for cell death in the center is most probably a combination of high tungsten load, high shock and gas blast.

The amount of callus formation after one month was not the same for all genotypes. The following sequence could be observed: Arabusta clone 1312 > *C. arabica* cv. Catuai > *C. canephora* OP-KB9 > *C. canephora* OP-597. The latter genotype practically formed no callus. As shown later this genotype reacted differently on particle intrusion. Callus formation in the middle of leaf explants was only observed on bombarded treatments. On control explants the white compact callus was exclusively formed on the cut edges (Plate 1B). Callus of white compact type formed on biolistically treated leaves did not differ macroscopically from callus on cut edges of untreated material, which is capable of initiation of the HFSE process (Berthouly & Michaux-Ferrière, submitted).

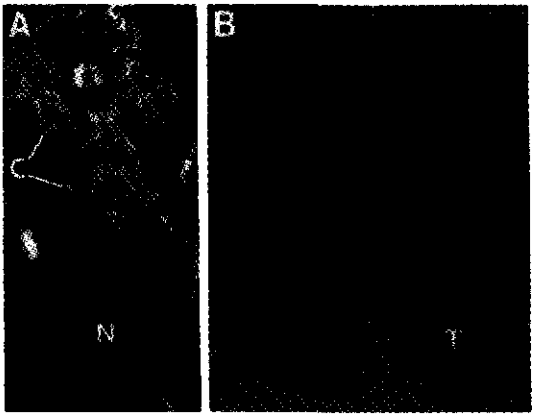


PLATE 1. Aspect of bombarded greenhouse grown coffee leaves. **(A)** Necrosed zones (N) surrounded by callus formation (C) on *C. arabica* cv Catuai explant 28 days after bombardment. **(B)** Primary callus formation on wounded excised edges of untreated explant (NT) and on surface of treated explant (T) of Arabusta clone 4302, 44 days after bombardment.

Microscopic observations: localization of tungsten particles and their impact on callus induction

Once a week during 2 months, callus development on the leaves bombarded with a plasmid-free mix was followed histologically. One day after bombardment tungsten particles can be observed in all of the about 12 cell layers of the coffee leaves. Complete crossing of particles through the leaf, traced by wounded cells, could also be observed (Plate 2A). Isolated particles were difficult to recover in the cross-sections, probably because most of the tungsten had aggregated before hitting the leaf surface, and could thus be observed as tungsten clumps. Clumps had provoked severe injuries which resulted in all cases in the death of cells in which they were found initially.

When tungsten-wounded cells were found close to callus-inducible cells (perivascular vein cells or receptive parenchyma cells), callus induction could be observed readily (day 7), as induced by dying neighbour cells (Plate 2B). Probably the callus-inducible cells are capable to react rapidly to the auxin content in the callus induction medium, because of their low differentiation level (Michaux-Ferrière *et al.* 1989). The callus cells divide rapidly and form firstly an endogenous callus clump (day 7), which becomes visible after eruption of the leaf epidermis (day 21, Plate 2C). At its base tungsten particles in dead cells, pressed together in a necrotic layer, have been observed. The callus, which increases in volume, consists of small cells of parenchymatic type, rich in starch and protein reserves. Callus formed on bombarded leaves is thus also in histological respect identical to the HFSE-preceeding callus, as described by Berthouly & Michaux-Ferrière (submitted).

The fact that living cells bearing tungsten were not observed, can be due to several reasons. Firstly, due to the impact of aggregated metal particles in cells, being a major cause for cell death, but also toxicity of tungsten itself may be a cause for cell stress or death. Tungsten toxicity was previously demonstrated by Russel *et al.* (1992a) on tobacco cell suspensions.

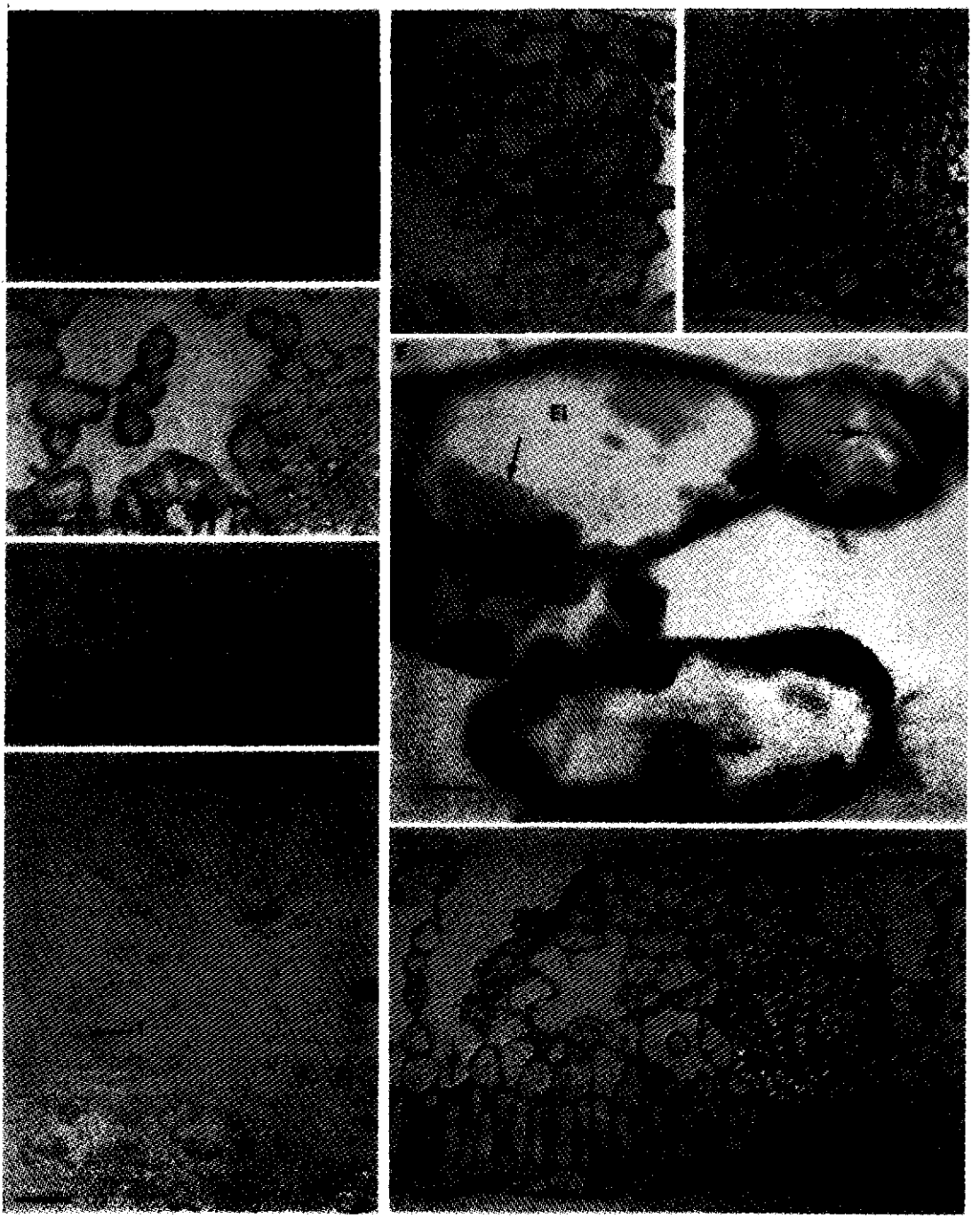
In other experiments the use of less toxic gold particles demonstrated a lower number of transient expression events in bombarded coffee leaves, compared to tungsten. In two experiments of each three replicates, number of blue units per plate was 100.1 ± 89.9 for tungsten, and 64.8 ± 47.9 for gold. However, this does not exclude the use of gold particles in studies for obtaining stable transgenic expression in coffee tissues.

Macroscopic observations of GUS-expressing units

At day 1, 7, 14, 21 and 28 after bombardment, leaves from 6 to 10 plates were screened in histochemical GUS assays. The average number of blue units per plate decreased respectively from 58.7 at day 1, to 2.0 at day 28. This might be an indication for degeneration of GUS-expressing cells. Although rather stable (Gaudin 1992), the initially synthesized β-glucuronidase is gradually lost. None of the unbombarded leaves, or controls bombarded without plasmid, showed GUS-expression.

It was noticed that GUS-expressing units were always located in non-necrosed leaf tissue. In more recent experiments, it was tried to reduce necrosis by immersion of tissues in citric acid/ascorbic acid solution, either during a short

PLATE 2. Histological description of bombarded greenhouse grown coffee leaves. The sections were PAS (periodic acid-Schiff) stained only, or double-stained with PAS + NBB (naphthol blue black). Ei, inferior epidermis; Es, superior epidermis; C, callus formation; V, vein; R, starch reserves (**A**) *C. canephora* OP-KB9; day 21 after bombardment (D21); PAS stained; 1 cm = 80 µm. Complete crossing of particles through the leaf showing a trace of wounded cells (large arrow). Endogenic callus is constituted of cells rich in starch reserves. Wounded degenerated cells surround the callus clump (small arrows). (**B**) *C. arabica* cv Catuai; D7; PAS + NBB; 1 cm = 40µm. Callus initiation from parenchyme cells near vein, provoked by wounding through aggregated tungsten particles (arrows). (**C**) *C. canephora* OP-KB9; D21; PAS; 1 cm = 80 µm. Growth of callus clump induced by intrusion of tungsten aggregates (t). Dead cells are pressed together in a necrotic layer (arrows). (**D**) *C. arabica* cv Catuai; D1; PAS + NBB; 1 cm = 40 µm. Intensely stained GUS-expressing unit near vein. Cells in the centre of the unit are more stained. No callus formation visible. (**E**) *C. arabica* cv Catuai; D1; PAS + NBB; 1 cm = 40 µm. Bursted cell with diX-indigo precipitated to vacuole and plastid membranes. (**F**) *C. arabica* cv Catuai; D7; PAS + NBB; 1 cm = 8 µm. Diverse diX-indigo precipitates (arrows) in GUS-expressing cells of inferior epiderm and lacunous parenchyme. (**G**) *C. arabica* KF2.1; D2; unstained; 1 cm = 20 µm. DiX-indigo precipitates in GUS-expressing unit (arrows). Precipitates in cytoplasm (1), nucleus (2) or on vacuolar membrane (3). These precipitates are shown in ultrastructure on PLATE 3. (**H**) *C. canephora* OP-597; D7; PAS + NBB; 1 cm = 40 µm. Intrusions of particles into leaf tissue without cell penetration.



moment just after bombardment, or contineously during two days following bombardment. Both treatments showed subsequently no effect on necrosis level compared to bombarded leaves without anti-oxidant treatment. However, an inhibitory effect on diX-indigo formation was observed (data not shown). This may be due to the fact that formation of diX-indigo is a chemical reaction in which oxygen is required. Anti-oxidants may therefore influence the subsequent X-gluc incubation in a negative manner.

In none of the 45 analyzed leaves, blue spots were observed in callus tissue. White callus near necrosed leaf tissue always turned brown during incubation in X-gluc solution. In order to clarify this, some non-necrosed control leaves bearing one month-old callus were bombarded. GUS assays carried out two days later showed that blue spots could be observed on the white callus, which proves that such cells are capable to express GUS. It may therefore be, that in the former experiments, GUS-expressing units on white callus had been masked, due to browning by surrounding dead tissue during X-gluc incubation. In consequence, either other inhibiters for polyphenolic oxidation have to be added to X-gluc solution, or it should be preferable to excise all necrosed tissue before X-gluc incubation.

Localization and characterization of GUS-expressing units

The histological study revealed in a more detailed manner the localization of the GUS-expressing cells in leaves. A blue unit was in general constituted of a group of about 5 to 20 diX-indigo-containing cells. They were found in all cell layers but more frequently in the lower epiderm and lacunuous parenchyma, layers closest to the particle impact. Differences in staining intensity were observed between blue units, possibly reflecting differences in cell activity (Plate 2D). In fact, the staining intensity of a blue unit depends on a combination of factors: differences in cell size, their metabolic activity (and thus more or less vacuolated), X-gluc substrate accessibility and the tissue specific expression of the promoter (Battraw & Hall 1990). In our study the critical factor for staining was rather determined by the fact whether or not the GUS gene-containing plasmid had been brought into a cell.

The larger part of blue cells was intact. Nevertheless, some bursted blue cells were found, indicating dead β-glucuronidase-containing cells (Plate 2E). Early cell death leaves the GUS-gene just a short moment for transcription of the β-glucuronidase enzyme, which itself is very stable. Thus, a diX-indigo precipitate formed after X-gluc incubation, at no matter what moment, does not reflect the present state of the expressing cells, but rather an amount of β-glucuronidase formed in their history (Gaudin 1992). Also inside a blue unit, different intensities of blue staining were noticeable: some intensely blue stained cells were surrounded by more diffusely stained cells (Plate 2D). Apparently the central cells had received the GUS gene and formed

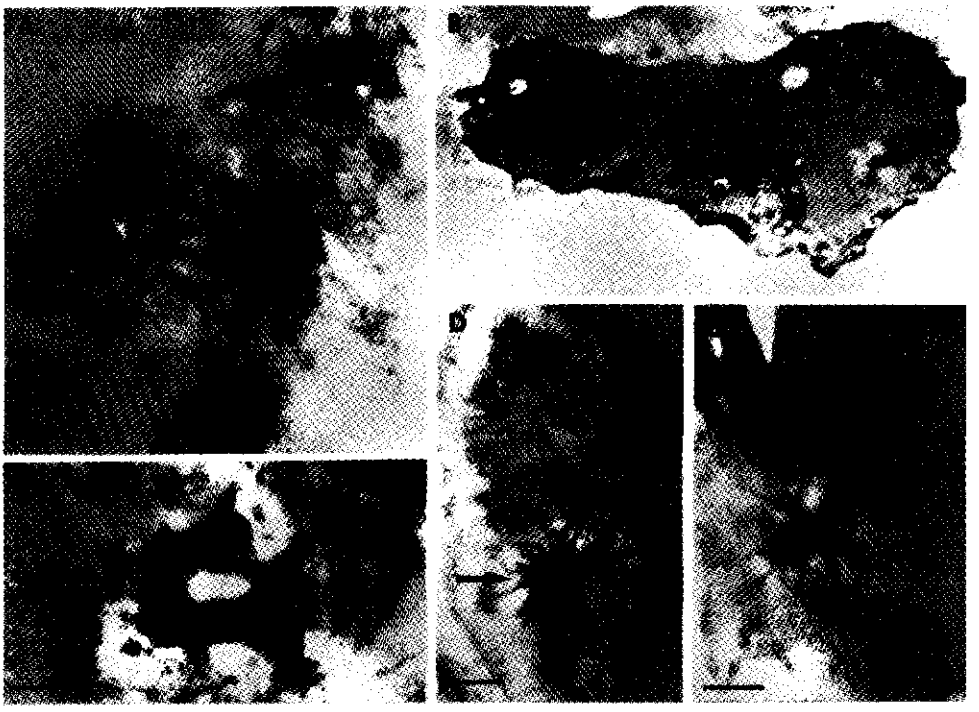


PLATE 3. Ultrastructural aspect of crystal-shaped diX-indigo precipitates in microcutting leaves of *C. arabica* KF2.1, two days after bombardment with GUS-containing plasmid. (**A**) 1 cm = 3.7 µm. Blue precipitate from cell 1, PLATE 2G. Note that crystals are not in vacuoles and plastids. (**B**) 1 cm = 4.7 µm. Stained nucleus from cell 2, PLATE 2G, containing crystal-shaped precipitate (arrow). (**C**) 1 cm = 4.3 µm. Part of nucleus marked by GUS precipitation. (**D**) 1 cm = 4.3 µm + (**E**) 1 cm = 3.7 µm. DiX-indigo precipitates in various crystal forms, external to plastids and vacuoles.

the enzyme β-glucuronidase, whereas the less stained cells may had received the soluble intermediar product in the X-gluc reaction, diffused away from the central cells. Several authors have described this diffusion of intermediar products (indoxyl derivatives), giving false impressions of GUS-expressing cells (De Block & Debrouwer 1992; Mascarenhas & Hamilton 1992; Caissard 1993).

For our study, the most interesting localization of a blue unit would be in callus-inducible sites (perivascular cells or receptive cells close to wounding). In fact, at day 7 two out of twelve blue units were observed in a perivascular site (Plate 2D), but callus had not been developed from these sites. Wounded cells, a requirement for callus induction, were not visible close to it. In the serial cross-sections remaining tungsten could not be traced close to the site.

Intracellular fine localization of GUS product

Differences in staining intensity between cells may also be due to different staining patterns, as the diX-indigo microcrystals differ in shape and colour. Needle-shaped, rod-shaped, granulous and amorph crystals were observed. Crystals were found associated to plastids, vacuoles and the nuclei, but more often precipitated to the organel membranes (Plate 2F). It could not be clarified whether or not the crystals were precipitated to the internal or external side of the membranes. Nor could the exact site of β-glucuronidase synthesis be determined following our procedure.

Transmission electron microscopy (TEM) revealed more details about the intracellular localization of GUS products. As indicated above, prior to each grid a semi-thin section was taken in order to precisely localize the cells examined on TEM. Plate 2G shows GUS-expressing cells of *C. arabica* KF2.1 two days after bombardment. DiX-indigo precipitates could be observed in about 10 cells belonging to a blue unit. The blue crystal-shaped form in cell n° 1 (Plate 2G), is shown in ultrastructure on Plate 3A. The coloured nucleus in cell n° 2 (Plate 2G) is shown enlarged on Plate 3B, and a crystal structure with similarity to the one in Plate 3A, can be observed in it. Plate 3C shows granular structures precipitated to the membrane of a degenerating nucleus. Plate 3D and 3E show examples of other diX-indigo crystals found close to plastids. Whereas the 3 µm semi-thin sections gave the impression of diX-indigo crystals localized inside organelles, TEM clearly indicated that crystals were external to plastids and vacuoles, but may be observed inside nuclei. Small electron-dense bodies (presumed as being diX-indigo) were never observed in vacuoles of TEM-treated tissues, contrarily to vacuoles in light microscopy-treated tissues. It must be noted however, that the difference may be due to the presence of the ferri/ferro-potassium complex in the X-gluc solution for TEM tissues, inhibiting diffusion of intermediary products, whereas it was absent in light microscopy treatments. These observations are not in agreement with Murakami *et al.* (1992), who localized GUS products in the vacuole by using the A-immunogold method.

