PRIMARY AND CYCLIC SOMATIC EMBRYOGENESIS IN CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

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PRIMARY AND CYCLIC SOMATIC EMBRYOGENESIS IN CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

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in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
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in het openbaar te verdedigen
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STELLINGEN

1. Voor het verkrijgen van getransformeerde cassave dient het aanbeveling om de in dit proefschrift beschreven regeneratie methode aanzienlijk aan te passen.
   Dit proefschrift.

2. De voor walnoot beschreven methode voor het verkrijgen van genetisch gemodificeerde planten door middel van cyclische somatische embryogenese biedt onvoldoende perspectief in cassave.
   McGranahan en medewerkers (Plant Cell Reports 8:512-516, 1990).

3. De wetenschappelijke waarde van veel artikelen over somatische embryogenese wordt verminderd door een gebrek aan duidelijke definities.
   Dit proefschrift

4. Het cyclisch vermeerderen van somatische embryo's maakt toepassing van technieken zoals transformatie minder genotype afhankelijk.
   Dit proefschrift.

5. Het feit dat nog steeds wordt beweerd dat de ééncellige oorsprong een kenmerk is van somatische embryogenese wijst op selectief lezen.
   Dit proefschrift.

6. Genetisch gemodificeerde gewassen zullen eerder door het publiek geaccepteerd worden als het ziet welke milieuproblemen hiermee kunnen worden opgelost.

7. De empirie komt voor de theorie in de celbiologie.

8. Niet geplande promoties lopen volgens plan.
   Dit proefschrift.

9. De frequentie van reclame van maandverband zou aangepast moeten worden aan het individuele behoeftepatroon.
   NRC-Handelsblad, 3 november 1993.

10. Het werken met recalcitrante planten geeft in de regel minder problemen dan het werken met recalcitrante mensen.

Stellingen behorende bij het proefschrift "Primary and cyclic somatic embryogenesis in cassava (*Manihot esculenta* Crantz)" door C.J.J.M. Raemakers, in het openbaar te verdedigen op dinsdag 30 november 1993, te Wageningen.

VOORWOORD.

Dit was een niet geplande promotie. Het onderzoek hiervoor begon in juni 1989 als een NOP-project op het laboratorium van Dr. Staritsky van de vakgroep Tropische Plantenteelt. Daar heb ik ruim twee jaar gewerkt. Daarna ben ik verhuisd naar de vakgroep Plantenveredeling.
Het onderzoek werd voor het grootste gedeelte gefinancierd uit fondsen van de vakgroepen Tropische Plantenteelt en Plantenveredeling. Voorts werd financiering voor de periode van een jaar verkregen van DGIS (Directoraat Generaal Internationale Samenwerking van het Ministerie van Buitenlandse Zaken).

Alle collega's van beide vakgroepen wil ik bedanken voor hun plezierige samenwerking.
Vier mensen wil ik in het bijzonder noemen. Dit zijn Dr. Staritsky en Professor Wessel van de vakgroep Tropische Plantenteelt en Professor Jacobsen en Dr. Visser van de vakgroep Plantenveredeling. Zonder de hulp van deze vier mensen was het NOP-project niet uitgemond in dit proefschrift. Richard Visser was de dagelijkse begeleider. Hij was er altijd, of het nu was voor werkoverleg, correctie van manuscripten of gewoon voor een praatje.

Vele mensen hebben op een of andere manier aan dit onderzoek meegeholpen. In het bijzonder wil ik de studenten Ester Abad, Janette Bessembinder, Simone Dikmoet, Sien Kwee, Moniek Lammertink, Joy Maikoe, Peter Plantinga, Vinood Premchand, Astrid van Rooy, Tineke Schavenmaker, Bizuaha Tesfaya, Anja van Veen, Hanneke Bergsma en de WEP-per Ir. Mario Amati vermelden voor hun bijdrage. Iedereen zal een gedeelte van zijn of haar onderzoek terugvinden in dit proefschrift.

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LIST OF ABBREVIATIONS

2,4-D = 2,4-dichlorophenoxyacetic acid

AB = abaxial side

ABA = abscisic acid

AD = adaxial side

AUX = auxin

BA = 6-benzylaminopurine

BM = Basal Medium

C = cycle

CIAT = Centro Internacional de Agricultura Tropical

CMM = callus proliferation from meristematic mass

CTC = callus proliferation from cotyledon of explanted embryo

CYT = cytokinin

Dicamba = 3,6-dichloro-2-methoxybenzoic acid

F = fragmentation

FAO = Food and Agricultural Organization

IAA = [3-indoly]acetic acid

IBA = 4-[3-indoly]butyric acid

IBPGR = International Board of Plant Genetic resources

IITA = International Institute of Tropical Agriculture

GA = gibberellic acid

GB = globular embryo

GE = germinating embryos

GE/IE = germinating embryos per initial explant

GE/ILL = germinating embryos per initial leaf lobe

LSD = least significant difference

M = medium type

MC = meristematic or embryogenic cells

MIE = medium volume per initial embryo

MM = meristematic mass

NAA = α-napthaleneacetic acid

NEC = non embryogenic cells

Picloram = 4-amino-3,5,6,-trichloropicolinic acid

PS = procambium strands

S = suspensor

T = tracheid

TDZ (thidiazuron) = N-phenyl-N^1(1,2,3 thiadiazolyl)ureu

TS = torpedo shaped embryo

VC = vascular tissue

zeatin = (6-[4-hydroxy-3-methylbut-2-enylamino]purine).

CHAPTER 1

GENERAL INTRODUCTION

THE CROP CASSAVA

Cassava (*Manihot esculenta* Crantz) is a woody shrub, grown for its starchy tuber roots in the lowland tropics. It belongs to the Euphorbiaceae, the same plant family as other important tropical crops as *Hevea brasiliensis* and *Ricinus communis*. The crop has originated in South America [178] and during the sixteenth to eighteenth century it spread to Africa, Asia and Oceania [69]. Nowadays it is one of the most important sources of calories in the tropics for some 500 million people [53], ranking ninth on world scale [26]. About 65 percent of the world production is used for human consumption, 19 percent for cattle feed, 6 percent for industrial starch and 10 percent are losses [26].