Genotype specific reaction

The histological study of the treated leaves of *C. canephora* OP-597 revealed a somewhat different reaction to the bombardment than the other genotypes. Tungsten particles seemed not to have penetrated into the leaf cells. Rather than end up in the cell interior, particles provoked craters, by sort of pressing the cells together without being able to penetrate them (Plate 2H). Apparently cell walls of these leaves are too 'elastic' to allow the particles to enter. A rapid initiation of leaf necrosis after bombardment, led to complete browning without any callus formation, in spite of the quite often observed tungsten-induced wounding near perivascular cells. Necrosis of wounded coffee leaves is due to increase of polyphenolic content and their subsequent

decompartimentation and oxidation, in stressed cells (Monaco *et al.* 1977). It is presumed that the difference in necrosis between Arabusta and OP-597 is explained by the higher oxidation of polyphenols in the latter. Practically no blue units could be perceived in these leaves, which is logic since blue units appear only in unnecrosed tissue as shown before. Due to their physiological character, greenhouse grown leaf explants of OP-597 may be considered as recalcitrant for genetic transformation by particle gun bombardment.

Conclusions

This study deals with the more fundamental problems that are in general encountered when genetic transformation procedures are being developed for less studied species, as is coffee. Our aim was to explore the competence for stable transformation from coffee leaves, with the applied experimental conditions.

Using somatic embryogenesis from greenhouse grown coffee leaves for stable transformation purposes by the biolistic method is, as demonstrated, a delicate matter. In fact, somatic embryogenesis is provoked by the biolistic treatment, as the intrusion of particles wounds the leaf cells. A transformation event arising from perivascular vein cells or receptive cells is committed to some requirements:

1. plasmids, carried by non-aggregated tungsten particles, need to penetrate callus-inducible cells, and may not leave unrepairable wounding,
2. aggregated tungsten particles may not remain too close to the perivascular site, by this generating death and non-expression of cells, due to their presumed toxic effect,
3. a certain wounding close to the site is nevertheless required for inducing the division of the plasmid-containing inducible cells.

In our study these requirements were not observed in combination. It might be noticed that the total amount of analyzed blue units was 306 and that the number of transient expressions required for one stable event is unknown for our biolistic system. McCabe & Christou (1993) demonstrated a 15% "conversion" of transient expressions into stable ones for the ACCELL system, Russel Kikkert (1993) showed a 10% conversion for the Biorad PDS1000 helium-improved device, and Vain *et al.* (1993) obtained a 4% conversion using the PIG. Although, level of transient expression is not necessarily related to stable expression, it can be used as a guide to develop systems for stable expression.

Obtaining stable expression events in our system may be achieved by optimization of several factors. The strength of our device induces a high level of cell death; adaptations for avoiding tissue damage have to be incorporated. Avoidance of tungsten particle aggregation during the plasmid precipitation procedure, is a critical step for satisfactory use of the biolistic system on sensitive tissues, as are coffee leaves.

Several authors have tried to improve the plasmid precipitation procedure, by using other precipitation agents (Perl *et al.* 1992) or microcarriers (Sautter *et al.* 1991). However, in our system, use of gold particles showed a decrease of transient expression compared to tungsten, whereas level of leaf necrosis was similar.

The physiological state of plant tissue is of main importance. Results were negatively influenced by leaves showing rapid polyphenolic oxidation (necrosis) and by tissue showing non-penetrance of particles in cells. Besides, necrosis is a barrier for recognition of GUS-expressing cells during X-gluc incubation. Use of anti-oxidants could not inhibit browning of tissue during incubation, responsible for masking of blue coloration. Use of microcutting-derived leaves (less sensitive to polyphenolic oxidation) has shown to increase transient expression by 3 to 5 fold, compared to greenhouse leaves (Van Boxtel *et al.* 1993). Moreover, transient GUS expression on leaves from microcuttings was shown to be inversely related to level of polyphenolic oxidation. Craig (1992) suggested the use of polyvinyl pyrollidone (MW 40,000) to prevent tissue browning, and recently Beeckman & Engler (1994) described clearing of histochemically stained plant tissue with chloral lactophenol (CLP). But despite the availability of these measures for necrosis prevention, it is preferable for coffee to use in vitro cultured leaves instead of greenhouse grown leaves.

Finally, it is of importance to target the appropiate cells that are competent for both transformation and regeneration. In order to make plant tissue more prone to transformation, cells have to be in a metabolically active state. Sangwan *et al.* (1992) reported an enhancing effect on *Agrobacterium*-mediated transformation frequency by a preculture on phytohormone medium, resulting in cellular dedifferentiation and subsequent cell divisions. However, extensive histology remains needed in order to ascertain the origin of regenerating tissue in a particular transformation study.

Although a study by light microcopy of GUS-expressing cells gave the impression of intra-organel precipitation of diX-indigo crystals, TEM study clearly indicated that crystals were localized external to plasmids and vacuoles. On the other hand, nucleus-harboured crystals were observed. But this does not prove presence of β-glucuronidase inside the nucleus, which is supposed to enhance achievement of stable expression (Yamashita *et al.* 1991). For intracellular localization of β-glucuronidase itself, the histochemical assay as described here is not appropriate. Use of *in situ* hybridization of mRNA's and immunocytochemical methods has been suggested (Gaudin 1992).

Although commited to several requirements, obtaining of transgenic coffee tissue through high frequency somatic embryogenesis from biolistically treated *in vitro* leaves, seems achievable. But bombardment of primary callus, whichs subsequently gives rise to HFSE-callus, seems to be preferable in that case.

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CHAPTER 7

Inhibitory effect of selective agents on callus development for selection of genetically transformed coffee tissues

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Abstract

In order to improve selection of transgenic coffee (*Coffea* sp.) tissue after transformation treatments, effect of the selective agents chlorsulfuron, glufosinate, glyphosate, hygromycin and kanamycin was studied on callus development on leaf explants (from greenhouse grown plants and somaplants) and in embryogenic suspension cultures. Studied genotypes were from *C. arabica*, *C. canephora* and the interspecific hybrids Arabusta and Congusta. Different levels of sensitivity to the agents were found, the hybrid genotypes being more tolerant. The process of direct somatic embryogensis on leaf explants proved to be more inhibited than high frequency somatic embryogenesis. With regard to the selective effect, chlorsulfuron and hygromycin provoked strong inhibition and severe necrosis, whereas glyphosate and kanamycin showed variable growth inhibition properties. Glufosinate appeared to inhibit efficiently growth of callus on leaves and in suspension cultures of all genotypes tested, without inducing disturbing necrosis. These properties may be an advantage for use of glufosinate in a selective growth system for detection of transformed coffee tissues.

Introduction

In order to enhance the efficiency of procedures for obtaining transgenic plants, a selectable marker gene is cointroduced with the gene of interest. The selection gene, usually coding for antibiotic or herbicide resistance, allows convenient and easy selection of the few transformed cells in large populations of untransformed cells (Nap *et al.* 1992).

One of the most frequently applied selective agents is kanamycin, to which resistance in plants is conferred by the *npt* gene from *Escherichia coli*. Other selectable marker genes, proven to confer resistance to selective agents in plants, are available, among others the *hpt* gene (resistance to hygromycin), the *crs1-1* gene (sulfonylureas), the *bar* gene (phosphinothricin) and the *aroA* gene (glyphosate) (Bowen 1993).

The few reports dealing with transgenic coffee (*Coffea* spp.) research, mention low transformation frequencies and would therefore require efficient procedures for selection of transgenic tissue. So far, the results with kanamycin have been variable.

Barré (1990) described inhibitory kanamycin concentrations for "direct" somatic embryogenesis (Yasuda *et al.* 1985) from leaves of microcuttings. A concentration of 125 $mg.l^{-1}$ was sufficient for inhibition of callus formation during the first 5 days of induction, but application in later stages of embryogenesis required higher concentrations (150-200 $mg.l^{-1}$). Barton *et al.* (1991) regenerated *C. arabica* protoplasts, electroporated in presence of a *nptII*-containing plasmid, in culture medium in which 100 $mg.l^{-1}$ of kanamycin was supplied three weeks after electroporation. Southern analysis confirmed the presence of *nptII* in one regenerated plantlet.

Spiral & Pétiard (1993) infected somatic embryos of *C. arabica*, *C. canephora*

and the interspecific hybrid, Arabusta, with binary *Agrobacterium rhizogenes*, containing a wild type Ri-plasmid. Although the vector was carrying the *npt* gene, they used root formation in the presence of kanamycin and GUS staining as selection criteria, because somatic embryos of coffee had shown tolerance to high kanamycin concentrations (400 $mg.l^{-1}$), as well as to geneticin and hygromycin..

Unpublished studies in our laboratory have also indicated tolerance of regenerating embryogenic suspension cultures of *C. arabica* to a relatively high kanamycin concentration (400 $mg.l^{-1}$).

So far we have demonstrated transient expression of introduced DNA in coffee leaves after biolistic treatment (Van Boxtel *et al.* 1993). GUS gene under control of the EF1α-A1 promoter of *Arabidopsis thaliana* (Axelos *et al.* 1989) showed highest expression. Next step should be the selection of transgenic tissue arising from somatic embryogenesis, induced after bombardment. Therefore, the objective was to develop a reliable selective growth system for detection of tranformed coffee tissue. Due to the variable results with kanamycin, minimal concentrations of other agents for efficient inhibition of callus proliferation were determined in three culture systems. For our purpose, a selective agent showing maximal inhibition of untransformed cell proliferation in an early stage seems preferable, thus giving a rapid advantage to transformed cells, which have been stressed by introduction of exogenous DNA. In coffee tissues, a second requirement for a selective agent, would seem to be its property to leave transformed cells some time for proliferation, without the toxic influence of polyphenolic oxidation from surrounding stressed or dying tissue. Monaco *et al.* (1977) described that rapid necrosis of tissue was due to oxidation of polyphenols, which on its turn may inhibit growth or kill surrounding tissue. Transformed cell clumps resistant to the selective agent may as yet die because of the toxic influence of oxidized phenols (chapter 6). Thus, beside inhibition of callus proliferation, reduced tissue necrosis is considered as an important criterion for the use of selective agents.

Materials & methods

Plant material

Greenhouse leaf explants. Healthy greenhouse grown plants (GGP) were selected as source for leaf explants. Selected genotypes were *C. arabica* cv. Catuai, *C. canephora* clone 197 and Arabusta clone 1312. Sterilized young leaves from orthotropic nodes were cut into pieces of 0.5 to 1 cm^2 and placed with upper surface down on callus induction media: *C. arabica* and Arabusta on C-medium (Berthouly & Michaux-Ferrière, submitted), *C. canephora* on Y-medium (Yasuda *et al.* 1985). The explants were cultured in dark at 27°C in ⌀ 10 cm plates (OPTILUX, Falcon). After 5 days explants were transferred to fresh callus induction media, containing antibiotics or herbicides in various

concentrations. Because previous reports had demonstrated that first cell divisions from perivascular cells occur after 3 to 5 days of culture initiation (Berthouly & Michaux-Ferrière, submitted; Michaux-Ferrière *et al.* 1989), selective agents for early selection were supplied at day 5. One treatment consisted of three culture plates, with in each plate 5-6 explants. Also control treatments were transferred to fresh media. Cultures were kept in dark at 27°C, and transferred to low light (2-3 $\mu mol.m^{-2}.s^{-1}$) after 5 weeks of total culture. Once a week during 7 weeks the number of explants with callus formation and the percentage of necrosed tissue surface was scored.

Microcutting leaf explants. Healthy leaves of 3 months-old somatic embryo-derived microcuttings (SEM) were grown on medium **DEV** for plantlet development (Dublin 1984), at 27°C and 50 $\mu mol.m^{-2}.s^{-1}$ of irradiation (12h/d). Explants of 0.5 to 1 cm^2 of *C. arabica* KF2.1 and Arabusta clone 1312 were placed on **C**-medium, *C. canephora* clone 197 on **Y**-medium. Culture conditions and supply of selective agents was as mentioned above.

Suspension cultures. Procedure for the obtainment of embryogenic suspension cultures from HFSE-callus is previously described by Van Boxtel & Berthouly (submitted). Two 4 month-old suspensions maintained in liquid medium **CP** for callus proliferation, were used for testing: open pollinated progeny of *C. canephora* clone 597 (OP-597) and Congusta clone HA, the interspecific hybrid between *C. congensis* and *C. canephora*. Therefore, 0.5 ml of packed cell volume (PCV) was cultured in 25 ml **CP**-medium in 100 ml erlenmeyer flasks, supplied with selective agents in various concentrations. Each treatment was carried out with 3 or 4 replicates. PCV of callus mass was measured each 6 days, by absorbing it in a pipette. After 12 days callus mass was transferred to fresh medium with selective agents. Twelve days later the replicates of each treatment were mixed together and callus was distributed over 4 culture replicates: two with selective agent, two without. Ten to 12 days later final PCV was measured. Level of tissue necrosis was followed all along the 35 days of culture.

Applied selective agents

Chlorsulfuron-Glean (99.7% active substance; DuPont de Nemours, France) inhibits the acetolactate synthase (ALS) enzyme which catalyses the biosynthesis of the amino acids valine, leucine and isoleucine (Brasileiro *et al.* 1992). Glufosinate-Basta (15% active substance; Hoechst) contains phosphinothricin which intervenes by competitive inhibition of glutamin synthase, resulting in toxic accumulation of ammonia (De Block *et al.* 1987). Glyphosate (N-phosphonomethylglycine, 96%; Aldrich) interferes with aromatic amino acid biosynthesis by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which synthesizes secondary metabolites in plants (Shah *et al.* 1986). Both hygromycin-B hydrochloride (80%, SIGMA) and kanamycin monosulphate (78.3%, SIGMA) are aminoglycosides produced by soil-borne bacteria, respectively *Streptomyces hygroscopicus* and *S. kanamyceticus*. Hygromycin inhibits the biosynthesis of proteins (Bowen 1993). Kanamycin is responsible in plant cells for impaired chloroplast synthesis resulting in chlorosis (Nap *et al.* 1992).

Evaluation of inhibitory effect

According to the consideration given in the Introduction, a selective agent concentration in callus induction medium was considered promising when a) 7 days after supply of selective agent 90% inhibition of callus formation in relation to control was observed, b) the surviving 10% of callus had

not further developed after 25 days of selective agent supply, and c) tissue necrosis at day 15 was not higher than with the control.

Results

Callus formation on leaves of control treatments

Leaves were cultured on two different media for callus induction, which are suitable for respectively, so-called "direct" somatic embryogenesis (Yasuda *et al.* 1985), and "indirect" high frequency somatic embryogenesis (HFSE) (Berthouly & Michaux-Ferrière, submitted).

In our study, application of the first procedure on control treatments was characterized by a short callus phase and little callus proliferation. After about 40 days of culture, somatic embryos developed from small amounts of callus. Percentage of *C. canephora* explants with callus formation after 30 days of culture on **Y**-medium was 48% for GGP-leaves (Table 1) and 96% for SEM-leaves (Table 2).

The HFSE-procedure is characterized by formation of a primary white compact callus on control leaf explants, during one month culture on **C**-medium. Callus formation on GGP-leaves and SEM-leaves was observed on respectively 91% and 98% for Arabusta, and 86% and 98% for *C. arabica* (Table 1 and 2). After transfer of explants onto medium **E** for embryo induction at day 45, the primary callus degenerated giving rise to friable HFSE-callus 2 to 3 months later.

Inhibition of callus formation on leaves of greenhouse grown plants

In Table 1 the results of inhibition of callus formation by selective agents on GGP-leaves are shown. At inhibiting doses, hygromycin, chlorsulfuron and, to a lesser extend, glyphosate induced severe necrosis. Kanamycin was efficient for *C. arabica* cv Catuai at 100 $mg.l^{-1}$ and at 200 $mg.l^{-1}$ for *C. canephora* clone 197. For Catuai and the Arabusta clone, 6 $mg.l^{-1}$ glufosinate was sufficient for inhibiting callus formation, without provoking severe tissue necrosis. For the more sensitive "direct" somatic embryogenesis system with clone 197 even a concentration of 1 $mg.l^{-1}$ glufosinate was sufficient. The data show that glufosinate and kanamycin offer best perspectives according to the criteria indicated in Materials and methods.

Genotypes showed different sensivity to the selective agents. Arabusta clone 1312 had most callus formation and this was difficult to inhibit. Clone 197 on the other hand, was most sensitive to all selective agents and for suppression of "direct" somatic embryogenesis lower concentrations than studied here, especially of chlorsulfuron, glyphosate and hygromycin, might have given better results.