Cassava has many advantages. It has a high yield potential and can be grown even under poor conditions because it can tolerate environmental stresses such as drought, pests and disease attacks and recover readily [26]. The tubers can be harvested from 6 to 36 months after planting. However, the crop has also some disadvantages. The tuber roots are difficult to store after harvest, they have a low protein content and are toxic because of high levels of cyanogenic glycosides [26]. The feasibility of overcoming these problems by only classical breeding is hampered because of several problems such as a high degree of heterozygosity, low fertility, poor seed set and low rates of seed germination [17, 86, 120]. Therefore, emphasis is being placed on the application of biotechnology to overcome some of these problems. For the improvement of crops by molecular and celbiological breeding techniques efficient methods for plant regeneration from cells must become available.

MODES OF REGENERATION

Plant regeneration can be accomplished by two different pathways: organogenesis and somatic embryogenesis. Organogenesis is defined as the process in which an unipolar structure is formed, with a multicellular origin and which is connected with its vascular system to that of the parental tissue [58, 176, 207, 210]. The unipolar structure is either a shoot or a root. The formation of shoots by organogenesis has been described in more than 300 species [50, 58].

Analogous to zygotic embryogenesis, somatic embryogenesis is defined as the process in which a bipolar structure [7, 189, 207] arises through a series of stages characteristic for zygotic embryo development [74, 189, 207, 229] from one somatic cell [74, 176, 189, 207, 218] and having no vascular connection with the parental tissue [74, 190, 207]. Somatic embryogenesis has been described in at least 200 species [50, 213, 218, 231].

ORIGIN OF REGENERATION

In both organogenesis [58, 210] and embryogenesis [19, 50, 189, 229, 231] the regenerated structures either originate directly from the explant or indirectly from callus induced on the explant. In somatic embryogenesis the differences between direct and indirect regeneration are best

studied. Sharp et al. [189] and Evans et al. [50] argued that direct somaticembryogenesis proceeds from already pre-embryogenic determined cells. In indirect somatic embryogenesis cells require the redifferentiation of differentiated cells. As a consequence, callus proliferation will precede the acquisition of the embryogenically competent state. In direct somatic embryogenesis, growth regulators are regarded as activators of development while in indirect somatic embryogenesis they are seen as the primary agent of determination. Direct and indirect somatic embryogenesis should be considered as two extremes of a continuum [19, 189, 229, 231]. Cells capable of direct somatic embryogenesis are physiologically similar to those in zygotic embryos. They are frequently found in tissue associated with zygotic embryogenesis; either in tissue before the onset of embryogenesis (i.e. in the flower organ) or in the developing zygotic embryo. In the cells of these organs, the genes necessary for zygotic embryogenesis and their products are active in varying degrees [19, 189]. The ease at which induction of somatic embryogenesis occurs, can be seen as a "memory" of pathways either previously or just after [19]. Cells of tissue which are in time or space more diverged from zygotic embryo explants need a greater amount of reprogramming of previously active developmental pathways before they reach the embryogenic ground state and as a consequence callus is formed [19, 189, 218].

In plant-regeneration, a distinction can be made between the mode of regeneration (somatic embryogenesis or shoot organogenesis) and the origin of the regenerated structures (direct or indirect). The possible application of these different types of regeneration for plant transformation will be discussed in the next section.

REGENERATION AND TRANSFORMATION

The most widely used method of gene transfer into plant cells is based on the soil bacteria *Agrobacterium tumefaciens* [recently reviewed in 232]. In species not amenable for *Agrobacterium*-mediated gene transfer, ballistic techniques are used in which DNA coated on metallic particles is delivered into cells of plant tissue [recently reviewed in 94]. This is called particle bombardment. Transformed plants, confirmed by molecular analysis, have been obtained in 27 species [232]. Most transformation systems rely on indirect shoot organogenesis. The callus phase is used as a stage to sort out, by selective growth, transformed from untransformed cells followed by multiplication of the former [129]. After this stage, calli are transferred to media for plant regeneration. Indirect embryogenesis can be used in the same way as indirect organogenesis, as was shown for *Apium graveolens* [20], *Beta vulgaris* [40], *Citrus sinensis* [79], *C. reticulata* [79], *Gossypium hirsutum* [219], *Helianthus annuus* [52], *Medicago sativa* [154], *Medicago varia* [21], *Oryza sativa* [24], *Solanum melongena* [54], *S. muricalum* [9] and *Zea mays* [6].

In direct regeneration, a callus phase is absent. Therefore, it can not be used to select transformed from untransformed cells. To our knowledge, there is no report on the successful use of direct organogenesis in conjunction with transformation. However, direct embryogenesis has been used in for example *Brassica napus* [204], *Daucus carota* [183], *Glycine max* [57] and *Juglans regia* [124, 125]. In these four species primary somatic embryos form secondary embryos. Repeated subculture of somatic embryos allows continuous proliferation of somatic embryos. Somatic embryos were transformed by either particle bombardment [57] or with *Agrobacterium tumefaciens* [124, 125, 204]. The phase of embryo proliferation was used, instead of the callus phase in indirect regeneration, to select and multiply transformed cells. The chimeric nature of transformed embryos was changed after several cycles of embryo proliferation under selective conditions into solid transformed embryos and plants.

It is clear that indirect regeneration offers the best opportunities for plant transformation using both organogenesis and embryogenesis. However, in direct regeneration, culture regimes which allow the continuous proliferation of embryos or shoot organs might be successful for the recovery of transgenic plants from genetically modified cells.