Table 1. Effect of different concentrations of herbicides and antibiotics on callus formation and necrosis of greenhouse grown leaves of *Coffea.* Leaves were cultured from day 0 (d0) on callus induction medium (C or Y), agents were applied at d5. Data were evaluated as indicated in Materials & methods: low levels of callus formation and tissue necrosis are given in bold figures; low levels in one combination are in italics.

concentration of selective agent		High frequency SE[1] procedure (C-medium)						Direct SE[1] procedure (Y-medium)		
		C. arabica cv Catuai			Arabusta clone 1312			*C. canephora* clone 197		
		callus[2]		necrosis[3]	callus[2]		necrosis[3]	callus[2]		necrosis[3]
		d12	d30	d20	d12	d30	d20	d12	d30	d20
chlorsulfuron	2	22	50	79	76	94	6	**0**	**0**	50
(µg.l^{-1})	10	**0**	29	79	**0**	82	35	**0**	**0**	50
	45	**0**	**0**	88	**0**	33	33	**0**	**0**	80
glufosinate	0.2	22	86	86	72	78	2	**0**	**0**	18
	1	11	100	71	78	57	17	***0***	***0***	***10***
(mg.l^{-1})	3	47	**0**	**10**	29	**0**	**5**	***6***	***0***	***5***
	6	***6***	***0***	***25***	***0***	***0***	***5***	***0***	***0***	***10***
	9	**0**	**0**	90	12	**0**	15	***0***	***0***	***10***
glyphosate	30	**0**	**0**	50	33	67	2	**0**	**0**	29
(mg.l^{-1})	60	***0***	***0***	***33***	17	21	5	**0**	**0**	38
	90	***0***	***0***	***39***	**6**	17	10	**0**	**0**	59
hygromycin	2	28	71	79	89	94	6	**0**	**0**	45
	10	**0**	**0**	85	**0**	17	28	**0**	**0**	100
(mg.l^{-1})	30	**0**	**0**	85	**6**	**0**	50	**0**	**0**	100
	60	**0**	**0**	100	**0**	**0**	75	**0**	**0**	100
	90	**0**	**0**	100	**0**	**0**	90	**0**	**0**	100
kanamycin	100	***0***	***0***	***5***	33	41	2	19	**0**	**5**
(mg.l^{-1})	200	***0***	***0***	***6***	11	23	2	***0***	***0***	***10***
	300	***0***	***0***	***11***	***11***	***12***	***2***	***0***	***0***	***10***
control		39	86	42	89	91	3	30	48	15

[1] SE, somatic embryogenesis
[2] % of explants with callus formation
[3] % of necrosed explant surface

Inhibition of callus formation in leaves of somatic embryo-derived microcuttings

Results on SEM-leaves are shown in Table 2. In general SEM-leaves showed more reaction to the callus induction media than GGP-leaves, especially for the *C. arabica* and *C. canephora* genotypes. Results of inhibiting agents were similar to those shown in Table 1. Callus inhibition without at the same time occurrence of severe necrosis was not obtained with hygromycin, chlorsulfuron or glyphosate. Application of 30 mg.l^{-1} hygromycin on *C. arabica* KF2.1 was the only exception. Kanamycin was efficient at 100 mg.l^{-1} for both KF2.1 and clone 197. Again glufosinate was efficient on all three genotypes: 6 mg.l^{-1} was sufficient for both KF2.1 and clone 1312, and 1 mg.l^{-1} could do for clone 197. Necrosis effect of hygromycin and glufosinate on SEM-

Table 2. Effect of different concentrations of herbicides and antibiotics on callus formation and necrosis of leaves of somatic embryo-derived microcuttings of *Coffea*. Leaves were cultured from day 0 (d0) on callus induction medium (C or Y), agents were applied at d5. Data were evaluated as indicated in Materials & methods: low levels of callus formation and tissue necrosis are given in bold figures; low levels in one combination are in italics.

concentration of selective agent		High frequency SE[1] procedure (C-medium)						Direct SE[1] procedure (Y-medium)		
		C. arabica KF 2.1			Arabusta clone 1312			*C. canephora* clone 197		
		callus[2]		necrosis[3]	callus[2]		necrosis[3]	callus[2]		necrosis[3]
		d12	d30	d20	d12	d30	d20	d12	d30	d20
chlorsulfuron	2	61	100	**25**	59	100	20	20	60	70
(µg.l^{-1})	10	6	100	75	12	65	70	**0**	**7**	90
	45	**0**	39	45	**0**	**6**	70	**0**	**0**	85
glufosinate	0.2	50	100	**30**	65	82	**10**	40	47	60
	1	28	50	**20**	12	**0**	**15**	**0**	**0**	50
(mg.l^{-1})	3	17	**6**	**15**	24	**6**	**10**	***2***	***0***	***10***
	6	***0***	***0***	***10***	***0***	***0***	***10***	***0***	***0***	***10***
	9	***0***	***0***	***10***	***0***	***0***	***10***	***0***	***0***	***10***
glyphosate	30	**0**	17	72	11	83	67	**0**	**0**	24
(mg.l^{-1})	60	**0**	**6**	56	**0**	**0**	47	**0**	**0**	44
	90	**0**	**0**	56	**0**	**0**	67	**0**	**0**	24
hygromycin	2	50	94	**20**	59	88	25	53	20	85
	10	**6**	50	**20**	**0**	47	50	**0**	**0**	90
(mg.l^{-1})	30	***0***	***0***	***20***	**0**	0	40	**0**	**0**	90
	60	**0**	0	60	**0**	0	95	**0**	**0**	95
	90	**0**	0	90	**0**	0	100	**0**	**0**	95
kanamycin	100	***0***	***0***	*22*	22	11	33	***0***	***0***	*12*
(mg.l^{-1})	200	***0***	***0***	*6*	11	6	22	***0***	***0***	*12*
	300	***0***	***0***	*8*	*11*	*6*	*17*	**0**	**0**	29
control		81	98	**35**	92	98	17	88	96	**18**

[1] SE, somatic embryogenesis
[2] % of explants with callus formation
[3] % of necrosed explant surface

leaves is visualized in Plate 1a + b. Use of glufosinate or kanamycin meets the requirements mentioned in Materials and methods.

Growth inhibition of embryogenic callus suspensions by selective agents

Both *C. canephora* OP-597 and Congusta clone HA were cultured in CP-medium. Growth inhibition of clone HA was measured in presence of chlorsulfuron, glufosinate and hygromycin. All five selective agents were tested on OP-597. Results of the experiments are shown in Fig. 1A-H.

Level of tissue necrosis at day 18 was described subjectively and the following

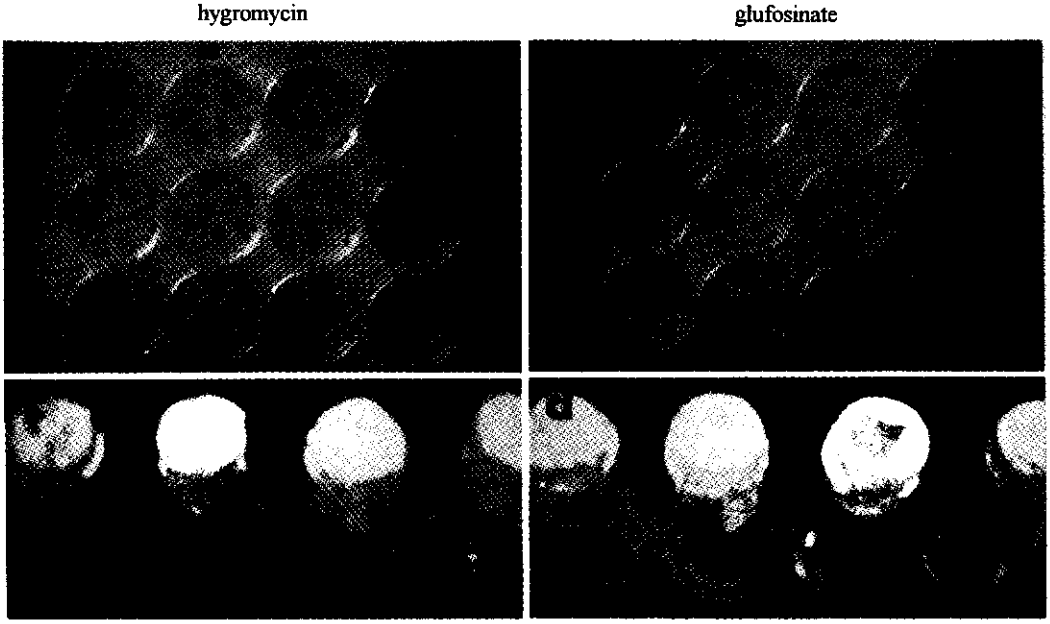


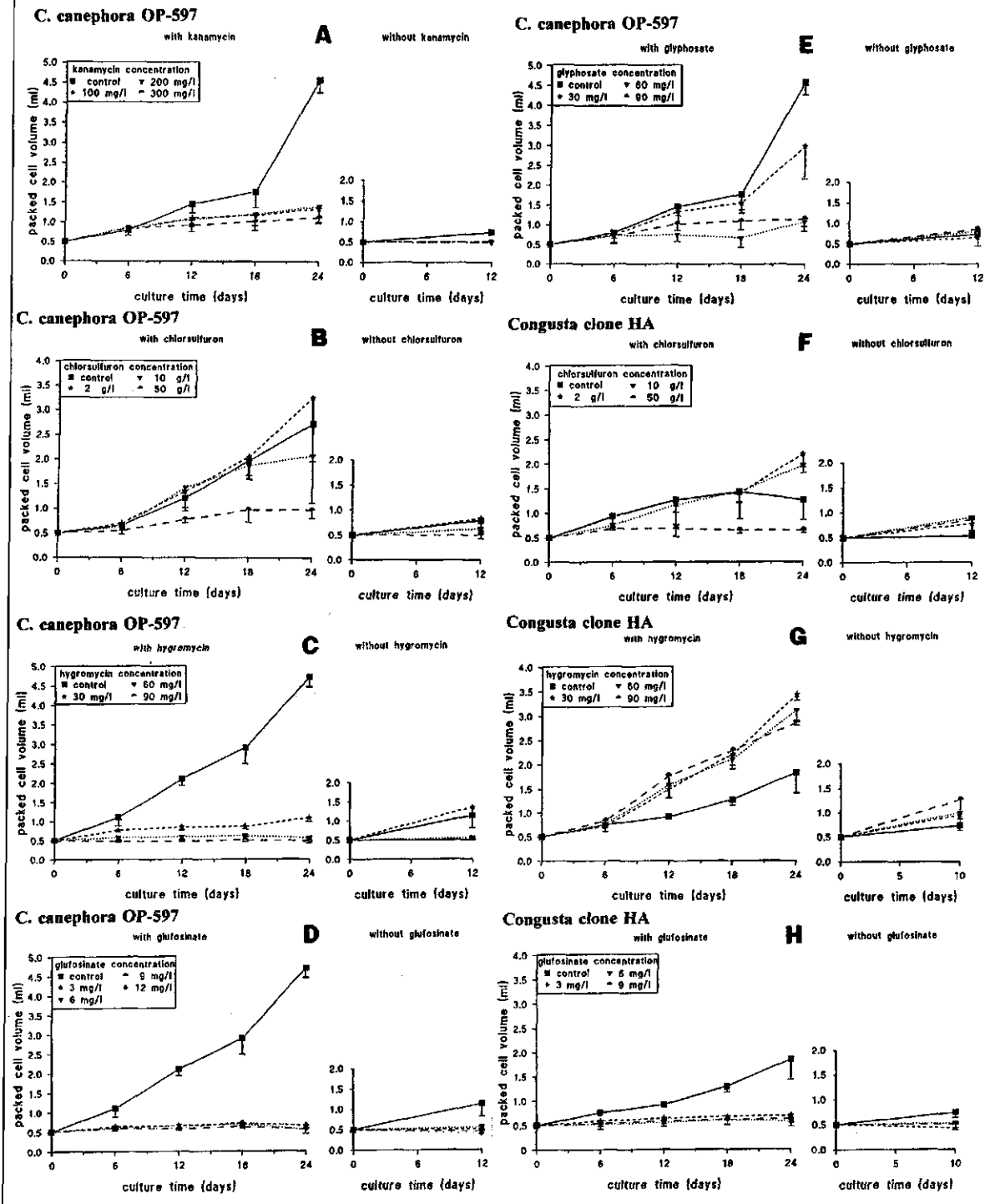
Plate I. Necrosis effect of selective agents on coffee tissues. Leaf explants of somatic embryo-derived microcuttings of *C. canephora* clone 197, *C. arabica* KF 2.1 and Arabusta clone 1312 cultured during 18 days on callus induction media (**C** or **Y**), containing different concentrations of **a)** hygromycin and **b)** glufosinate. Embryogenic callus suspensions of *C. canephora* OP-597 cultured during 18 days in **CP**-medium, containing different concentrations of **c)** hygromycin and **d)** glufosinate.

order could be observed (from dark brown colored to cream colored): hygromycin > chlorsulfuron > glyphosate > kanamycin > glufosinate. Polyphenolic oxidation in necrosing tissue is problematic especially in liquid cultures. The toxic substances are released throughout in the medium.

Growth of OP-597 suspensions was inhibited by all kanamycin concentrations and neither of them could recover and regain normal growth after kanamycin removal (Fig. 1A). Tissue necrosis led to moderate browning of culture medium.

A glyphosate concentration of 60 $mg.l^{-1}$ was inhibiting growth of OP-597 suspensions, but after removal of the herbicide growth appeared to resume, an indication for tissue survival (Fig. 1B). Higher glyphosate concentrations are probably needed for efficient inhibition of callus suspension growth.

Fig. 1. (page 91). Growth curves of embryogenic coffee callus suspensions in selective agent-containing **CP**-medium during 24 days, and subsequent culture in selective agent-free **CP**-medium during 10-12 days. Initial density was 0.5 ml pcv in 25 ml medium; subculture was performed at day 12. *C. canephora* OP 597 with **A)** kanamycin, **B)** glyphosate, **C)** chlorsulfuron, **E)** hygromycin and **G)** glufosinate. Congusta clone HA with **D)** chlorsulfuron, **F)** hygromycin and **H)** glufosinate.


C. canephora OP-597
with kanamycin
A
without kanamycin
kanamycin concentration
control
100 mg/l
200 mg/l
300 mg/l
packed cell volume (ml)
culture time (days)
C. canephora OP-597
with glyphosate
E
without glyphosate
glyphosate concentration
control
30 mg/l
60 mg/l
90 mg/l
C. canephora OP-597
with chlorsulfuron
B
without chlorsulfuron
chlorsulfuron concentration
control
2 g/l
10 g/l
50 g/l
Congusta clone HA
with chlorsulfuron
F
without chlorsulfuron
C. canephora OP-597
with hygromycin
C
without hygromycin
hygromycin concentration
control
30 mg/l
60 mg/l
90 mg/l
Congusta clone HA
with hygromycin
G
without hygromycin
C. canephora OP-597
with glufosinate
D
without glufosinate
glufosinate concentration
control
3 mg/l
6 mg/l
9 mg/l
12 mg/l
Congusta clone HA
with glufosinate
H
without glufosinate

Chlorsulfuron inhibited efficiently suspension growth of both OP-597 and clone HA at a concentration of 50 $\mu g.l^{-1}$ (Fig. 1C, D). Contrarely to OP-597, growth of clone HA restarted after chlorsulfuron removal. Tissue necrosis provoked severe browning of culture medium in all cases.

An unexpected result was obtained with the hygromycin test. For OP-597 all hygromycin concentrations were inhibitory, being 60 and 90 $mg.l^{-1}$ most efficient (Fig. 1E). However, for clone HA, presence of hygromycin stimulated suspension growth, in relation to the control treatments (Fig. 1F). Besides the fact that hygromycin provoked the most severe browning of medium (Plate 1c), this selective agent appeared therefore not to induce similar growth inhibition in different genotypes.

Best results were obtained with glufosinate. Besides the observation that suspension growth was efficiently inhibited for both genotypes and did not regain growth after glufosinate removal (Fig. 1G, H), only slight tissue necrosis was observed during the first 3 weeks of culture (Plate 1d). In case of selection after successful transformation, this should allow resistant calli to survive without the toxic influence of oxidized polyphenols.

Discussion & conclusions

To develop a selective growth system for genetically transformed coffee tissues, in a first approach the effect of several selective agents on callus formation from leaf explants, and on growth of embryogenic suspension cultures was studied.

Primarily, the nature of the target tissue should be taken in consideration. Green tissues as leaf explants may be more sensitive to agents which affect plastid-localized processes than callus cells. Subsequent survival of transformed cells may then be affected by the release of toxic compounds as phenolics, from surrounding untransformed cells. Furthermore, exposure of callus cells to high levels of these agents during the selection procedure can affect regeneration of green plants or subsequent plastid development (Bowen 1993).

Inhibition of callus development on leaves

Among five selective agents tested (chlorsulfuron, glufosinate, glyphosate, hygromycin and kanamycin) glufosinate showed most efficient inhibition of callus formation on leaves of *C. arabica*, Arabusta and *C. canephora*, without provoking rapid tissue necrosis. Despite of results being comparable for inhibition of callus induction on GGP-leaves and on SEM-leaves, the first are in general more sensitive to polyphenolic oxidation-induced necrosis. In a histological study, optimization of procedures for obtaining transgenic coffee tissue after biolistic treatment on GGP-leaves, were already discussed (chapter 6). GUS-containing cells seemed to be

hindered in their multiplication by abundant necrosis of surrounding tissue.

It was shown that leaves of *C. canephora* were more sensitive to action of selective agents. Possibly due to leaf morphology (thin and weak), a dose of 1 $mg.l^{-1}$ glufosinate was sufficient to inhibit callus formation with *C. canephora*, whereas 6 $mg.l^{-1}$ was needed for *C.arabica* KF2.1 and Arabusta. The sensitive character of leaves of certain *C. canephora* genotypes (clone 197 and OP-597) to bombardment has earlier be noted (chapter 5 and 6). Because of the tendency to rapid necrosis, lower amount of tungsten had to be used for biolistic treatment of clone 197 leaves, in order to avoid massive leaf injury.