REGENERATION IN CASSAVA

The first attempts to regenerate plants via organogenesis in cassava were unsuccessful [49,109, 141 149]. In 1978, Tilquin [212] obtained adventitious shoots from callus induced on stem explants and a year later Shahin and Shephard [187] reported regeneration of plants from leaf derived protoplasts. In both cases the frequency of shoot organogenesis was low and genotype specific. Other researchers were not able to repeat these results.

Somatic embryogenesis in cassava was published for the first time in 1982 [196]. In a two step procedure, embryos were induced from zygotic embryo explants [196] or from young leaves [201]. In the first step 2,4-D, was used to induce somatic embryos. The embryos passed through all the typical stages of zygotic embryogenesis and were not connected with their vascular system to the explant [199]. The embryos originated directly, without an intervening callus phase, on both leaves and zygotic embryos [199]. For further development of the embryos, the explants were transferred to a medium with a much lower 2,4-D concentration (step 2). On this medium embryos developed into structures with a distinct hypocotyl and large green cotyledons and some developed into shoots. Shoot formation was improved by separate culture of embryos isolated from the explant on step 3 medium.

In cassava, secondary embryogenesis has been reported from primary embryos derived from both zygotic embryos [200] and from leaf explants [205]. Embryogenic capacity was maintained for more than one year by reculturing somatic embryos every 4 to 6 weeks on fresh 2,4-D supplemented medium. Unfortunately the authors [200, 205] did not give detailed descrip-

tions of the procedure that was followed. Procedures for both organogenesis and embryogenesis have been described in cassava, however, organogenesis was reported infrequently and only for specific genotypes. Somatic embryogenesis was reported more frequently and for at least 15 genotypes [196, 201, 205]. Therefore, the regeneration work as an important first step for plant transformation, described in this thesis, was focused on somatic embryogenesis and in particular on conditions which allow continuous proliferation of somatic embryos (cyclic embryogenesis) and shoot conversion.

OUTLINE OF THE THESIS

The next chapter gives an overview of the process of somatic embryogenesis (primary and cyclic embryogenesis and shoot conversion). In Chapters 3 to 8 experiments on different aspects of somatic embryogenesis in cassava are described. Chapter 3 describes the induction and outgrowth in shoots of primary embryos derived from leaf explants of greenhouse grown donor plants. Cyclic embryogenesis and the advantages of using liquid culture medium are discussed in Chapters 4 and 5, respectively. Shoot conversion of embryos of different embryogenic cycles is investigated and discussed in Chapters 4 to 6. In Chapter 6 explants of in vitro instead of greenhouse grown donor plants are used in order to ensure the availability of source material with a high response for primary embryogenesis during the whole year. The morphogenesis and histogenesis of cyclic embryogenesis are described in Chapter 7. Chapter 8 describes the occurrence and degree of somaclonal variation of embryo derived plants from up to 17 embryogenic cycles, equalling 2½ year of culture.

Finally, in Chapter 9 prospects of somatic embryogenesis for transformation of cassava are discussed.

CHAPTER 2

PRIMARY AND CYCLIC SOMATIC EMBRYOGENESIS

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Abstract. *The explants successfully used to obtain primary somatic embryos can be divided into zygotic embryos, floral and vegetative explants. In general, primary somatic embryogenesis from vegetative explants is indirect and mostly driven by auxine (AUX) or auxine and cytokinin (AUX/CYT) supplemented media and primary somatic embryogenesis from zygotic embryos is direct and driven, to a larger extent, by CYT or growth regulator free media. Primary somatic embryogenesis from floral explants is between these two extremes. Independent of the origin and of the explant used, primary somatic embryos are an excelent source to be used for a new cycle of somatic embryogenesis. Repeated subculture of somatic embryos (cyclic embryogenesis) allows the development of continuous proliferating somatic embryo cultures. Cyclic embryogenesis has been described in at least 80 Gymnosperm and Angiosperm species. In general, in species with CYT driven primary embryogenesis, cyclic embryogenesis is characterized by proliferation of new embryos on growth regulator free medium (habituation) whereas in species with CYT/AUX or AUX driven primary embryogenesis, cyclic embryogenesis needs continuous exposure to growth regulators.*

In most species somatic embryos can be converted into shoots. However, the frequencies are mostly low and the process is accompanied with malformations. In general, embryos induced by growth regulator free or CYT supplemented media meet more difficulties in shoot development than embryos induced by AUX supplemented media. Furthermore it can be concluded that, in spite of the fact that detailed data on the occurrence of somaclonal variation is limited, cyclic embryogenesis can lead to aberrant genotypes.

INTRODUCTION

Somatic embryogenesis is one of the two pathways of plant-regeneration. In this chapter the processes which allow continuous proliferation of somatic embryos and their subsequent development into plants, are reviewed. Proliferating embryogenesis is defined by Williams and Maheswaran [231] as the formation of somatic embryos from somatic embryos. It is associated with loss of integrated group control of organized cells. Some cells act independently, break away from group control and initiate a new cycle of somatic embryos. The most common name for this phenomenon is secondary somatic embryogenesis [13, 34, 82, 164, 209], but it has also been named accessory somatic embryogenesis [5, 7, 13, 172], cleavage polyembryony [72, 163], cyclic somatic embryogenesis [110, 192], adventive somatic embryogenesis [70, 93, 142, 177], somatic embryo cloning [129, 163], recurrent somatic embryogenesis [39, 115, 129, 152] and repetitive somatic embryogenesis [6, 7, 43, 218, 225].

In this overview, the term primary somatic embryogenesis is used to describe the formation of first cycle embryos from plant explants. Secondary somatic embryogenesis is used occasionally to describe the formation of embryos from primary embryos. Here, the term cyclic somatic embryogenesis will be used to describe continuous embryo proliferation. In Table 1 ten

Gymnosperm and seventy-two Angiosperm species are listed in which cyclic embryogenesis has been described. In the next section the induction of primary embryos will be discussed.

PRIMARY EMBRYOGENESIS

EXPLANTS USED

Primary somatic embryos can be obtained from three sources: zygotic embryos, floral explants (nucellus, ovule, anther, pollen) and vegetative parts (leaves, petioles and apexes). In the Gymnosperm species listed in Table 1, primary embryos were solely initiated from zygotic embryos. Although vegetative explants [179] and floral explants [140] have also been reported to be embryogenic.

In 51 percent of the Angiosperm species zygotic embryos, in 23 percent floral explants and in 3 percent vegetative explants were used to initiate primary somatic embryos. In the remaining 23 percent both zygotic embryos with either vegetative or floral explants were used to initiate primary somatic embryos. Usually this induction was accomplished on the same media, except in *Aesculus hippocastanum* [93], *Brassica napus* [111, 163, 208, 209] and *Quercus rubra* [63, 64] where floral explants required auxin supplemented and zygotic embryos auxin free medium. In many species primary somatic embryos can be induced on a wider range of explants than shown in Table 1 (for references see literature) and most likely all these embryos can be used for cyclic embryogenesis.

GROWTH REGULATORS USED

In most cases primary somatic embryos are formed on the explant in a two step procedure [10, 27, 43, 65, 70, 88, 93, 95, 127, 130, 152]. In the first step somatic embryos (direct embryogenesis) or embryogenic competent callus (indirect embryogenesis) are induced on the explant. In the second step, usually on a medium with a lower concentration of or without the inductive growth regulator(s), the induced embryos develop further (direct) or somatic embryos are formed from the embryogenic competent callus cells (indirect).

Primary somatic embryogenesis is achieved in different plant species on media with quite different compositions. On the basis of the growth regulator(s) used, cytokinin (CYT), cytokinin plus auxin (CYT/AUX), auxin (AUX) and growth regulator free induced embryogenesis can be discerned. In the Gymnosperm species listed in Table 1, primary embryogenesis is driven by CYT/AUX [12, 45, 56, 73, 98, 133, 177, 214] or CYT [144] supplemented media. Although, in the genus Pinus also AUX supplemented medium had that capacity [72].

In Angiosperm monocots, primary embryogenesis is exclusively induced by AUX supplemented media. Furthermore, only auxins with strong effects such as Picloram, Dicamba or 2,4-D initiate embryogenesis, except in *Asparagus officinalis* [34], where also NAA has that capacity.

Table 1: Occurrence of cyclic somatic embryogenesis in Gymnosperm and Angiosperm species.

CYCLE OF EMBRYOGENESIS	PRIMAIRY		CYCLIC		GROWTH REGULATORS USED						
	EXPLANT	ORI-GIN	EXPLANT	ORI-GIN	NO	CYT	AUX	CYT AUX	CYCLES	SHOOTS (%)	REF.
GYMNOSPERMS											
Abies nordmanniana	z.emb	I	g.emb	D	-	+	-	-	> 3	no	144
Larix decidua	z.emb	I	emb	D	-	-	+	-	> 3	30	95
x leptolepis											
Picea abies	z.emb	I	g.emb	D	-	-	+	-	4	5	133
	z.emb	I	m.emb	D	-	-	+	-	4	5	8
P. glauca	z.emb	I	g.emb	D	-	-	+	-	>10	low	98
P. mariana	z.emb	I	g.emb	D	-	-	+	-	>10	low	214
P. glauca x engelmanni	z.emb	I	g.emb	D	-	-	+	-	>10	low	45
P. rubens	z.emb	I	g.emb	D	-	-	+	-	>10	low	214
P. sitchensis	z.emb	I	g.emb	D	-	-	+	-	>10	no	177
Pinus strobus	z.emb	I	g.emb	D	-	-	+	-	> 5	no	56
P. taeda	z.emb	I	g.emb	D	-	-	+	-	> 5	low	73
	z.emb	I	m.emb	D	-	-	+	-	> 5	low	
ANGIOSPERMS (MONOCOTS)											
Asparagus officinalis	male apex	I	emb	D	+[c]	-	-	+	>10	yes	34
Hemerocallis sp.	ovary	I	g.emb	D	-	-	-	+	>10	yes	100
Dactylis glomerata	leaf	B	emb	I	-	-	-	+	2	95	27
Oryza sativa	z.emb	I	emb	D	-	-	-	+	3	yes	89
Panicum maximum	z.emb	I	g.emb	I	-	-	-	+	3	yes	113
Pennisetum americanum	inflor.	I	g.emb	D	-	-	-	+	>10	yes	223,224
Zea mays	apex	B	emb	I	-	-	+	+	2	80	47,236
	z.emb										
ANGIOSPERMS (DICOTS)											
Acanthopanax senticosus	z.emb(c,e)	D	t.emb(c,e,h)	D	-	-	+[c]	+	2	75	70
Aesculus hippocastanum	z.emb(r)	D	c.emb(r)	D	-	-	+	-	3	1	93
	filament	B	c.emb(r)	D	-	-	+[c]	+			
Apium graveolens	leaf	I	g.emb	D	-	-	+	+	> 3	100	137,138
Arachis hypogaea	z.emb(c,e)	I	g.emb	D	-	-	-	+	> 5	5	43,185
	leaf	I									10
A. paraguariensis	z.emb(c,e)	I	g.emb	D	-	-	-	+	> 5	yes	185
Atropa beladonna	anth.	D	emb(h)	D	+	-	+	-	3	yes	175
Beta vulgaris	z.emb(h)	I	emb(h,c,r)	D	-	-	-	+	2	yes	206
Brassica campestris	z.emb(h)	D	emb(c,h)	D	+[p]	+	-	-	2	yes	118
B. napus	m.sp./anth.	D	emb.(c)	D	+[c]	+[c]	+[c]	+[p]	>10	yes	111,208,209
	z.emb(h)	D	emb(h)	D	+[c]	+	-	-	> 3	yes	163
B. juncea	m.sp.	D	emb	D	-	-	+	-	> 5	yes	161
Camellia japonica	z.emb(c,e)	D	emb(h,e,c)	D	+	+	+	+	>10	72	90,226
	root										225
C. reticulata	z.emb(c,e)	D	emb(h)	D	+	+	+	+	>10	yes	157,158
C. sinensis	z.emb(c)	D	emb(h)	D	+	+	+	+	>10	21	87
Carica papaya	ovule	I	g.emb	?	-	-	-	+	> 5	yes	106