For all studied conditions, leaves of Arabusta produced most callus, and its proliferation was most difficult to inhibit by selective agents. Probably the hybrid character is responsible for this more vigorous behaviour.

Beside the genotypic effect, also the effect of different somatic embryogenesis procedures has to be considered. In all cases, the so-called "direct" somatic embryogenesis, according to Yasuda *et al.* (1985), appeared to be more sensitive to selective agent supply, than the HFSE-procedure. This implies that selective agent concentrations have to be reduced, when used in a selective growth system for the "directly" obtaining of somatic embryos from biolistically treated leaves. On the other hand, following the HFSE-procedure, the development of primary nodular callus is necessary for the subsequent HFSE-callus formation. Thus, biolistic treatment can be applied directly on leaves or on the primary callus, needed for obtaining HFSE-callus. A selective growth system based on inhibition of HFSE-callus formation from primary callus, may require other agent concentrations than needed for inhibition of primary callus induction from leaves. The applied transformation procedure will be decisive for the glufosinate concentration that is needed.

Our results show that in agreement with results of Barré (1990) and Spiral & Pétiard (1993), use of kanamycin as selective agent may be applicable in coffee. But kanamycin should then only be applied to inhibit multiplication of untransformed cells in an early phase of embryogenesis, since somatic embryos, developing later on, show tolerance and unequal responses to high doses. In our study, 100 to 200 $mg.l^{-1}$ kanamycin was sufficient to inhibit primary callus formation without rapid tissue necrosis.

Inhibition of callus proliferation in embryogenic suspensions

Selection of transformed tissue in suspension cultures showed similar aspects. Rapid tissue necrosis is not desired, but to allow manual selection, well growing transgenic calli have to be distinguishable from normal calli. Selective growth may therefore be more appropriate under regeneration conditions. In this way, somatic embryos developing in liquid regeneration medium (**R**), may be considered as potentially being transgenic. However, also under these conditions polyphenolic oxidation has to be retarded as long as possible, because the regeneration of embryogenic coffee calli in

liquid medium takes about 6 to 8 weeks (chapter 2).

In the present study, both chlorsulfuron and hygromycin provoked severe necrosis in tested callus suspensions. Studied glyphosate concentrations were not sufficient to inhibit growth. Only kanamycin in a concentration of 100 $mg.l^{-1}$ or glufosinate at 3 $mg.l^{-1}$ were sufficient to inhibit growth of embryogenic suspensions of respectively *C. canephora*, and *C. canephora* and Congusta.

As a conclusion, two selective culture systems may be preferable. Firstly, SEM-leaves are submitted to a 2 days-pretreatment on callus induction medium (**Y** or **C**) prior to transformation. Subsequent selective pressure of 1 to 3 $mg.l^{-1}$ glufosinate is applied 3 to 5 days later. It is not yet clear if such a glufosinate concentration, applied in a later stage of the somatic embrogenesis process has a similar inhibitory effect. Therefore doses have to be tested only after 1 month, when the induction of HFSE-callus initiates on the degenerating primary callus cultured on **E**-medium.

Secondly, embryogenic callus suspensions are exposed to a one week-pretreatment in liquid **R**-medium prior to transformation, and 3 $mg.l^{-1}$ glufosinate is added 3 to 5 days later. Also in this system, inhibitory glufosinate concentration has to be determined in later developmental stages of somatic embryogenesis.

Use of glufosinate resistance genes in a selective growth system may also have agronomical importance. Glufosinate spraying for weeding in plantations of young coffee plants is hampered by its lethal effect on the fragile young plants (Bouharmont, pers. comm.). Glufosinate resistant plants may facilitate spraying without loss of coffee plants.

In previous experiments promoter EF1α-A1 from *A. thaliana* has proven to be a strong enhancer for transient expression in bombarded coffee leaves (chapter 5). For stable expression assays, it is recommended to place *bar* gene, which confers to glufosinate resistance (De Block *et al.* 1987), under control of EF1α-A1 promoter.

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CHAPTER 8

General discussion

1. Recapitulation

Genetic transformation has the potential to overcome barriers present in conventional breeding methods and to accelerate breeding of new varieties. Overcoming of genetic barriers, like insertion of bacterial genes in the plant genome, may be of benefit to the entire plant kingdom. A considerable acceleration can be achieved especially in breeding of perennial crops. For the cultivated species of the genus *Coffea*, both arguments justify the use of genetic transformation. To encounter the needs of producers and consumers, coffee cultivars should be enriched with valuable agronomic or quality traits. Firstly, introduction of the bacteria-derived genes for male sterility (Mariani *et al.* 1990) and for restoration of fertility (Mariani *et al.* 1992) may potentially be carried out by genetic transformation, rather than by conventional breeding. Integration of these genes can accelerate breeding by facilitating the production of hybrid *C. arabica* seeds. Also introduction of bacteria-derived Bt-genes conferring resistance to insects represents a potential application of genetic transformation. Finally, introgression of cloned natural resistance genes, either being active against fungi, bacteria, insects or nematodes, from wild into cultivated coffee species, should be realized more rapidly by genetic transformation methods.

The 25 years of experience in coffee biotechnology since Staritsky (1970) supports strongly the application of genetic transformation. The availability of well-controlled regeneration systems is a requirement. A method which allows the introduction of agricultural interesting genes into the coffee genome may thus intervene at any moment in the regeneration system, because subsequent regeneration is a potential acquisition. Four regeneration systems have been described in coffee: the so-called "direct" somatic embryogenesis (Yasuda *et al.* 1985), high frequency somatic embryogenesis (Söndahl & Sharp 1977), protoplast regeneration (Schöpke *et al.* 1987) and androgenesis (Ascanio & Asdrubal Arcia 1994). Gene transfer methods, either direct or indirect, should be integrated in one of these developed procedures. The principal aim is to discover the combined regeneration system and gene transfer method giving best perspectives for achievement of stably transformed coffee plants. The work of this thesis consisted of studies on three of such combinations: a) electroporation of protoplasts, b) biolistics on tissue prior to induction of "direct" somatic embryogenesis and c) biolistics before high frequency somatic embryogenesis.

It was demonstrated that problems related to the occurrence of polyphenolic oxidation arise as a "leitmotiv" all along the development of a genetic transformation procedure for coffee.

2. Regeneration systems

2.1 Direct somatic embryogenesis (DSE)

Somatic embryogenesis has proven to be a powerful tool as a coffee regeneration system (Söndahl & Lauritis 1992). Direct somatic embryogenesis (DSE), being the more rapidly, is able to produce easily germinating somatic embryos and considered to be less subject to somaclonal variation. However, embryo yield is low and response is genotype-specific (Bieysse *et al.* 1993; Ramos *et al.* 1993). The somatic embryos arise from dedifferentiation of perivascular leaf cells and are principally of multicellular origin (Michaux-Ferrière *et al.* 1989). DSE can be induced from explants on culture medium with cytokinin as the sole growth regulator (Yasuda *et al.* 1985; Hatanaka *et al.* 1991), but it can also occur during auxin-induced HFSE-callus formation, when somatic embryos develop directly (undesired) from primary callus (Michaux-Ferrière *et al.* 1987). Söndahl & Sharp described the latter process as low frequency somatic embryogenesis (LFSE). Induction of DSE from explants after genetic transformation treatment might be a possibility for obtaining solid transformants. Critical concentrations of selective agents for inhibition of non-transformed cell proliferation in a DSE-system have to be determined. Doing so, it must be taken into account that tissue-wounding transformation methods like biolistics, *Agrobacterium* vectors or whole tissue electroporation, in general provoke oxidation of polyphenols. In pilot experiments, leaf explants bombarded with *bar*-containing plasmids were cultured on DSE-induction medium supplemented with glufosinate. It appeared that the glufosinate concentration which was only 60% inhibiting for DSE on untreated control tissue, was completely inhibiting DSE on bombarded tissue: the effect of biolistic shock had provoked severe tissue necrosis and thus introduced a supplementary unforseen stress. All DSE, either from touched or untouched cells, was inhibited, which proves its sensitivity for tissue wounding and subsequent intervention of selective agents, expressed by polyphenolic oxidation.

2.2 High frequency somatic embryogenesis (HFSE)

Also HFSE appeared to be genotype specific and senstive to polyphenolic oxidation (chapter 2). But its advantage is being found in the production of highly embryogenic friable callus, which is an ideal source for biotechnological procedures, such as rapid multiplication in liquid medium, regeneration and large-scale embryo production in bioreactors, and use for gene transfer manipulations. Since somatic embryos developing from HFSE-callus are of unicellular origin, gene introgression in this tissue may avoid subsequent formation of chimeras. HFSE-callus can also be used indirectly for genetic transformation, by using it for protoplast isolation and subsequent electroporation (chapter 4). Due to their physiological state, HFSE-callus cells, either on semi-solid or in liquid medium, are less hindered by polyphenolic oxidation than cells in organized

tissues. Polyphenolic contents in cell compartiments of HFSE-callus (Monaco *et al.* 1977) may be of a lesser extend and thus of less influence during cell stress.

2.3 Protoplasts

Coffee protoplast regeneration procedures have been described (Spiral & Pétiard 1991; Acuna & de Pena 1991; Tahara *et al.* 1994). These procedures, but also those not resulting in regeneration, have proved that isolation of viable protoplasts in large quantities can be achieved. The regeneration, however, is often hampered by the physical method of culture (Orozco & Schieder 1984; Schöpke 1989). The in chapter 3 tested culture conditions, in liquid medium or by embedding in agarose, resulted in the formation of protocolonies, consisting of 20-30 cells, two months after isolation. In our hands, protoplasts could thus not be used as a reliable source for a coffee regeneration system in stable transformation studies. Barton *et al.* (1991) gave evidence for this obstacle; they apparently obtained only one transgenic plantlet, which subsequently died, after four years of studies on protoplast regeneration and electroporation. Due to the long regeneration time (10-14 months), long term studies on coffee protoplast transformation and regeneration will be necessary, for giving better insight in solving bottle-necks.

3. Genetic transformation

3.1 Transient expression

Transient expression studies are of interest to obtain rapidly information on effects of physicical transformation conditions, choise of plant tissue, promoters and gene expression. Since regeneration is not required, there is less restriction to the choise of employed tissue.

We have seen that high yields of viable protoplasts could be obtained from non-embryogenic suspension cultures (chapter 4). Physical electroporation conditions were optimized and high levels of transient expression of the GUS reporter gene were demonstrated. Testing of promoter strength or expression of genes of interest is thus a potential application. However, some disadvantages were perceived. A relative high level of background "GUS-activity" was always present when transient expression was assayed fluorometrically. Some possible causes were proposed for explaining this phenomenon. It also appeared that the level of GUS activity in protoplasts increased during time, upto a maximum at day 9 after electroporation. This may be due to the persistent nature of the β-glucuronidase enzyme, combined with a presumed low metabolic activity of coffee protoplasts. Hence, discrimination between background "GUS activity" and plasmid-related GUS activity was best only 6 to 9 days after electroporation, which is different from the normally proposed 2 days for optimized

transient expression studies (Jefferson 1987). Furthermore, the fluorometric GUS assay, which is better adapted to testing of protoplasts, lacks the simplicity of the histochemical GUS assay. But on the other hand, fluorometry gave a clearer quantification of differences in effects of tested treatments.

An alternative is the application of biolistics on intact coffee tissue. Analysis of transient expression after only two days was sufficient for obtaining of significant differences between treatments. It was demonstrated that *in vitro* cultured leaves were most appropriate for this goal, with regard to their availability, handiness and assay-easiness (chapter 5). The occurrence of biolistically-induced polyphenolic oxidation was a success-reducing factor. The use of genotypes which are more sensitive to polyphenolic oxidation, like *C. canephora* clone 197 and OP-597, should be avoided. Also necrosis during X-gluc incubation has to be limited for maintaining perception of GUS-expressing spots. Use of some oxidation reducers was proposed (PVP-10 or -40, metabisulfite, caffeine). Very recently Beeckman & Engler (1994) described the use of chlorallactophenol for clearing of histochemically stained tissue. But from a sanitary point of view, manipulation with phenol-derived chemicals may better be avoided.

3.2 Stable expression

Avoidance or reduction of polyphenolic oxidation is the guideline for obtaining stable transformation of coffee. It is preferable to employ transformation methods which do not provoke severe tissue wounding, or, if so, use of tissue with low polyphenolic content. A histological study excluded the use of greenhouse grown tissues for biolistics (chapter 6). Use of *in vitro* cultured tissue is recommended, but it still leaves the question, which kind of tissue has to be used and in what developmental stage. When bombarding leaves, subsequent induction of DSE might be preferable over induction of HFSE-callus-preceeding primary callus, since DSE leads more rapidly to somatic embryo formation. Results in chapter 7 however, revealed the sensitive nature of DSE to selective agents. Another option is bombardment of primary callus. Beside the advantage of its low sensitiveness for polyphenolic oxidation, the desired friable HFSE-callus develops directly from it. On the other hand ensuring the availability of high quantities of primary callus for treatment is a not so trivial task.

Consequently, use of HFSE-callus is more advantageous: although initially formed in limited quantity on leaf explants, its multiplication in liquid medium allows a constant availability of tissue. In chapter 7, the inhibitory effect of selective agents on callus suspensions in multiplication phase was described. But it may be preferable to develop a selective growth system based on callus suspensions in their regeneration phase. This would simplify selection, since the criterion for selection of transformants would be somatic embryo formation. Moreover, the unicellular origin of the developing somatic embryos avoids the potential chimeric character of transformants.

Finally, the use of somatic embryos for bombardment may be considered. Since subsequent secondary somatic embryogenesis is possible (Berthouly & Michaux-Ferrière submitted), stable transformants can potentially be obtained. However, the development of a selective growth system will be a complicated task, since studies have demonstrated high tolerance of this tissue to some selective agents (Spiral & Pétiard 1993). In our study best perspectives were obtained with glufosinate and in less amount with kanamycin (chapter 7).

4. Prospects and concluding remarks

The specific advantages of HFSE-callus in liquid culture (easy manipulation, highly embryogenic character, production of high amounts of somatic embryos of unicellular origin) should be used in other transformation systems. Electroporation of partially digested callus, according to D'Halluin *et al.* (1992) is a potential alternative. An electroporation device with extended capacitor is therefore needed.

It was demonstrated that the histochemical GUS assay was not ideal for analyzing GUS presence in phenolics-containing coffee tissues (chapter 6). The X-gluc incubation at 37°C proved to be a step responsible for occurence of tissue necrosis. It is recommended to perform screening of small tissue parts of regenerants (supposed to be transgenic) by the shortened fluorometric assay (Jefferson 1987). For the protein extraction step, it is important to add metabisulfite. Metabisulfite has also proven to be indispensable for DNA-extraction, prior to PCR amplification on coffee tissue (Lashermes, pers. comm.). If a reliable selective growth system is available, the use of the luciferase reporter gene (Ow *et al.* 1986) should deserve careful consideration. Spraying of luciferine and measurement of light emission by a photo-illuminometer *in vivo* is possible. With regard to the genetic transformation of coffee, use of the luciferase reporter gene may demonstrate advantages of *in vivo* selection over the *gus* reporter gene.

Actually, results of this thesis are used for further ongoing studies. Attempts are being made to construct transformation vectors which give best perspectives for the obtainment of transgenic coffee plants. The EF1α-A1 promoter of *Arabidopsis thaliana*, which has proven to give high expression in coffee tissues (chapter 5), is most appropriate for controlling the expression of a selection gene. The *bar* gene, conferring to phosphinotricin resistance seems to be the best candidate for this aim (chapter 7).

The particle gun device used during this study was not optimal. The powder-driven device described by Zumbrunn *et al.* (1989) delivered often too powerful shots and adjustment of technical device parameters was difficult. This is one of the reasons why the device was less appropriate for achieving stable expression from the senstive

coffee tissues. Preliminary experiments concerning transient expression of GUS in coffee leaves by using a simplified helium-driven device, according to Takeuchi *et al.* (1992), demonstrated less GUS activity. However for stable expression such a device, which is more subtile and easier to adjust, should be more suitable.

Although to our knowledge, no studies on the biolistic transformation of coffee are actually carried out, it deserves consideration of continued study. Doing so, the results from this thesis will serve as a guideline.

Summary

Background

With a value of 7-12 billion dollar yearly, coffee is world's first commercial agricultural export product, expressed in trade value. The two species that are cultivated, *Coffea arabica* (80%) and *Coffea canephora* (20%), are mainly grown in South America (50%), Africa (25%) and Asia (17%). Breeding strategies are mainly focussed on disease and pest resistance. Since one breeding cycle (from seed to seed-producing plant) takes 5 years, usually 25 to 35 years are needed for developing new cultivars by application of classical coffee breeding methods. For this kind of crops, genetic transformation may significantly accelerate the breeding process.

This study started in May 1990 at CIRAD/IRCC, and was focussed on the development of a reliable genetic transformation method for coffee. A possible first application of this technique in coffee breeding is the introduction of available genes that encode for insect resistance (*Bacillus thuringiensis* toxin genes). *Agrobacterium tumefaciens*-mediated transformation was not considered, because earlier experiments that were performed at CIRAD, had shown absence of transformation ability of coffee by this method. Therefore, research was focussed on the use of electroporation of protoplasts and on the biolistic method (particle gun bombardment). Simultaneously, complementary research which was carried out on tissue culture of coffee is also reported.