CONTINUED

TABLE 1 CONTINUED

CYCLE OF EMBRYOGENESIS	PRIMAIRY		CYCLIC		GROWTH REGULATORS USED						
		ORI-		ORI-			CYT			SHOOTS	
	EXPLANT	GIN	EXPLANT	GIN	NO	CYT	AUX	AUX	CYCLES	(%)	REF.
Carum carvi	petiole	I	g.emb	I	+	-	-	-	3	yes	5
Citrus microcarpa	nucellus	D	g.emb	D	+	+	-	-	2	yes	171
C. paradisi	nucellus	D	emb	D	+	+	+	-	2	yes	96
Citrus sinensis	ovule	D	emb	D	+	+	+	-	2	yes	96
Clitoria ternatea	z.emb(r,h,c)	B	emb(r,h)	D	+	+	+	-	>10	72	39,102
Cucurbita pepo	z.emb	I	emb	D	-	-	+	+	> 5	yes	85
Daucus carota	z.emb(c,r,h)	B	emb(r,c,h)	D	+	-	-	+	>10	94	192
Elaeis guineensis	leaf	I	g.emb	D	-	-	-	+	>10	18	36
Euphoria longan	leaf	I	c.emb(r)	D	-	-	+	-	> 2	yes	108
Eucalyptus citriodora	z.emb	D	t.emb	D	-	-	-	+	>10	50	134,135
Euphorbia pulcherrima	apex	I	g.emb	I	-	-	+	-	2	yes	162
Fagopyrum esculentum	z.emb	B	emb(c,h)	I	-	-	+	-	2	low	142
Fagus sylvatica	z.emb	I	g.emb	D	-	-	+	-	>10	10	227
Glycine max	z.emb(c)	B	g.emb	D	-	-	-	+	3	90	110,185
G. canescens	z.emb(c)	B	g.emb	D	-	-	-	+	3	90	185
Helianthus annuus	z.emb(c)	D	emb	D	-	-	-	+	3	low	55
Hevea brasiliensis	z.emb	I	g.emb(c,e)	D	-	-	+	-	2	no	131
Ilex aquifolium	z.emb(c,e)	D	c.emb(c)	D	+	-	-	-	3	yes	81
Juglans hindsii	z.emb	D	c.emb(r,c)	D	+[c]	+	-	-	>10	low	217
J. major	z.emb	D	c.emb(r,c,h)	D	+[c]	+	-	-	> 5	low	28
J. nigra	z.emb	D	c.emb(r,c,h)	D	+[c]	+	-	-	>10	low	28,35
J. nigra x regia	z.emb	D	c.emb(r,c,h)	D	+[c]	+	-	-	>10	low	28,217
J. regia	z.emb	D	c.emb(r,c,h)	D	+[c]	+	-	-	>10	low	217
Limnantes alba	z.emb(c,h)	D	emb(h)	D	-	+	+	-	3	no	195
Liquidambar styraciflua	z.emb	I	emb(h,c)	?	+	+	-	-	> 3	yes	194
Magnolia sps.	z.emb	B	t.emb(r)	D	-	-	+	+	> 5	25	130
Malus domestica	nucellus	D	emb(c)	D	+	-	-	-	2	no	46
M. pùmila	nucellus	D	emb(c)	D	+	+	+	-	2	no	83
Manihot esculenta	z.emb(c,e)	D	emb(c,e)	D	-	-	-	+	>10	32	205
	leaf										201
Mangifera indica	nucellus	I	g.emb	D	-	-	+[c]	+	2	yes	37,38
Medicago sativa	z.emb(h)	I	t.emb(c,e,h)	D	+[c]	-	+	-	>10	yes	114,115
	leaf										152
Myrciaria cauliflora	ovule	I	g.emb	?	-	-	-	+	2	yes	107
Pharbitis nil	z.emb(h)	D	emb(r)	D	-	-	-	+	3	yes	88
Poncirus trifoliata	nucellus	D	g.emb	D	-	+	+	+[c]	> 3	yes	123
Populus ciliata	leaf	I	g.emb(h,c)	D	-	-	-	+	> 5	yes	22
Prunus persica	z.emb(r)	D	emb	B	-	-	+	-	>10	yes	13
P.incisa x P. serula	z.emb	I	emb	D	+[c]	+	-	-	3	yes	41
Pterocarya spp.	z.emb	D	emb(r,c)	D	+[c]	+[1]	-	-	>10	low	217
Quercus alba	z.emb	B	emb(c)	B	+[c]	+	+	-	3	3	63
Q. bicolar	♂ flower	I	emb(c)	D	+[c]	-	-	+	> 5	yes	64
Q. rubra	z. emb	B	emb(c)	D	+[c]	+	+	-	> 5	yes	63
	♂ flower	I	emb(c)	D	+[c]	-	-	+			116
Q. suber	stem	I	emb(r)	D	+[c]	-	+	-	?	no	116

CONTINUED

TABLE 1 CONTINUED

CYCLE OF EMBRYOGENESIS	PRIMAIRY		CYCLIC		GROWTH REGULATORS USED						
		ORI-		ORI-			CYT		SHOOTS		
	EXPLANT	GIN	EXPLANT	GIN	NO CYT		AUX	AUX	CYCLES	(%)	REF.
Ranunculus sceleratus	floral buds	I	plant	B	-	-	-	+	6	44	97
			emb(h)	I							
Rauvolfia vomitoria	leaf	I	c.emb	I	-	-	-	+	> 3	yes	215,221
Ribes rubrum	ovule	?	c.emb	?	+	+	-	-	2	low	235
Theobroma cacao	z.emb	B	emb(c,e)	D	+	+	+	+	>10	yes	2,3,99,153
Trifolium repens	z.emb(h)	D	t.emb(a,c)	D	-	+	-	-	2	yes	117
Vitis longii	z.emb(c,e)	D	g.emb	D	-	-	+	+	>10	36	202
	anther	I	emb(r,h)	B	+°	-	+	-	>10	50	68
V. rupestris	anther	D	g.emb	D	-	-	+	+	>10	41	203
	leaf	I	g.emb	D	-	-	+	+°	>10	yes	121
V. rupestris x vinifera	anther	I	g.emb	D	-	-	+	-	>10	yes	203
V. vinifera	z.emb(c,e)	D	g.emb	D	-	-	+	-	>10	36	202
	leaf	B	g.emb								203
	anther	I	g.emb								203

ABREVIATIONS:

EXPLANT: c: cotyledon, c.emb: cotyledonary embryo, e: embryogenic axis, g.emb: globular embryo, h: hypocotyl, inflor.: inflorescence, m.sp.: micro spores, r: root t.emb: torpedo-shaped embryo and z.emb: zygotic embryo

ORIGIN OF EMBRYOGENESIS: B: both direct and indirect, D: direct, I: indirect, ?: unknown

GROWTH REGULATORS USED °: only in cyclic embryogenesis, ': only in primary embryogenesis

In the Angiosperm dicot *Camellia* sps [87, 90, 157, 158, 225, 226] and in *Theobroma cacao* [2, 3, 99, 153] primary somatic embryogenesis was reported to be induced by growth regulator free, CYT, CYT/AUX and AUX supplemented media. In 17 of the 65 dicot Angiosperm species listed in Table 1, primary somatic embryogenesis was induced on growth regulator free medium and in 13 of these species also CYT and/or CYT/AUX had that capacity. CYT supplemented media were used in 25 species of which BA was used most frequently (57 percent) followed by kinetin (37 percent), zeatin (3 percent) and TDZ (3 percent). AUX supplemented media were applied in 29 dicot species in which 2,4-D was most frequently used (49 percent) followed by NAA (27 percent), IAA (6 percent), IBA (6 percent), Picloram (5 percent) and Dicamba (5 percent). In most species more than one of the different auxins or cytokinins had the capacity to induce primary embryogenesis. AUX/CYT media were used in 31 species and in 18 of them also either AUX or CYT media were capable of inducing primary embryogenesis. Combinations of growth regulator free and/or CYT media with AUX media were only found in *Camellia* sps. [157, 226], *Daucus carota* [192] and *Theobroma cacao* [99, 153].

The different growth regulators are not equally used to initiate primary somatic embryogenesis in different explant types of the Angiosperm dicot species (Fig. 1).

Zygotic embryos and floral explants initiate embryos in a greater proportion of species on CYT supplemented media than vegetative explants. In the latter explant type AUX and AUX/CYT supplemented media are used to a greater extent.

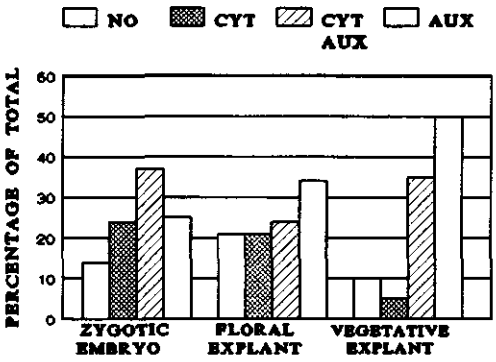

Figure 1. Influence of growth regulators on induction of primary somatic embryogenesis of different explant types in dicot Angiosperm species.

ORIGIN OF EMBRYOS

In all Gymnosperm species and most monocot Angiosperm species, primary embryos originated from callus induced on the explant (indirect embryogenesis). In the monocots *Dactylis glomerata* [27] and *Zea mays* [236] the origin of embryos was both indirect and direct. In 63 percent of the dicot Angiosperm species the origin of primary embryos from zygotic embryos was direct, in 23 percent indirect and in 14 percent both direct and indirect (Fig. 2). For vegetative explants this figure was respectively 15, 77 and 8 percent. The origin of embryos from floral explants was in between these two extremes.

The frequency of direct or indirect embryogenesis depended on growth regulators used during the induction of embryos.

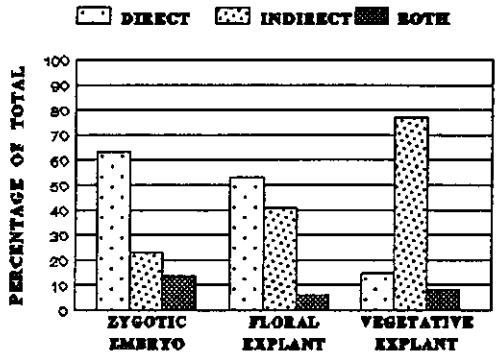

Figure 2. Influence of explant type on the origin of primary somatic embryos in dicot Angiosperm species.

In about 70 percent of the species where growth regulator free or CYT supplemented media were used, the embryos developed directly from the explants and in 13 percent the origin was indirect (Fig. 3).

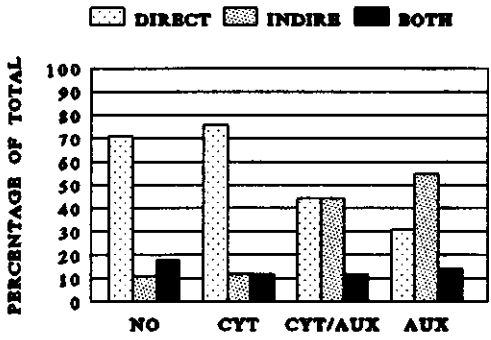

Figure 3. Influence of growth regulators on the origin of primary somatic embryos in Angiosperm dicot species.