High Frequency Somatic Embryogenesis

Various research groups in the world have revealed the potential of somatic embryogenesis of *Coffea* spp. Research that is described in this thesis contributed to establish an improved procedure for High Frequency Somatic Embryogenesis (HFSE) of several *C. arabica*, *C. canephora*, Arabusta and Congusta genotypes. During this process a primary "nodular" callus was formed on explants which were cultured on callus induction medium. After transfer to embryo induction medium, the callus degenerated and gave rise to Low Frequency embryo formation (LFSE) and generally later on, to friable highly embryogenic HFSE-callus. The combined or sole occurrence of these two types of somatic embryogenesis seemed to be genotype-dependent. The friable HFSE-callus served subsequently for the establishment and multiplication of embryogenic callus suspensions. Culture conditions, such as light intensity, subculture interval, culture density and 2,4-D concentration, were determined for both rapid multiplication and long-term maintenance of such suspensions. The suspensions could be directed at will to the development of high numbers of somatic embryos of unicellular origin by omission of auxin and lowering of culture density. The duration of the complete procedure, from leaf explant to plantlet regeneration passing through callus multiplication in liquid medium, was about 9 months. This is 4-6 months shorter than other described procedures.

The presented research for genetic transformation, both by electroporation and biolistics, took advantage of this improved HFSE procedure.

Protoplast isolation and electroporation conditions

The most appropriate tissue for protoplast isolation, with regard to yield and viability, appeared to be suspension cultures. Using embryogenic suspensions of *C. arabica*, 2.2 x 10^7 protoplasts per gram fresh weight were obtained with a viability of 52%. Protoplasts were able to form cell walls and divided till small colonies, two months after isolation. Up to 3.7 x 10^7 $P.g^{-1}$ fresh weight with 90% viability could be obtained by using non-embryogenic suspensions. Such protoplasts were used for transient expression assays, in order to define optimal physical electroporation conditions. The β-glucuronidase (GUS) marker, that was used for this purpose, was best expressed between 6 to 9 days after electroporation, which is more than the two days needed for expression in other coffee tissues. GUS activity was highest (12 nM 4-MU.mg^{-1} protein.min^{-1}) when protoplasts were electroporated with 250 μF capacitor discharge under 250-350 $V.cm^{-1}$ field strength. Viability of the protoplasts treated this way, however, decreased considerably to about 30%. The readily available non-embryogenic suspension-derived protoplasts may nevertheless be used to test transient expression and strength of promoters in a coffee-electroporation system.

Considering the long interval needed for work with coffee protoplasts and lack of success with regeneration, the studies on this transformation method were abandoned. Due to promising results obtained in pilot experiments using particle gun bombardment on coffee tissue, this method became subject of investigation.

Transient expression studies with biolistics

After optimization of technical conditions of the powder driven particle gun device, the suitability of different coffee tissues for transient expression of the GUS marker was tested firstly. Bombardment of leaves of microcuttings allowed most easily detectable GUS expression, in comparison to greenhouse grown leaves and callus suspensions. Use of somatic embryos for this aim was hindered by disturbing endogenous GUS-like expression in this tissue. Differences in sensitivity to biolistic treatment appeared between genotypes; some *C. canephora* genotypes showed intens necrosis and consequently low GUS expression. GUS activity could be increased 2- to 3-fold by placing the GUS gene under control of the EF1α-A1 promoter of *Arabidopsis thaliana*, in comparison to three other promoters (CaMV-E35S, UBQ1 and LTR). Highest level of transient expression (1300 GUS expressing units per plate) was obtained using leaves of *C. arabica* microcuttings and the EF1α-A1 promoter. Actually, plantlets regenerated from bombarded somatic embryos and leaves of microcuttings are being tested for their transgenic character by PCR.

Histological study of bombarded leaves

The competence of cells of greenhouse grown coffee leaves for stable expression after integration of introduced genes was analysed. Therefore, callus development and GUS expression was investigated in bombarded leaves with a histological study. GUS activity was not traced in callus induced by the intrusion of the tungsten particles, but only in non-regenerating leaf parts. All cells receiving aggregated tungsten particles were found to be dead one month after bombardment. Stable integration seems to require: a) penetration of plasmid-carrying non-aggregated particles in callus-inducible cells (perivascular cells) which recover

after wounding, and b) permanently wounded neighbour cells for triggering callus induction. Because of their high sensitivity to polyphenolic oxidation leading to necrosis, greenhouse grown leaves appear less appropriate for transformation by biolistics.

By means of a study with transmission electron microscopy on bombarded leaves, GUS products (diX-indigo crystals) were traced in cell nuclei, but not in organelles. The crystals precipitated by preference on lipid membrane structures.

Selective growth

Three herbicides (chlorsulfuron, glufosinate and glyphosate) and two antibiotics (hygromycin and kanamycin) were tested in medium, for potential selective growth of transgenic coffee cells. Genes that encode for resistance against these agents have been isolated and can be used as selection markers in transformation experiments. Glufosinate appeared to provide the most efficient inhibition of coffee callus proliferation. With leaf explants, 6 $mg.l^{-1}$ glufosinate in culture medium was sufficient for the inhibition of primary "nodular" callus formation, while only 1 $mg.l^{-1}$ inhibited so-called "direct" somatic embryogenesis. Inhibition of embryogenic callus proliferation in liquid medium was obtained by the addition of 3 $mg.l^{-1}$ glufosinate. Besides the efficient inhibition of callus growth, no severe necrosis was observed. Absence of necrosis is considered favorable for permitting contineous growth of resistant tissue. Use of plasmids containing the *bar* gene, conferring resistance to glufosinate, might therefore contribute to the selection of stably transformed coffee tissue. This *bar* gene is preferably driven by the EF1α-A1 promoter.

Conclusion

Avoidance of polyphenolic oxidation seems to be the keyword in the development of a genetic transformation system for coffee. Tissue treated for gene transfer, should either be less sensitive to this phenomenon or precautions need to be taken to avoid it. The applied selective growth system should fulfil the same criteria for potential outgrowth of transgenic tissue. For these reasons, the most appropriate starting tissue for achieving stably transformed coffee appeared to be highly embryogenic HFSE-callus, cultured in liquid medium. Beside the fact that this tissue is less sensitive to necrosis when cultured contineously in liquid medium, somatic embryos developing from it are of unicellular origin. Use of glufosinate in a selective growth system may stimulate the obtaining of solid transgenic coffee. Finally, the prefered method of direct gene transfer appeared to be biolistics when using an improved device. However, electroporation of partially digested callus or *A. rhizogenes*-mediated transformation must also be held in consideration.

The study on genetic transformation of coffee comprised fundamental and applied aspects. Physical, physiological and histological parameters analyzed in parallel led us to reveal and partially resolve bottle-necks in two genetic transformation methods for coffee. The results obtained improve the knowledge on genetic transformation mechanisms, an interest that goes beyond the coffee research environment.

Résumé

Introduction

Avec des échanges qui varient entre 7 à 12 milliards de dollars par an, le commerce du café se place au premier rang mondial des produits d'exportation agronomique. Les deux espèces cultivées, *Coffea arabica* (80%) et *C. canephora* (20%), le sont principalement en Amérique Latine (50%), en Afrique (25%) et en Asie (17%). Les stratégies d'amélioration sont surtout concentrées sur la résistance aux maladies et aux ravageurs. Etant donné la durée du cycle de reproduction (5 ans depuis la semence jusqu'à la plante produisant les graines), 25 à 35 ans sont nécessaires pour le développement de nouvelles variétés, par les méthodes classiques de sélection. La transformation génétique pourrait accélérer significativement ce processus d'amélioration.

Cette étude a débuté en mai 1990 au CIRAD/IRCC, et avait comme objectif le développement d'une méthode fiable de transformation génétique du caféier. Une première application potentielle de cette technique pour l'amélioration du caféier, est l'introduction des gènes disponibles, codant pour la résistance aux insectes (gènes de toxine de *Bacillus thuringiensis*). La transformation par *Agrobacterium tumefaciens* n'a pas été considérée, parce que des expériences préliminaires réalisées au CIRAD ont montré une absence d'aptitude du caféier à la transformation par cette méthode. Pour cette raison, les efforts ont été concentrés sur l'éléctroporation de protoplastes et sur la méthode biolistique (canon à particules). Simultanément, des recherches complémentaires concernant la culture *in vitro* du caféier, ont été réalisées.

Embryogenèse Somatique Haute Fréquence

Divers groupes de recherches dans le monde ont révélé la potentialité d'embryogenèse somatique de *Coffea* spp.. Les résultats décrits dans cette thèse ont contribué à établir un procédé amélioré pour l'Embryogenèse Somatique Haute Fréquence (HFSE) de plusieurs génotypes de *C. arabica*, *C. canephora*, Arabusta et Congusta. Pendant ce processus de HFSE, un cal primaire "nodulaire" est formé sur des explants cultivés sur milieu de callogenèse. Après transfert des explants sur milieu d'expression de l'embryogenèse, le cal dégénère pour donner lieu à la formation d'embryons de type Basse Fréquence (LFSE). Plus tard, un cal de type HFSE, friable et hautement embryogène, est généralement formé. La concomitance ou pas de ces deux types d'embryogenèse somatique, semble être génotype-dépendant. Le cal friable de type HFSE sert par la suite à l'établissement et la multiplication de suspensions de cals embryogènes. Des conditions optimales de culture, concernant l'intensité de lumière, la fréquence de repiquage, la densité de culture et la concentration en 2,4-D, ont été déterminées à la fois pour la multiplication rapide et pour le maintien à long-terme de telles suspensions. Les suspensions ont été orientées à volonté vers la formation de nombreux embryons somatiques d'origine unicellulaire, par le retrait des auxines et la réduction de la densité de culture. La durée totale du procédé, depuis l'explant foliaire jusqu'à la régénération de plantules, en passant par la multiplication de cal en milieu liquide, est de 9 mois. Ceci représente 4 à 6 mois de moins que les autres procédés décrits.

Les recherches sur la transformation génétique présentées ici, à la fois par électroporation et par voie biolistique, ont pu profiter de ce procédé amélioré de HFSE.

Isolement de protoplastes et conditions d'électroporation

Le tissu le plus apte à l'isolement de protoplastes, en ce qui concerne la quantité et la viabilité, est apparu comme étant les suspensions de cals. En utilisant des suspensions embryogènes de *C. arabica*, 2.2 x 10^7 protoplastes par gramme de matière fraîche ont été obtenus, avec une viabilité de 52%. Les protoplastes ont été capables de former des parois cellulaires, et se sont divisé jusqu'au stade protocolonie, deux mois après leur isolement. Jusqu'à 3.7 x 10^7 $P.g^{-1}$ de matière fraîche avec une viabilité de 90% ont été obtenus en utilisant des suspensions non-embryogènes. De tels protoplastes ont été utilisés pour des études d'expression transitoire afin de mettre au point les conditions physiques optimales d'électroporation. Le meilleur moment pour évaluer l'expression du gène marqueur β-glucuronidase (GUS) se situe entre 6 et 9 jours après l'électroporation, ce qui est plus que les 2 jours demandés pour l'expression dans d'autres tissus de caféier. L'activité GUS la plus élevée (12 nM 4-MU.mg^{-1}protéine.min^{-1}) a été obtenue suite à des décharges électriques générées par une capacité de 250 μF et un voltage de 250-350 $V.cm^{-1}$. La viabilité des protoplastes ainsi traités a diminuée jusqu'à environ 30%. Néanmoins, le fait que des protoplastes dérivés de suspensions non-embryogènes soient facilement disponibles, en font un tissu plus approprié pour des études d'expression transitoire et des tests de puissance de promoteurs avec un système d'électroporation du caféier.

Compte-tenu du temps exigé par les manipulations et l'absence de régénération, les études d'éléctroporation ont été abandonnées. Grâce aux résultats prometteurs obtenus à partir des expériences préliminaires avec le canon à particules sur des tissus de caféier, cette méthode a été, par la suite, privilégiée.

Etudes d'expression transitoire par voie biolistique

Après la mise au point des paramètres techniques du canon à poudre, l'aptitude de différents tissus de caféier, pour l'expression transitoire du marqueur GUS, a d'abord été testée. L'expression GUS a été plus facilement évaluée sur des feuilles de microboutures que sur des feuilles issues de serre et des suspensions de cals. L'utilisation d'embryons somatiques a été gênée par une expression endogène troublante, ressemblant à celle du GUS. Des différences en sensibilité pour le traitement biolistique ont été mises en évidence entre les génotypes; quelques génotypes de *C. canephora* ont montré une nécrose intense et par conséquent peu d'expression GUS. L'activité GUS a pu être augmentée 2 à 3 fois en plaçant le gène GUS sous le contrôle du promoteur EF1α-A1 d'*Arabidopsis thaliana*, en comparaison avec 3 autres promoteurs (CaMV-35S, UBQ1 et LTR). Le niveau le plus élevé d'expression transitoire (1300 unités GUS par boîte de pétri) a été obtenu en utilisant des feuilles de *C. arabica*, issues de microboutures, et le promoteur EF1α-A1. Actuellement, des plantules régénérées à partir d'embryons somatiques et de feuilles de microboutures bombardés, sont en cours d'analyse par PCR pour vérifier leur caractère transgénique.

Etude histologique des feuilles bombardées

La compétence des cellules de feuilles issues de plantes en serre pour l'expression stable après

intégration des gènes introduits, a été analysée. Ainsi, le développement de cal et l'expression GUS ont été suivis dans des feuilles bombardées, par une étude histologique. L'activité GUS n'a pas été détectée dans du cal induit par l'intrusion de particules de tungstène, mais exclusivement dans des parties de feuilles non-régénérantes. Un mois après le bombardement, toutes les cellules qui avaient reçu des particules de tungstène agglomérées, sont mortes. Pour une intégration stable, deux conditions semblent être requises: a) une pénétration de particules non-agglomérées portant des plasmides, dans des cellules capables de se rétablir après blessure et d'induire du cal (cellules périvasculaires); b) des cellules voisines blessées pour déclencher l'induction de cal. A cause de leur sensibilité plus élevée à l'oxydation polyphénolique, menant à la nécrose, les feuilles provenant de serre semblent être moins aptes à la transformation par voie biolistique.

Par une étude en microscopie électronique à transmission sur des feuilles bombardées, les produits du gène GUS (cristaux de diX-indigo) ont été mis en évidence dans les noyaux, le cytosol et autour des organelles, sur les structures membranaires lipidiques.

Culture sélective

Trois herbicides (chlorsulfuron, glufosinate et glyphosate) et deux antibiotiques (hygromycine et kanamycine) ont été testés pour la culture sélective de cellules de caféier potentiellement transgéniques. Des gènes codant pour la résistance à ces agents sont isolés et peuvent être utilisés comme marqueurs de sélection dans des expériences de transformation. Le glufosinate a montré l'inhibition la plus efficace pour la prolifération de cal. Pour les explants foliaires, 6 $mg.l^{-1}$ de glufosinate dans le milieu de culture ont été nécessaires pour inhiber la formation de cal primaire "nodulaire", alors que seulement 1 $mg.l^{-1}$ a inhibé le processus d'embryogenèse somatique "directe". L'inhibition de la prolifération de cal embryogène en milieu liquide a été obtenue par l'addition de 3 $mg.l^{-1}$ de glufosinate. De plus, aucune nécrose sévère n'a été observée. L'absence de nécrose est considérée comme favorable pour permettre la croissance continue du tissu résistant. L'utilisation de plasmides contenant le gène *bar*, attribuant la résistance au glufosinate, pourrait ainsi contribuer à la sélection de tissus de caféier transformés de façon stable. Ce gène *bar* doit être, de préférence, sous le contrôle du promoteur EF1α-A1.

Conclusions

Eviter l'oxydation polyphénolique semble être l'étape clef pour le développement d'un système de transformation génétique du caféier. Ceci peut être réalisé soit en choisissant des tissus peu sensibles à ce phénomène, soit en prenant des précautions physico-chimiques. Le système de culture sélective appliqué doit répondre aux mêmes critères, pour permettre le développement du tissu transgénique. Pour ces raisons, le tissu de départ le plus apte pour l'obtention de caféiers transformés de façon stable, est apparu comme étant le cal HFSE hautement embryogène, cultivé en milieu liquide. Outre le fait que ce tissu soit moins sensible à la nécrose en milieu liquide, les embryons somatiques qui s'y développent sont d'origine unicellulaire. L'utilisation du glufosinate dans le milieu de culture pourrait favoriser la sélection de tissus transgéniques. D'après cette étude, la méthode de transfert direct la plus prometteuse est, sans doute, la voie biolistique, mais de préference avec utilisation d'un appareil amélioré. Cependant, l'électroporation de cal partiellement digéré ou la transformation

par *A. rhizogenes* doivent également être considérés.

L'étude sur la transformation génétique du caféier a inclus des aspects fondamentaux et appliqués. Des paramètres physiques, physiologiques et histologiques analysés en parallèle, nous ont mené à révéler et partiellement résoudre des problèmes inhérents aux deux méthodes de transformation génétique. Les résultats obtenus améliorent la connaissance des mécanismes de transformation génétique, un intérêt qui va au delà du domaine de la recherche sur le caféier.

Samenvatting

Achtergrond

Met een jaarlijkse bedrag van 7-12 miljard dollar, plaatst koffie zich aan kop van de wereldranglijst van landbouwexportproducten, uitgedrukt in handelswaarde. De twee geteelde soorten, *C. arabica* (80%) en *C. canephora* (20%), worden voornamelijk verbouwd in Zuid-Amerika (50%), Afrika (25%) en Azië (17%). De practische veredeling is hoofdzakelijk gericht op het verkrijgen van ziekte- en insektenresistentie. Aangezien één veredelingscyclus (van zaad tot zaad-producerende plant) vijf jaar duurt, zijn gewoonlijk zo'n 25-35 jaar nodig voor het ontwikkelen van een nieuw ras in het geval klassieke koffie-veredelingsmethoden worden toegepast. Bij dit soort gewassen kan de toepassing van genetische transformatie het veredelingsproces aanzienlijk versnellen.

Dit onderzoek ging in mei 1990 van start op het CIRAD/IRCC en was gericht op de ontwikkeling van een betrouwbare genetische transformatiemethode voor koffie. Het introduceren van reeds beschikbare genen voor insektenresistentie (*Bacillus thuringiensis* toxine genen) zou hiervan een eerste potentiele toepassing kunnen zijn. Transformatie d.m.v. *Agrobacterium tumefaciens* werd niet in de beschouwing mee genomen, omdat voorbereidend onderzoek op het CIRAD had aangetoond dat er bij koffie kennelijk van een afwezigheid van transformatie-vermogen sprake was via deze methode. Om deze reden werd voor transformatie alle aandacht gericht op protoplasten-electroporatie en "particle gun bombardment" van weefsels (biolistische methode). Gelijktijdig uitgevoerd, aanvullend onderzoek aan weefselkweek van koffie werd eveneens beschreven.

"High Frequency Somatic Embryogenesis"

De potentie van somatische embryogenese bij *Coffea* sp. werd reeds door verscheidene groepen in de wereld beschreven. Dit promotie-onderzoek heeft bijgedragen aan het ontwikkelen van een verbeterde methode van "High Frequency Somatic Embryogenesis" (HFSE) voor verschillende *C. arabica*, *C. canephora*, Arabusta en Congusta genotypen. Tijdens dit HFSE-proces wordt een primair "nodulair" callus gevormd aan explantaten op callusinductie-medium. Na het overzetten van de explantaten op embryoinductie-medium degenereert het callus en ontwikkelt zich hierop vervolgens een "Low Frequency" embryo-vorming (LFSE), en naderhand meestal een bros, zeer embryogeen HFSE-callus. Het al dan niet gecombineerd of afzonderlijk optreden van deze twee processen bleek genotype-afhankelijk te zijn. Het bros HFSE-callus diende vervolgens voor het opzetten en vermeerderen van embryogene callus suspensies. Condities voor de cultuur van zulke suspensies, zoals licht intensiteit, frekwentie van subcultuur, dichtheid van cultures en 2,4-D concentratie, werden bepaald zowel voor de snelle vermeerdering als voor de instandhouding. De suspensies konden naar wens geconditioneerd konden worden tot de vorming van grote hoeveelheden somatische embryos van unicellulaire oorsprong, door het wegnemen van auxine en een verlaging van cultuur dichtheid. De duur van de totale procedure, vanaf bladexplantaat tot aan regeneratie van scheutjes, via een callusvermeerderingsfase in vloeibaar medium, was circa 9 maanden. Dit is 4 tot 6 maanden korter in vergelijking met andere beschreven

procedures.

In het beschreven onderzoek naar genetische transformatie, zowel via electroporatie als "particle gun bombardment", is van deze verbeterde HFSE-procedure gebruik gemaakt.

Protoplastenisolatie en electroporatie-condities

Het meest geschikte uitgangsmateriaal voor protoplastenisolatie, m.b.t. opbrengst en kwaliteit, bleken suspensiecultures te zijn. Gebruik van embryogene *C. arabica* suspensies leverde 2.2 x 10^7 protoplasten per gram vers gewicht op, waarvan 52% vitaal was. De protoplasten waren vervolgens in staat om celwanden te vormen en zich te delen tot kleine kolonies, zo'n 2 maanden na isolatie. Van niet-embryogene suspensies werden tot 3.7 x 10^7 $P.g^{-1}$ vers gewicht verkregen, waarvan 90% vitaal was. Deze protoplasten werden gebruikt in transiente expressie studies, ten einde de meest optimale electroporatie-condities te kunnen definiëren. De voor dit doel gebruikte β-glucuronidase (GUS) marker, kwam het best tot expressie tussen 6 en 9 dagen na electroporatie, hetgeen langer is dan de 2 dagen benodigd voor expressie in andere koffieweefsels. De hoogste GUS activiteit (12 nM 4-MU.mg^{-1} proteïne.min^{-1}) werd gemeten wanneer protoplasten werden geëlectroporeerd met een 250 μF condensator-ontlading bij een electrische veldsterkte van 250-350 $V.cm^{-1}$. De kwaliteit van de behandelde protoplasten verminderde aanzienlijk onder deze omstandigheden (tot 30% vitaal). De ruime beschikbaarheid van protoplasten van niet-embryogene oorsprong is desalnietemin van belang voor het testen van de mate van promoter-expressie na introductie in koffie.

Als gevolg van het lange tijdsbestek nodig voor het werken met koffieprotoplasten en het uitblijven van succes met regeneratie, werd het onderzoek naar deze transformatiemethode gestaakt. Dankzij bemoedigende resultaten die behaald werden tijdens vóóronderzoek met de "particle gun" op koffieweefsel, werd deze methode het onderzoeksobject.

Transiente expressie studies d.m.v. "particle gun bombardment"

Na de technische proefomstandigheden van het door kruit aangedreven "particle gun" apparaat geoptimaliseerd te hebben, werd eerst de bruikbaarheid van verschillende koffieplantweefsels voor transiente expressie onderzocht. In vergelijking met blaadjes van kasmateriaal en met callussuspensies, kon GUS expressie eenvoudiger aangetoond worden op beschoten blaadjes van *in vitro* gekweekte scheuten. Het gebruik van somatische embryos voor dit doel werd belemmerd door het optreden van hinderlijke endogene GUS expressie in dit weefsel. Verschillen tussen genotypen m.b.t. gevoeligheid voor "particle gun" behandeling kwamen naar voren; enkele *C. canephora* genotypen vertoonden intense necrose en als gevolg daarvan een lage GUS expressie. Wanneer het GUS gen onder controle van de EF1α-A1 promoter van *Arabidopsis thaliana* werd geplaatst, werd GUS activiteit met een factor 2-3 verhoogd ten opzichte van die met drie andere promoters (CaMV-E35S, UBQ1, LTR). Het hoogste niveau van transiente expressie (1300 GUS "expressiespots" per schot) werd verkregen wanneer blaadjes van *C. arabica* scheutculturen en de EF1α-A1 promoter werden gebruikt. Op dit moment worden scheutjes, geregenereerd na beschieting van blaadjes en van somatische embryos, door middel van PCR getest op hun al dan niet transgene aard.

Histologisch onderzoek van beschoten bladeren

Teneinde de mogelijkheid te evalueren van een stabiele expressie na integratie van

geïntroduceerde genen in koffiebladeren afkomstig uit de kas werd callusontwikkeling en GUS expressie in deze bladeren gedurende 2 maanden na beschieting gevolgd met behulp van histologische analyses. GUS activiteit kon niet aangetoond worden in het door wolfraam partikels-geïnduceerde callus, maar slechts in niet-callusvormende bladdelen. Een maand na beschieting bleek dat cellen het binnendringen van samengeklonterde wolfraam brokstukken nooit overleefden. Er kan gesteld worden dat de kans op stabiele gen-integratie verhoogd wordt, wanneer a) plasmide-dragend wolfraam in niet-samengeklonterde vorm in callus-induceerbare cellen penetreert (o.a. perivasculaire cellen) die hierna in staat zijn zich te herstellen, en b) blijvende verwonding van hun buurcellen optreedt, wat nodig is voor de aanzet tot callus-inductie. Als gevolg van hun gevoeligheid voor polyfenolische oxydatie, die tot necrose leidt, bleken bladeren van in de kas gekweekte planten niet geschikt te zijn voor transformatie via de "particle gun".

Onderzoek met behulp van transmissie electronenmicroscopie op beschoten bladeren toonde aan dat GUS producten (diX-indigo kristallen) in celkernen gevonden konden worden, maar niet in de organellen. De kristallen precipiteerden bij voorkeur op lipide membraanstructuren.

Selectieve groei

Drie herbiciden (chloorsulfuron, glufosinaat en glyfosaat) en twee antibiotica (hygromycine en kanamycine) werden voor selectieve groei van transgene cellen in medium getest. Genen die coderen voor resistentie tegen deze middelen zijn reeds geisoleerd en kunnen gebruikt worden als selectie markers in transformatie-experimenten. Glufosinaat liet de meest efficiënte remming van koffiecallusgroei zien. Op bladexplantaten was 6 $mg.l^{-1}$ glufosinaat voldoende om de vorming van primair "nodulair" callus te remmen, terwijl slechts 1 $mg.l^{-1}$ een volledige remming van zogenaamde "directe" somatische embryogenese veroorzaakte. Groeiremming van embryogeen callus in vloeibaar medium werd verkregen door toevoeging van 3 $mg.l^{-1}$ glufosinaat. Benevens de efficiënte remming van callusgroei was er weinig sprake van necrose, hetgeen als bevorderlijk beschouwd moet worden voor de mogelijke uitgroei van resistent weefsel. Het gebruik van plasmiden die het *bar*-gen bevatten (dat glufosinaat resistentie induceert) voor genetische transformatie onder selectieve groeicondities kan bijdragen in het selecteren van stabiel getransformeerd koffieweefsel. Het is daarbij aan te bevelen dit *bar*-gen van de EF1α-A1 promoter te voorzien.

Conclusie

Het vermijden van polyfenolische oxydatie lijkt het sleutelwoord te zijn voor de ontwikkeling van een genetisch transformatiesysteem voor koffie. Weefsel dat genetisch getransformeerd wordt, moet ofwel weinig gevoelig zijn voor dit fenomeen, of, ter voorkoming ervan, met voorzorg behandeld worden. Ook het toegepaste selectieve groeisysteem moet aan deze criteria voldoen, wil uitgroei van transgeen weefsel een optimale kans hebben. Om deze redenen lijkt het zéér embryogene HFSE-callus in vloeibaar medium het meest geschikte uitgangsmateriaal te zijn. Naast het feit dat dit weefsel minder gevoelig is voor necrose tijdens continue kweek in vloeibaar medium, zijn de zich hieruit ontwikkelende somatische embryos van unicellulaire oorsprong. Het gebruik van glufosinaat in een selectief groeisysteem kan dan het verkrijgen van volledig getransformeerde planten bevorderen. Tenslotte, genoverdracht bij koffie kan via

"particle gun" succesvol toegepast worden als een geoptimaliseerd apparaat gebruikt wordt. Maar ook electroporatie van gedeeltelijk verteerd callus en *A. rhizogenes*-geïnduceerde transformatie mogen niet buiten beschouwing gelaten worden.

Dit onderzoek aan de genetische transformatie van koffie omvatte fundamentele en toegepaste aspecten. De in parallel geanalyseerde fysische, fysiologische en histologische parameters hebben bij twee genetische transformatiemethoden de knelpunten en hun oplossingen blootgelegd. De verkregen resultaten dragen bij tot het verbeteren van de kennis van transformatie-mechanismen, een belang dat verder reikt dan alleen transformatie van koffie.

References

Acuna R, de Pena M. 1987. Isolation and culture of coffee protoplasts from cell suspensions of *Coffea arabica* L. cv. Caturra. In: Zerda A, ed. *Abstracts of the International Congress of Plant Tissue Culture, Tropical Species*. Bogota: Universidad Nacional de Colombia, 34-35.

Acuna R, de Pena M. 1991. Plant regeneration from protoplasts of embryogenic cell suspensions of *Coffea arabica* L. cv. Caturra. *Plant Cell Reports* **10**, 345-348.

Alvard D, Cote F, Teisson C. 1993. Comparison of methods of liquid medium culture for banana propagation. Effects of temporary immersion of explants. *Plant Cell Tissue & Organ Culture* **32**, 55-60.

Aponte Acuna M-E. 1993. Somatic embryogenesis induced by culture on single media in coffee plants from crosses of *Coffea arabica* by Timor Hybrid. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 82-88.

Ascanio C, Arcia Asdrubal M. 1994. Efecto del estado de desarrollo de las anteras y de un shock térmico sobre la androgénesis en *Coffea arabica* L. var. Garnica. *Café Cacao Thé* **38**, 75-80.

Axelos M, Bardet C, Liboz T, Le Van Thai A, Curie C, Lescure B. 1989. The gene family encoding the *Arabidopsis thaliana* translation elongation factor EF-1α: molecular cloning, characterization and expression. *Molecular & General Genetics* **219**, 106-112.

Barré M. 1990. Mise au point d'un protocole de transformation génétique chez le caféier (*Coffea arabica* L.) grace à la bactérie vectrice *Agrobacterium tumefaciens*. Mémoire DEA, Institut National Agronomique, Paris-Grignon, France, pp 33.

Barton C, Adams T, Zarowitz M. 1991. Stable transformation of foreign DNA into *Coffea arabica* plantlets. In: *14ème Colloque Scientifique International sur le Café* (San Francisco). Paris: ASIC, 460-464.

Bates G, Carle S, Piastuch W. 1990. Lineair DNA introduced into carrot protoplasts by electroporation undergoes ligation and recicularization. *Plant Molecular Biology* **14**, 899-908.

Battraw M, Hall T. 1990. Histochemical analysis of CaMV 35S promoter-β-glucuronidase gene expression in transgenic rice plants. *Plant Molecular Biology* **15**, 527-538.

Beeckman T, Engler G. 1994. An easy technique for the clearing of histochemically stained plant tissue. *Plant Molecular Biology Reporter* **12**, 37-42.

Bellini C, Guerche P, Spielmann A, Goujaud J, Lesaint C, Caboche M. 1989. Genetic analysis of transgenic tobacco plants obtained by liposome-mediated transformation: absence of evidence for the mutagenic effect of inserted sequences in sixty characterized transformants. *Journal of Heredity* **80**, 361-367.

Berthouly M, Guzman N, Chatelet P. 1987. Micropropagation *in vitro* de différentes lignées de *Coffea arabica* v. Catimor. In: *12ème Colloque Scientifique International sur le Café* (Montreux). Paris: ASIC, 462-467.

Berthouly M, Alvard D, Carasco C, Teisson C. 1994. *In vitro* micropropagation of coffee sp. by temporary immersion. In: *Abstracts 8th International Congress of Plant Tissue & Cell Culture* (Firenze). IAPTC, 162.

Berthouly M, Michaux-Ferrière N. High frequency somatic embryogenesis in *Coffea canephora* (var. Robusta): induction conditions and histological evolution. Submitted.

Bevan M, Flavell R, Chilton M-D. 1983. A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* **304**, 184-187.

Bieysse D, Gofflot A, Michaux-Ferrière N. 1993. Effect of experimental conditions and genotypic variability on somatic embryogenesis in *Coffea arabica*. *Canadian Journal of Botany* **71**, 1496-502.

Blaydes D. 1966. Interaction of kinetin and various inhibitors in the growth of soybean tissue. *Physiologia Plantarum* **19**, 748-753.

Bonga J. 1982. Vegetative propagation in relation to juvenility, maturity and rejuvenation. In: Bonga J, Durzan D, eds. *Tissue Culture in Forestry*. Dordrecht: Martinus Nijhoff Publishers, 387-412.

Bowen B. 1993. Markers for plant gene transfer. In: Kung S-D, Wu R, eds. *Transgenic plants 1. Engineering and utilization*. San Diego: Academic Press, 89-123.

Brasileiro A-C, Tourneur C, Leplé J-C, Combes V, Jouanin L. 1992. Expression of the mutant *Arabidopsis thaliana* acetolactate synthase gene confers chlorsulfuron resistance to transgenic poplar plants. *Transgenic Research* **1**, 133-141.

Butt A. 1985. A general method for the high-yield isolation of mesophyll protoplasts from deciduous tree species. *Plant Science* **42**, 55-59.

Caissard J. 1993. Repérage ultrastructural de la β-glucuronidase sur des plantes transgéniques comportant le gène GUS: méthodologies et études de cas. Thèse de doctorat, Université Paris VI, France, pp .

Callis J, Raasch J, Vierstra R. 1990. Ubiquitin extension proteins of *Arabidopsis thaliana*. Structure, localization, and expression of their promoters in transgenic tobacco. *The Journal of Biological Chemistry* **265**, 12486-12493.

Capot J. 1972. L'amélioration du caféier en Côte d'Ivoire. Les hybrides 'Arabusta'. *Café Cacao Thé* **16**, 3-17.

Carasco C, Dufour M, Berthouly M, Michaux-Ferrière N. 1994. Ontogenèse comparative de l'embryogenèse zygotique et somatique chez *Coffea* sp.. *Café Cacao Thé* **38**, 11-18.

Carneiro M. 1993. Induction of double haploids on *Coffea arabica* cultivars via anther or isolated microspore culture. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 133.

Carvalho A. 1985. Principles and practice of coffee plant breeding for productivity and quality factors: *Coffea arabica*. In: Clarke R, Macrae R, eds. *Coffee 4. Agronomy*. London: Elsevier, 129-65.

Chang D, Chassy B, Saunders J, Sowers A. 1992. *Guide to electroporation and electrofusion*. San Diego, Academic Press, pp. 581.

Charrier A, Berthaud J. 1985. Principles and methods in coffee plant breeding: *Coffea canephora* PIERRE. In: Clarke R, Macrae R, eds. *Coffee 4. Agronomy*. London: Elsevier, 167-198.

Chee P, Cantliffe D. 1989. Composition of embryogenic suspension cultures of *Ipomoea batatas* Poir. and production of individualized embryos. *Plant Cell Tissue & Organ Culture* **17**, 39-52.

Christou P, McCabe D, Swain W. 1988. Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiology* **87**, 671-674.

Christou P, Swain W, Yang N-S, McCabe D. 1989. Inheritance and expression of foreign genes in transgenic soybean plants. *Proceedings of the National Academy of Sciences USA* **86**, 7500-7504.

Christou P, Ford T, Kofron T. 1991. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Bio/Technology* **9**, 957-962.

Colonna J-P. 1972. Contribution à l'étude *in vitro* d'embryons de caféiers. Action de la caféine. *Café Cacao Thé* **16**, 193-203.