The use of AUX, alone or in combination

with CYT, resulted in a shift to indirect embryogenesis. One exception is *Clitoria ternatea* [102] where growth regulator free medium gave indirect and CYT/AUX media direct somatic embryogenesis.

GENERAL CONCLUSIONS

The explants successfully used to obtain primary somatic embryos can be divided into zygotic embryos, flowering associated and vegetative explants.In most species there was no evidence that primary somatic embryos of a particular explant were better suited to be used as the source material for cyclic embryogenesis than primary somatic embryos of another explant. In general, primary somatic embryogenesis from vegetative explants is indirect and mostly driven by AUX or AUX/CYT supplemented media. Primary somatic embryogenesis from zygotic embryos was characterized by a direct origin of the embryos and was driven, to a larger extent, by CYT or growth regulator free media. Primary somatic embryogenesis from floral explants was between these two extremes. In the next section different aspects of cyclic somatic embryogenesis are presented.

CYCLIC EMBRYOGENESIS

EXPLANTS USED

In most species embryos develop up to the globular or younger stages of somatic embryogenesis before the loss of group control of cells causes a new cycle of embryogenesis from these embryos. If these cultures are performed in liquid medium, the newly formed embryos slough of into the medium and initiate a new cycle of embryogenesis. In some cases a new cycle is started from free single cells [7]. Suspension cultures are often highly asynchronous. Synchronity can be improved when sieved fractions of the embryogenic material are used to start a new cycle of somatic embryogenesis.

In *Acanthopanax senticosus* [70], *Manihot esculenta* [201, 205], *Ribes rubrem* [235] and *Trifolium repens* [117] more advanced stages of embryos are used to initiate a new cycle. In these cultures a new cycle can be started by the transfer of more or less mature embryos to fresh induction medium. As a consequence these cultures are often more synchronized. Embryo proliferation is not always restricted to specific developmental stages. In *Juglans* sps. [28, 35, 217], *Quercus* sps. [63, 64, 116] and *Theobroma cacao* [2] globular to mature embryos are source material to a new cycle and in *Brassica napus* [208, 209], *Medicago sativa* [115, 152], *Ranunculus sceleratus* [97] embryo derived plants are even subjected to this process. In *Fagus sylvatica* [227] and *Populus ciliata* [22] embryos developed in liquid culture up to the globular stage and in solid culture to maturity before a new cycle began. This was not observed in *Picea glauca* [98] and *Pinus taede* [73]. In these two species continuous subculture either in liquid or on solid CYT/AUX supplemented medium allowed development up to the globular stage. Transfer of globular embryos to medium with ABA ensured maturation which gave a new cycle of embryogenesis after transfer to CYT/AUX

medium [73, 133]. In *Daucus carota* [192], development of embryos to the globular stage or to more mature stages depended on the NH_4 concentration.

GROWTH REGULATORS USED

Usually, the transfer of isolated embryos or clumps of embryos to the same induction medium as used for primary embryogenesis is sufficient to start a new cycle. However, in *Trifolium repens* [117] higher concentrations of BA and in *Arachis hypogaea* [43] lower concentrations of 2,4-D were required for cyclic than for primary somatic embryogenesis. In *Helianthus annuus* [55] and *Hemerocallis* sps. [100] Dicamba was not capable of inducing primary embryogenesis, but did so in cyclic embryogenesis.

All species with growth regulator free driven primary somatic embryogenesis, except *Brassica campestris* [118], required also no growth regulators for further cycles of embryogenesis (Fig. 4).

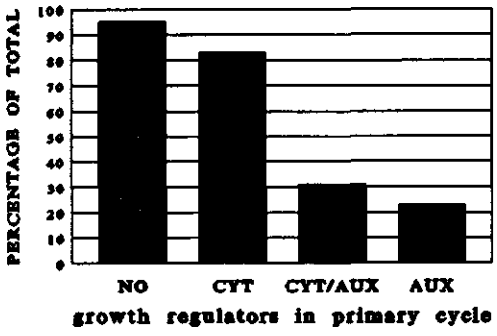

Figure 4. Relation between growth regulators used to initiate primary embryogenesis and the occurence of growth regulator free cyclic embryogenesis (habituation) of dicot Angiosperm species.

In *B. campestris* CYT supplemented media were necessary for cyclic embryogenesis [118].

In 83 percent of the species with CYT induced primary embryogenesis, cyclic embryogenesis required no growth regulators (Fig. 4).

For the AUX and AUX/CYT groups this was respectively 31 and 23 percent. With the exception of *Asparagus officinalis* all the 26 species, where primary embryogenesis was restricted to AUX supplemented media also cyclic embryogenesis required AUX supplemented medium. In *Asparagus officinalis* [34] cyclic embryogenesis was driven by growth regulator free medium.

ORIGIN OF CYCLIC SOMATIC EMBRYOS

In almost all Gymnosperm and Angiosperm species, the origin of embryos in cyclic embryogenesis is direct regardless of the growth regulators used and of the origin of the embryos in primary embryogenesis.

Cyclic embryos are formed from cotyledons [29, 92, 117], hypocotyls [90, 92, 157], roots [116] or combinations of these organs [110, 159, 225]. Embryos originate from specific cells in the epidermis and/or subepidermis, either only from single cells [159, 209], from multiple cells [117, 157] or from both [29, 110, 153]. In cyclic embryogenesis of *Dactylis glomerata* [216] embryos originated from both epidermal and vascular bundle parenchym cells.