Constabel F. 1975. Isolation and culture of plant protoplasts. In: Gamborg O, Wetter L, eds. *Plant tissue culture methods*. Ottawa: National Research Council of Canada, 11-21.

Coste R. 1989. *Techniques agricoles et productions tropicales 40: Caféiers et Cafés*. Paris: Maisonneuve et Larose et ACCT, pp 373.

Craig S. 1992. The GUS reporter gene - Application to light and transmission electron microscopy. In: Gallagher S, ed. *GUS protocols. Using the GUS gene as a reporter of gene expression.* San Diego: Academic Press, 115-124.

Crocomo O, Carvalho F, Sharp W, Bandel G, Carvalho P. 1979. Hormonal control of cellular phenotype and induction of globular embryos in tissue cultures of *Coffea arabica* cv. Catuai. *Energia Nuclear e Agricultura Piracicaba* **1**, 41-47.

Crossway A, Oakes J, Irvine J, Ward B, Knauf V, Shewmaker C. 1986. Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. *Molecular & General Genetics* **202**, 179-185.

Curie C, Axelos M, Bardet C, Atanassova R, Chaubet N, Lescure B. 1993. Modular organization and developmental activity of an *Arabidopsis thaliana* EF-1α gene promoter. *Molecular & General Genetics* **238**, 428-436.

Custers J, Vanee G, Buijs L. 1980. Clonal propagation of *Coffea arabica* L. by nodal culture. In: *9ème Colloque Scientifique International sur le Café* (London). Paris: ASIC, 589-596.

Daniell H, Vivekananda J, Nielsen B, Ye G, Tewari K, Sanford J. 1990. Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proceedings of the National Academy of Sciences USA* **87**, 88-92.

De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gosselé V, Rao Movva N, Thompson C, van Montagu M, Leemans J. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *The EMBO Journal* **6**, 2513-2518.

De Block M, Debrouwer D. 1992. *In situ* enzyme histochemistry on plastic-embedded plant material. The development of an artefact-free β-glucuronidase assay. *The Plant Journal* **2**, 261-266.

de Garcia E, Menendez A. 1987. Embriogénesis somatica a partir de explantes foliares del cafeto 'Catimor'. *Café Cacao Thé* **31**, 15-22.

Dekeyser R, Claes B, de Rycke R, Habets M, van Montagu M, Caplan A. 1990. Transient gene expression in intact and organized rice tissues. *The Plant Cell* **2**, 591-602.

de la Pena A, Lorz H, Schell J. 1987. Transgenic rye plants obtained by injecting DNA into young floral tillers. *Nature* **325**, 274-276.

de Pena M. 1983. Somatic embryo induction from *Coffea canephora* and *Coffea arabica*. In: *Simposio sobre Ferrugens do Cafeeiro* (Oeiras). Oeiras: CIFC, 493-512.

Deshayes A, Herrera-Estrella L, Caboche M. 1985. Liposome-mediated transformation of tobacco mesophyll protoplasts by an *Escherichia coli* plasmid. *The EMBO Journal* **4**, 2731-2737.

de Touchet B. 1991. Micropropagation du palmier à huile (*Elaeis guineensis* Jacq.) en milieu liquide. Thèse de doctorat, Université de Paris-Sud/Orsay, pp 153.

D'Halluin K, Bonne E, Bassut M, De Beuckeleer M, Leemans J. 1992. Transgenic maize plants by tissue electroporation. *The Plant Cell* **4**, 1495-1505.

Duan X, Chen S. 1985. Variation of the characters in rice (*Oryza sativa*) induced by foreign DNA uptake. *China Agricultural Science* **3**, 6-9.

Dublin P, Parvais J-P. 1975. Note sur les premiers haploïdes spontanés découverts chez le *Coffea canephora* var. robusta. *Café Cacao Thé* **19**, 191-196.

Dublin P. 1980a. Induction de bourgeons néoformés et embryogenèse somatique. Deux voies de multiplication végétative *in vitro* des caféiers cultivés. *Café Cacao Thé* **24**, 121-130.

Dublin P. 1980b. Multiplication végétative *in vitro* de l'Arabusta. *Café Cacao Thé* **24**, 281-291.

Dublin P. 1981. Embryogenèse somatique directe sur fragments de feuilles de caféier Arabusta. *Café Cacao Thé* **25**, 237-42.

Dublin P. 1984. Techniques de réproduction végétative *in vitro* et amélioration génétique chez les caféiers cultivés. *Café Cacao Thé* **28**, 231-244.

Ducos J-P, Zamarripa A, Eskes A, Pétiard V. 1993. Production of somatic embryos of coffee in a bioreactor. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 89-96.

Dudits D, Maroy E, Praznovszky T, Olah Z, Gyorgyey J, Cella R. 1987. Transfer of resistance traits from carrot into tobacco by assymetric somatic hybridization: Regeneration of fertile plants. *Proceedings of the National Academy of Sciences USA* **84**, 8434-8438.

d'Utra Vaz F, dos Santos A, Manders G, Cocking E, Davey M, Power J. 1993. Plant regeneration from leaf mesophyll protoplasts of the tropical woody plant, passionfruit (*Passiflora edulis* fv flavicarpa Degener.): the importance of the antibiotic cefotaxime in the culture medium. *Plant Cell Reports* **12**, 220-225.

Eskes A. 1989. Resistance. In: Kushalappa A, Eskes A, ed. *Coffee rust: epidemiology, resistance and management.* Boca Raton: CRC Press, 171-292.

Feder N, O'Brien T. 1968. Plant microtechnique: some principles and new methods. *American Journal of Botany* **55**, 123-142.

Finer J, McMullen M. 1990. Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. *Plant Cell Reports* **8**, 586-589.

Finer J, Vain P, Jones M, McMullen M. 1992. Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Reports* **11**, 323-328.

Fisher D, Jensen W, Ashton M. 1968. Histochemical studies of pollen: storage pockets in the enoplasmic reticulum (ER). *Histochemie* **13**, 169-182.

Fraley R, Rogers S, Horsch R, Sanders P, Flick J, Adams S, Bittner M, Brand L, Fink C, Fry C, Galluppi G, Goldberg S, Hoffmann N, Woo S. 1983. Expression of bacterial genes in plant cells. *Proceedings of the National Academy of Sciences USA* **80**, 4803-4807.

Franche C, Bogusz D, Schöpke C, Fauquet C, Beachy R. 1991. Transient gene expression in cassava using high-velocity microprojectiles. *Plant Molecular Biology* **17**, 493-498.

Frearson E, Power J, Cocking E. 1973. The isolation, culture and regeneration of *Petunia* leaf protoplasts. *Developmental Biology* **33**, 130-137.

Fromm M, Taylor L, Walbot V. 1985. Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proceedings of the National Academy of Sciences USA* **82**, 5824-5828.

Gamborg O, Miller R, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**, 151-158.

Gaudin V. 1992. Etude de la régulation de l'expression des gènes de la voie de la biosynthèse de l'auxine d'*Agrobacterium tumefaciens*. Thèse de doctorat, Université Paris-Sud/Orsay, France, pp 123.

Gavish H, Vardi A, Fluhr R. 1991. Extracellular proteins and early embryo development in *Citrus* nucellar cell cultures. *Physiologia Plantarum* **82**, 606-616.

Gordon-Kamm W, Spencer T, Mangano M, Adams T, Daines R, Start W, O'Brien J, Chambers S, Adams W, Willets N, Rice T, Mackey C, Krueger R, Kausch A, Lemaux P. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. *The Plant Cell* **2**, 603-618.

Gray D, Mortensen J. 1987. Initiation and maintenance of long term somatic embryogenesis from anthers and ovaries of *Vitis longii* "Microsperma". *Plant Cell Tissue & Organ Culture* **9**, 73-80.

Grèzes J, Thomas D, Thomasset B. 1994. Factors influencing protoplast isolation from *Coffea arabica* cells. *Plant Cell Tissue & Organ Culture* **36**, 91-97.

Griesbach R. 1983. Protoplast microinjection. *Plant Molecular Biology Reporter* **1**, 32-37.

Guerche P, Bellini C, Le Moullec J-M, Caboche M. 1987. Use of transient expression assay for the optimization of direct gene transfer into tobacco mesophyll protoplasts by electroporation. *Biochimie* **69**, 621-628.

Guerche P. 1988. Transformation génétique du colza (*Brassica napus*). Thèse de doctorat, Université Paris-Sud/Orsay, France, pp 89.

Guerreiro Filho O, Denolf P, Frutos R, Peferoen M, Eskes A. 1993. L'identification des toxines de *Bacillus thuringiensis* active contre *Perileucoptera coffeella*. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 329-337.

Halperin W. 1966. Alternative morphogenetic events in cell supensions. *American Journal of Botany* **53**, 443-53.

Hatanaka T, Arakawa O, Yasuda T, Uchida N, Yamaguchi T. 1991. Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. *Plant Cell Reports* **10**, 179-182.

Herman E, Haas G. 1975. Clonal propagation of *Coffea arabica* L. from callus culture. *HortScience* **10**, 588-589.

Herrera-Estrella L, Depicker A, van Montagu M, Schell J. 1983. Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature* **303**, 209-213.

Hidano Y, Niizeki M. 1988. Protoplast culture of deciduous fruit trees. *Scientia Horticulturae* **37**, 201-216.

Hobbs S, Jackson J, Baliski D, DeLong C, Mahon J. 1990. Genotype- and promoter-induced variability in transient β-glucuronidase expression in pea protoplasts. *Plant Cell Reports* **9**, 17-20.

Horn M, Shillito R, Conger B, Harms C. 1988. Transgenic plants of orchard grass (*Dactylis glomerata* L.) from protoplasts. *Plant Cell Reports* **7**, 469-472.

Horsch R, Fry J, Hoffmann N, Eichholtz D, Rogers S, Fraley R. 1985. A simple and general method for transferring genes into plants. *Science* **227**, 1229-1231.

Hu C, Chee P, Chesney R, Zhou J, Miller P, O'Brien W. 1990. Intrinsic GUS-like activities in seed plants. *Plant Cell Reports* **9**, 1-5.

Huttly A, Baulcombe D. 1989. A wheat α-Amy2 promoter is regulated by gibberellin in transformed oat aleurone protoplasts. *The EMBO Journal* **8**, 1907-1913.

Iida A, Yamashita T, Yamada Y, Morikawa H. 1991. Efficiency of particle-bombardment-mediated transformation is influenced by cell cycle stage in synchronized cultured cells of tobacco. *Plant Physiology* **97**, 1585-1587.

Ingelbrecht I, Herman L, Dekeyser R, van Montagu M, Depicker A. 1989. Different 3' end regions strongly influence the level of gene expression in plant cells. *The Plant Cell* **1**, 671-680.

Jefferson R. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter* **5**, 387-405.

Jefferson R, Kavanagh T, Bevan M. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* **6**, 3901-3907.

Joersbo M, Brunstedt J. 1990. Direct gene transfer to plant protoplasts by mild sonication. *Plant Cell Reports* **9**, 207-210.

Kaeppler H, Weining G, Somers D, Rines H, Cockborn A. 1990. Silicon carbide fiber-mediated DNA delivery into plant cells. *Plant Cell Reports* **9**, 415-418.

Kartha K, Mroginsky L, Pahl K, Leung N. 1981. Germplasm preservation of coffee (*Coffea arabica* L.) by *in vitro* culture of shoot apical meristems. *Plant Science Letter* **22**, 301-307.

Kay R, Chan A, Daly M, McPherson J. 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* **236**, 1299-1302.

Keller H, Wanner H, Baumann T. 1972. Kaffeinsynthese und gewebekulturen von *Coffea arabica*. *Planta* **108**, 339-350.

Kisaka H, Kameya T. 1994. Production of somatic hybrids between *Daucus carota* L. and *Nicotiana tabacum*. *Theoretical & Applied Genetics* **88**, 75-80.

Klein T, Wolf E, Wu R, Sanford J. 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* **327**, 70-73.

Kung S-D. 1993. From green revolution to gene revolution. In: Kung S-D, Wu R, eds. *Transgenic plants 2, Present status and social and economic impacts*. San Diego: Academic Press, xvii-xxxi.

Kushalappa A. 1989. Introduction. In: Kushalappa A, Eskes A, eds. *Coffee rust: epidemiology, resistance and management*. Boca Raton: CRC Press, 1-12.

Lanaud C. 1981. Production de plantules de *C. canephora* par embryogenèse somatique réalisée à partir de culture *in vitro* d'ovules. *Café Cacao Thé* **25**, 231-235.

Langridge W, Li B, Szalay A. 1985. Electric field-mediated stable transformation of carrot protoplasts with naked DNA. *Plant Cell Reports* **4**, 355-359.

Lappartient A. 1993. Utilisation d'un canon à particules pour la transformation transitoire et stable d'un arbre actinorhizien: *Casuarinaglauca*. Memoire Diplome d'Ingénieur Agronome, Institut National Agronomique, Paris-Grignon, pp 26.

Larkin P, Scowcroft W. 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theoretical & Applied Genetics* **60**, 197-214.

Lashermes P, Charrier A, Couturon E. 1993. On the use of doubled haploids in genetics and breeding of *Coffea canephora* P. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 218-225.

Lee S. 1990. Successful gene-splicing for coffee. *Tea & Coffee Trade Journal* **162**, 3-4.

Leemans J. 1993. Genetic engineering for fertility control. In: Dattée Y, Dumas C, Gallais A, eds. *Reproductive biology and plant breeding*, **13**th Eucarpia Congress (Angers). Berlin: Springer-Verlag, 101-106.

Leroy T. 1993. Diversité, paramètres génétiques et amélioration par sélection récurrente réciproque du caféier *Coffea canephora* P. Thèse de doctorat, Ecole Nationale Supérieure Agronomique, Rennes, France, pp 147.

Leroy T, Montagnon C, Cilas C. 1993. Estimation de paramètres génétiques chez *Coffea canephora* PIERRE. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 191-198.

Lindsey K, Jones M. 1987. Transient gene expression in electroporated protoplasts and intact cells of sugar beet. *Plant Molecular Biology* **10**, 43-52.

Luo Z, Wu R. 1988. A simple method for the transformation of rice via the pollen-tube pathway. *Plant Molecular Biology Reporter* **6**, 165-174.

Manders G, d'Utra Vaz F, dos Santos A, Davey M, Power J. 1991. Plant regeneration from mesophyll protoplasts of passionfruit (*Passiflora edulis* var flavicarpa). *Physiologia Plantarum* **82**, A16.

Mariani C, de Beuckeleer M, Truettner J, Leemans J, Goldberg R. 1990. Induction of male sterility in plants by a chimaeric ribonuclease gene. *Nature* **347**, 737-741.

Mariani C, Gossele V, De Beuckeleer M, De Block M, Goldberg R, De Greef W, Leemans J. 1992. A chimaeric ribonuclease-inhibitor gene restores fertility to male sterile plants. *Nature* **357**, 384-387.

Marques D. 1993. Induction of somatic embryogenesis on *Coffea eugenioïdes* MOORE by *in vitro* culture of leaf explants. *Café Cacao Thé* **37**, 251-255.

Mascarenhas J, Hamilton D. 1992. Artifacts in the localization of GUS activity in anthers of petunia transformed with a CaMV 35S-GUS construct. *The Plant Journal* **2**, 405-408.

Mazzafera P, Eskes A, Parvais J-P, Carvalho A. 1989. Stérilité mâle détectée chez *Coffea arabica* et *C. canephora* au Brésil. In: *13ème Colloque Scientifique International sur le Café* (Paipa). Paris: ASIC, 466-473.

McCabe D, Swain W, Martinell B, Christou P. 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology* **6**, 923-926.

McCabe D, Christou P. 1993. Direct DNA transfer using electric discharge particle acceleration (ACCELL™ technology). *Plant Cell Tissue & Organ Culture* **33**, 227-236.

Menczel L, Nagy F, Kiss Z, Maliga P. 1981. Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. *Theoretical & Applied Genetics* **59**, 191-195.

Michaux-Ferrière N, Dublin P, Schwendiman J. 1987. Etude histologique de l'embryogenèse somatique à partir d'explants foliaires de *Coffea arabica* L. *Café Cacao Thé* **31**, 103-114.

Michaux-Ferrière N, Bieysse 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 differents. *Café Cacao Thé* **33**, 207-217.

Michaux-Ferrière N, Schwendiman J. 1992. Histology of somatic embryogenesis. In: Dattée Y, Dumas C, Gallais A, eds. *Reproductive biology and plant breeding*, 13th Eucarpia Congress (Angers). Berlin: Springer-Verlag, 247-259.

Mitchell H. 1985. Cultivation and harvesting of the Arabica coffee tree. In: Clarke R, Macrae R, eds. *Coffee 4. Agronomy*. London: Elsevier, 43-90.

Monaco L, Söndahl M, Carvalho A, Crocomo O, Sharp W. 1977. Applications of tissue culture in the improvement of coffee. In: Reinert J, Bajaj Y, eds. *Applied and fundamental aspects of plant cell, tissue, and organ culture.* Berlin: Springer-Verlag, 109-129.

Montoro P, Etienne H, Carron MP. 1994. Maintainable somatic embryogenesis in *Hevea brasiliensis*. *Revue de Cytologie & Biologie Végetale, le Botaniste* **17**, in press.

Morikawa H, Iida A, Yamada Y. 1989. Transient expression of foreign genes in plant cells and tissues obtained by a simple biolistic device (particle-gun). *Applied Microbiology & Biotechnology* **31**, 320-322.

Mueller W, Greenwood A. 1978. The ultrastructure of phenolic storing cells fixed with caffeine. *Journal of Experimental Botany* **29**, 757-764.

Murakami T, Hosokawa D, Ohashi Y. 1992. The possible final localization of GUS (β-glucuronidase) reporter gene product in transgenic plants is vacuole. *Japanese Journal of Crop Science* **61**, 503-510.

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

Murhalidharan E, Gupta P, Mascarenhas A. 1989. Plantlet production through high frequency somatic embryogenesis in long term cultures of *Eucalyptus cytriodora*. *Plant Cell Reports* **8**, 41-43.

Nadel B, Altman A, Ziv M. 1989. Regulation of somatic embryogenesis in celery cell suspensions. 1. Promoting effects of mannitol on somatic embryo development. *Plant Cell Tissue & Organ Culture* **18**, 181-189.

Nap J-P, Bijvoet J, Stiekema W. 1992. Biosafety of kanamycin-resistant transgenic plants. *Transgenic Research* **1**, 239-249.

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

Neuenschwander B, Dufour M, Baumann T. 1993. Haploid cell colony formation from mechanically isolated microspores of *Coffea arabica*. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 760-761.

Neuhaus G, Spangenberg G, Mittelsten-Scheid O, Schweiger H-G. 1987. Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryoids. *Theoretical & Applied Genetics* **7**, 30-36.

Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider P. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *The EMBO Journal* **1**, 841-845.

Noël M. 1992. Régénération de la courgette (*Cucurbita pepo*) et transformation génétique de deux *Cucurbitaceae*: la courgette et le concombre (*Cucumis sativus*). Thèse de doctorat, Université de Clermont Ferrand II, France, pp. 121.

Noriega C, Söndahl M. 1993. Arabica coffee micropropagation through somatic embryogenesis via bioreactors. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 73-81.

Oard J, Paige D, Simmond J, Gradziel T. 1989. Transient gene expression in maize, rice, and wheat cells using an airgun apparatus. *Plant Physiology* **92**, 334-339.

Ocampo C, Manzanera L. 1991. Advances in the genetic manipulation of the coffee plant. In: *14ème Colloque Scientifique International sur le Café* (San Francisco). Paris: ASIC, 378-382.

Ochatt S, Chand P, Rech E, Davey M, Power J. 1988. Electroporation-mediated improvement of plant regeneration from colt cherry (*Prunus avium* x *Pseudocerasus*) protoplasts. *Plant Science* **54**, 165-169.

Ohta Y. 1986. High efficiency genetic transformation of maize by a mixture of pollen and exogenous DNA. *Proceedings of the National Academy of Sciences USA* **83**, 715-719.

Orozco F, Schieder O. 1984. Isolation of mesophyll protoplasts of the genus *Coffea*. *Turrialba* **34**, 534-536

Ow D, Wood K, DeLuca M, De Wet J, Helinski D, Howell H. 1986. Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* **234**, 856-859.

Paszkowski J, Shillito R, Saul M, Mandak V, Hohn T, Hohn B, Potrykus I. 1984. Direct gene transfer to plants. *The EMBO Journal* **3**, 2717-2722.

Perl A, Kless H, Blumenthal A, Galili G, Galun E. 1992. Improvement of plant regeneration and GUS expression in scutellar wheat calli by optimization of culture conditions and DNA-microprojectile delivery procedures. *Molecular & General Genetics* **235**, 279-284.

Pierson E, van Lammeren A, Schel J, Staritsky G. 1983. *In vitro* development of embryoids from punched leaf discs of *Coffea canephora*. *Protoplasma* **115**, 208-216.

Potrykus I. 1990. Gene transfer to cereals: an assessment. *Bio/Technology* **6**, 535-543.

Pröls M, Töpfer R, Schell J, Steinbiß H-H. 1988. Transient gene expression in tobacco protoplasts: I. Time course of CAT appearance. *Plant Cell Reports* **7**, 221-224.

Pouteau S, Huttner E, Grandbastien M, Caboche M. 1991. Specific expression of the tobacco Tnt1 retrotransposon in protoplasts. *The EMBO Journal* **10**, 1911-1918.

Ramos L, Yokoo E, Gonçalves W. 1993. Direct somatic embryogenesis is genotype specific in coffee. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 763-766.

Ramulu K, Dijkhuis P, Blaas J, Verbeek W, Verhoeven H, Colijn-Hooymans C. (1994). Microprotoplast fusion technique: a new tool for gene transfer between sexually incongruent species. In: Eucarpia conference (eds.). *Genetic manipulation in plant breeding*. Cork, Ireland, in press.

Rech E, Ochatt S, Chand P, Davey M, Power J. 1987. Electro-enhancement of division of plant protoplast-derived cells. *Protoplasma* **141**, 169-176.

Roest S, Gilissen L. 1993. Regeneration from protoplasts: a supplementary literature review. *Acta Botanica Neerlandica* **42**, 1-23.

Russel J, McCown B. 1986. Culture and regeneration of *Populus* leaf protoplasts isolated from non-seedling tissue. *Plant Science* **46**, 133-142.

Russel J, Roy M, Sanford J. 1992a. Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. *Plant Physiology* **98**, 1050-1056.

Russel J, Roy M, Sanford J. 1992b. Major improvements in biolistic transformation of suspension-cultured tobacco cells. *In Vitro Cell Developmental Biology* **28P**, 97-105.

Russel Kikkert J. 1993. The biolistic ®PDS-1000/He device. *Plant Cell Tissue & Organ Culture* **33**, 221-226.

Sambrook J, Fritsch E, Maniatis T. 1989. *Molecular cloning, Laboratory manuel,* 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1.21-1.39.

Sanford J, Klein T, Wolf E, Allen N. 1987. Delivery of substances into cells and tissues using a particle bombardment process. *Particulate Science and Technology* **5**, 27-37.

Sangwan R, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel B. 1992 Characterization of competent cells and early events of *Agrobacterium*-mediated transformation in *Arabidopsis thaliana*. *Planta* **188**, 439-456.

Sautter C, Waldner H, Neuhaus-Url G, Galli A, Neuhaus G, Potrykus I. 1991. Micro-targeting: high efficiency gene transfer using a novel approach for the acceleration of micro-projectiles. *Bio/Technology* **9**, 1080-1085.

Schöpke C, Müller L, Kohlenbach H-W. 1987. Somatic embryogenesis and regeneration of plantlets in protoplast cultures from somatic embryos of coffee (*Coffea canephora* P. ex Fr.). *Plant Cell Tissue & Organ Culture* **8**, 243-248.

Schöpke C. 1989. *In vitro* kultur bei kaffee: versuche zur isolierung und kultivierung von protoplasten und zur regeneration von pflanzen. Doktorat dissertation, Universität Frankfurt am Main. Verlag Tanja Maraun, pp 124.

Shah D, Horsch R, Klee H, Kishore G, Winter J, Tumer N, Hironaka C, Sanders P, Gasser C, Aykent S, Siegel N, Rogers S, Fraley R. 1986. Engineering herbicide tolerance in transgenic plants. *Science* **233**, 478-481.

Shahin E. 1985. Totipotency of tomato protoplasts. *Theoretical & Applied Genetics* **69**, 235-240.

Sharp W, Caldas L, Crocomo O, Monaco L, Carvalho A. 1973. Production of *Coffea arabica* callus of three ploidy levels and subsequent morphogenesis. *Phyton* **31**, 67-74.

Shillito R, Saul M, Paszkowski J, Müller M, Potrykus I. 1985. High efficiency direct gene transfer to plants. *Bio/Technology* **3**, 1099-1103.

Shimamoto K, Terada R, Izawa T, Fujimoto H. 1989. Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* **338**, 274-276.

Smith P, Krohn R, Hermanson G, Mallia A, Gartner F, Provenzano M, Fujimoto E, Goeke N, Olson B, Klenk D. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76-85.

Smith S, Street H. 1974. The decline of embryogenic potential as callus and suspension cultures of carrot (*Daucus carota* L.) are serially subcultured. *Annals of Botany* **38**, 223-241.

Snoeck J. 1985. Cultivation and harvesting of the Robusta coffee tree. In: Clarke R, Macrae R, eds. *Coffee 4. Agronomy*. London: Elsevier, 91-128.

Söndahl M, Sharp W. 1977. High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L.. *Zeitschrift für Pflanzenphysiologie* **81**, 395-408.

Söndahl M, Spahlinger D, Sharp W. 1979. A histological study of high frequency and low frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L.. *Zeitschrift für Pflanzenphysiologie* **94**, 101-108.

Söndahl M, Chapman M, Sharp W. 1980. Protoplast liberation, cell wall reconstitution, and callus proliferation in *Coffea arabica* L. callus tissues. *Turrialba* **30**, 161-165.

Söndahl M, Monaco L, Sharp W. 1981. In vitro methods applied to coffee. In: Thorpe T, ed. *Tissue culture and its application in agriculture*. New York: Academic Press, 325-347.

Söndahl M. 1982. Tissue culture of morphological mutants of coffee. In: Fujimara A, ed. *Proceedings of the 5th International Congress on Plant Tissue & Cell Culture* (Tokyo). IAPTC, 417-418.

Söndahl M, Nakamura T, Sharp W. 1985. Propagation of coffee. In: Henke, Hughes, Constatin, Hollaender, eds. *Tissue culture in forestry and agriculture*. New York: Plenum Press, 215-232.

Söndahl M, Loh W. 1985. Coffee biotechnology. In: Clarke R, Macrae R, eds. *Coffee 4. Agronomy*. London: Elsevier, 235-262.

Söndahl M, Bragin A. 1991. Somaclonal variation as a breeding tool for coffee improvement. In: *14ème Colloque Scientifique International sur le Café* (San Francisco). Paris: ASIC, 701-710.

Söndahl M, Lauritis J. 1992. Coffee. In: Hammerschlag F, Litz R, eds. *Biotechnology of perennial fruit crops. Biotechnology in Agriculture 8*. Wallingford, Oxon: CAB International, 401-420.

Spiral J, Pétiard V. 1991. Protoplast culture and regeneration in *Coffea* species. In: *14ème Colloque Scientifique International sur le Café* (San Francisco). Paris: ASIC, 383-391.

Spiral J, Pétiard V. 1993. Développement d'une méthode de transformation appliquée à différentes espèces de caféier et régénération de plantules transgéniques. In: *15ème Colloque Scientifique International sur le Café* (Montpellier). Paris: ASIC, 115-122.

Spörlein B, Koop H-U. 1991. Lipofectin: direct gene transfer to higher plants using cationic liposomes. *Theoretical & Applied Genetics* **83**, 1-5.

--. 1992. *Standard methods for the examination of water and wastewater*, 18th ed. American Public Health Association, American Water Works Association, Water Environment Federation, 3.9-3.15.

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

Staritsky G, van Hasselt G. 1980. The synchronised mass propagation of *Coffea canephora in vitro*. In: *9ème Colloque Scientifique International sur le Café* (London). Paris: ASIC, 597-602.

Tahara M, Yasuda T, Uchida N, Yamaguchi T. 1994. Formation of somatic embryos from protoplasts of *C. arabica* L.. *HortScience* **29**, 172-174.

Takeuchi Y, Dotson M, Keen N. 1992. Plant transformation: a simple particle bombardment device based on flowing helium. *Plant Molecular Biology* **18**, 835-839.

Tautorus T, Bekkaoui F, Pilon M, Datla R, Crosby W, Fowke L, Dunstan D. 1989. Factors affecting transient gene expression in electroporated black spruce (*Picea mariana*) and jack pine (*Pinus banksiana*) protoplasts. *Theoretical & Applied Genetics* **78**, 531-536.

Töpfer R, Gronenborn B, Schell J, Steinbiss H-H. 1989. Uptake and transient expression of chimeric genes in seed-derived embryos. *The Plant Cell* **1**, 133-139.

Tör M, Mantell S, Ainsworth C. 1992. Endophytic bacteria expressing β-glucuronidase cause false positives in transformation of *Dioscorea* species. *Plant Cell Reports* **11**, 452-456.

Townsley P. 1974. Production of coffee from plant cell suspension cultures. *Canadian Institute of Food Science Technology Journal* 7, 79-81.

Ursin V, Irvine J, Hiatt W, Shewmaker C. 1991. Developmental analysis of elongation factor-1α expression in transgenic tobacco. *The Plant Cell* **3**, 583-591.

Vain P, McMullen M, Finer J. 1993. Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Reports* **12**, 84-88.

Vain P, Keen N, Murillo J, Rathus C, Nemes C, Finer J. 1993. Development of the Particle Inflow Gun. *Plant Cell Tissue & Organ Culture* **33**, 237-246.

van Boxtel J, Dufour M, Eskes A. 1991. Callus formation of isolated coffee protoplasts (*Coffea arabica* and *C. canephora*). *Physiologia Plantarum* **82**, A14.

van Boxtel J, Berthouly M, Carasco C, Eskes A. 1993. Transient expression of β-glucuronidase following biolistic delivery of foreign DNA into coffee tissues. In: *15ème Colloque Scientifique International sur le Café*. Paris: ASIC, 757-759.

van Boxtel J, Berthouly M. High frequency somatic embryogenesis from coffee leaves: factors influencing callus induction, and subsequent multiplication and regeneration in liquid medium. Submitted.

van Boxtel J, Berthouly M, Carasco C, Dufour M, Eskes A. Transient expression of β-glucuronidase following biolistic delivery of foreign DNA into coffee tissues. Submitted.

Vancanneyt G, Schmidt R, O'Conner-Sanchez A, Willmitzer L, Rocha-Sosa M. 1990. Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Molecular & General Genetics* **220**, 245-250.

van der Steege G. 1991. Direct gene transfer to plant protoplasts. Basic aspects and application. Doctorate thesis, Rijksuniversiteit Groningen, the Netherlands, pp 91.

van der Steege G, Tempelaar M. 1991. Direct gene transfer - Electroporation for transient expression. In: Negrutiu I, Gharti-Chhetri G, eds. *A laboratory guide for cellular and molecular plant biology.* Basel: Birkhäuser Verlag AG, 109-115.

Vardi A, Spiegel-Roy P, Galun E. 1975. Citrus cell culture: isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. *Plant Science Letter* **4**, 231-236.

Vasil V, Vasil I. 1986. Plant regeneration from friable embryogenic callus and cell suspension cultures of *Zea mays* L.. *Journal of Plant Physiology* **127**, 399-408.

Vasil V, Castillo A, Fromm M, Vasil I. 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. *Bio/Technology* **10**, 667-674.

Widholm J. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Staining Technology* **47**, 189-194.

Yamashita T, Iida A, Morikawa H. 1991. Evidence that more than 90% of β-glucuronidase-expressing cells after particle bombardment directly receive the foreign gene in their nucleus. *Plant Physiology* **97**, 829-831.

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

Yasuda T, Tahara M, Uchida N, Yamaguchi T. 1986. Somatic embryogenesis from coffee callus and protoplasts. In: Somers D, Gengenbach B, Biesboer D, Hackett W, Green C, eds. *Abstracts of the 6th International Congress on Plant Tissue & Cell Culture* (Minneapolis). IAPTC, 137.

Ye G-N, Daniell H, Sanford J. 1990. Optimization of delivery of foreign DNA into higher-plant chloroplasts. *Plant Molecular Biology* **15**, 809-819.

Zamarripa A, Ducos J-P, Tessereau H, Bollon H, Eskes A, Pétiard V. 1991 Développement d'un procédé de multiplication en masse du caféier par embryogenèse somatique en milieu liquide. In: *14ème Colloque Scientifique International sur le Café* (San Francisco). Paris: ASIC, 392-402.

Zamarripa A. 1993. Etude et développement de l'embryogenèse somatique en milieu liquide du caféier (*Coffea canephora* P., *Coffea arabica* L. et l'hybride Arabusta). Thèse de doctorat, Ecole Nationale Supérieure Agronomique, Rennes, France, pp 191.

Zhang W, Wu R. 1988. Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. *Theoretical & Applied Genetics* **76**, 835-840.

Zhang W, McElroy D, Wu R. 1991. Analysis of rice *Act1* 5' region activity in transgenic rice plants. *The Plant Cell* **3**, 1155-1165.

Zhou G-Y, Weng J, Zeng Y, Huang J, Qian S, Liu G. 1983. Introduction of exogenous DNA into cotton embryos. *Methods of Enzymology* **101**, 433-481.

Zumbrunn G, Schneider M, Rochaix J-D. 1989. A simple particle gun for DNA-mediated cell transformation. *Technique - Journal of Methods in Cell and Molecular Biology* **1**, 204-216.

Curriculum Vitae

Johannus Henricus Joseph (Jos) van Boxtel is geboren op 8 augustus 1961 in Ravenstein (Noord-Brabant). Na het behalen in 1980 van het Atheneum-B diploma aan het Maaslandcollege te Oss, vervulde hij eerst de militaire dienstplicht, alvorens in 1982 aan zijn studie Plantenveredeling te beginnen aan de Landbouwuniversiteit Wageningen. Deze studie bestond o.a. uit een stage bij het veredelingsbedrijf FT-Pesquisa e Sementes in Ponta Grossa (Brazilië), en de afstudeervakken Plantenveredeling (dr.ir. Ies Bos) en Entomologie (ir. Frans Dieleman). Na het afronden van deze studie in 1989 begon hij in augustus van dat jaar aan een NOP-project bij de vakgroep Erfelijkheidsleer (prof.dr.ir. Maarten Koornneef en dr.ir. Herman Schoenmakers). In mei 1990 pakte hij zijn spullen en reisde af naar Montpellier, waar hij aan het hier beschreven promotie-onderzoek begon. Dit werk werd onder leiding van dr.ir. Albertus Eskes en dr. Marc Berthouly uitgevoerd in het laboratorium Biotrop, en grotendeels gefinancierd door het departement Cultures Pérennes (CP), beiden deel uitmakend van het Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD). Per 1 januari 1995 is Jos van Boxtel aangesteld als post-doc op het John Innes Centre te Norwich, waar hij gaat werken aan de genetische transformatie van "cowpea".