EFFICIENCY OF CYCLIC EMBRYOGENESIS

The production of embryos is determined by the number of responding explants and

the number of embryos produced per responding explant. In *Arachis hypogaea* [10,43, 185], *Beta vulgaris* [206], *Camellia japonica* [225], *C. reticulata* [157], *Glycine max* [110, 185], *Picea abies* [133] and *Vitis* sp. [203] less than 50 percent of the primary explants produced embryos whereas more than 75 percent of the somatic embryo explants formed new embryos. In *Medicago sativa* [152] only 5 of 300 zygotic embryos produced primary embryos whereas all somatic embryo explants initiated new ones. In *Glycine max* [110] the production of embryos in the primary cycle varied from 0.2 (NAA supplemented medium) to 1.3 per explant (2,4-D supplemented medium) and in the higher cycles from 8 to 30, respectively. Although no exact numbers were given, it was evident that also in other species the production of embryos in primary embryogenesis was lower than in cyclic embryogenesis [34, 46, 63, 93, 96, 116, 118, 134, 135, 185, 217].

The process of embryo development, loss of group control and entrance into a new embryogenic cycle, enabled the maintenance of embryogenic capacity in vitro for more than 2 years in *Asparagus officinalis* [34], *Citrus sinesis* [171], *C. microcarpa* [171], *Camellia japonica* [225, 226], *Clitoria ternatea* [39], *Eucalyptus citriodora* [135], *Hemerocallis sp.* [100], *Juglans nigra* [35], *Picea glauca* [98] and *P. glauca x engelmannii* [45].

INFLUENCE OF NON HORMONAL FACTORS

Several factors other than growth regulators are either controlling or obligatory for somatic embryogenesis. A few will be discussed here. In *Daucus carota* [192] whole embryos cultured on growth regulator free medium grew into shoots; only wounding of embryos led to new embryos. In *Picea abies* [8] AUX/CYT and *Arachis hypogaea* [10] AUX induced somatic embryogenesis, wounding was not obligatory but it increased the embryogenic response. In *Magnolia* ssp. [130] and *Malus domestica* [46] light and in *Ilex aquifolium* [81] dark conditions inhibited embryogenesis.

Yeast extract was obligatory in cyclic culture of embryos of *Medicago sativa* [114, 152], in primary embryogenesis of *Lycopersicon peruvianum* [234], *L. esculenta* [234], *Trifolium repens* [117], and *Santalum album* [174]. In *Citrus microcarpe* [171] casein hydrolysate and in *Aesculus hipocastanum* [167] proline were necessary. These are only a few non hormonal factors which have been described in the literature to influence embryogenesis. Other factors are temperature, pretreatment of donor plants, light, subculture duration, nitrogen source, type of agar, type of sugar and so on. For a more detailed description of these factors the following reviews [6, 189, 213, 218] are of importance.

GENERAL CONCLUSIONS

In at least 80 species procedures have been developed to obtain a continuously proliferating embryogenic culture (cyclic embryogenesis). There is no evidence that cyclic embryogenesis is solely restricted to the species listed in Table 1. In numerous other plant species primary embryos cultured for maturation or shoot development

developed secondary embryos [29, 62, 65, 79, 91, 101, 143, 148, 155, 164, 169, 172, 173, 174, 184, 188, 220, 228, 233, 234]. In these species secondary embryogenesis was considered as an artefact of the procedure which resulted in a poor ability of the embryos to develop into shoots [7, 79, 128, 234]. Probably in most of these species cyclic embryogenesis can be accomplished by returning the somatic embryos to the medium that was used to initiate primary embryos. In general, in species with CYT driven primary embryogenesis, cyclic embryogenesis is characterized by proliferation of new embryos on growth regulator free medium (habituation) whereas in species with CYT/AUX or AUX driven primary embryogenesis, cyclic embryogenesis needs continuous exposure to growth regulators.

SHOOT CONVERSION

DEFINITIONS USED

The possible application of cyclic embryogenesis depends on the ability and the ease with which somatic embryos develop into shoots. In many articles the only fact reported was that whole plants were regenerated, but no further details were given. In most cases selected embryos were used for shoot recovery whereas in others random samples were taken. In *Fagus sylvatica* [227], 10 percent of the cotyledonary embryos developed into shoots, but the percentage from the total number of embryos was not mentioned. In somatic embryogenesis, the developmental stages like, globular, cotyledonary and torpedo shape are well described and generally accepted. But there is no general agreement in terminology for the stages beyond the torpedo shape stage. Mature embryos were on one extreme defined as embryos which have passed beyond the globular stage [227], and on the other end, as structures with a primary root, a hypocotyl and well developed cotyledons [121, 203, 217]. The latter definition was used by others to describe germination of embryos [36, 55, 68, 106, 114, 130]. In other examples only the formation of roots was used as characteristic of germination [64, 81, 127, 177]. In these latter three definitions, only a fraction of the germinated embryos developed into shoots. For example in *Magnolia* sps. [130] 25 percent and in *Mangifera indica* [38] 9 percent of the germinated embryos grew actually into shoots. In other cases germination of embryos was equivalent with shooting, i.e., the formation of a primary root, greening of cotyledons and hypocotyl and the formation of the first true leaves [15, 35, 39, 70, 121, 192, 202, 217].

It would be more convenient to standardize these definitions. Because root and/or cotyledon elongation are easily observed and can be quantified, these characteristics are generally applicable to describe germination in different species. The term mature embryos is in this thesis restricted to the developmental stage at the onset of germination i.e. the torpedo shaped stage and shoot development for the process leading to shoots which can be multiplied by established standard methods. More

uniform definitions allow comparison of shooting procedures in different species. In Table 1 the percentage of shoot development of individual embryos is given.

INTERRUPTION OF THE PROCESS OF CYCLIC SOMATIC EMBRYOGENESIS

For the development of somatic embryos into plants, first the cycle of embryo proliferation has to be stopped. In most species with AUX induced embryogenesis the process of embryo proliferation is easily stopped by omission of auxin or lowering of the concentration. In some species embryogenesis still continues for one or two cycles [22, 37, 38, 113, 214, 221, 223, 224,], probably because of a carry-over effect of the auxin.

It is a common observation of growth regulator free or CYT induced embryogenesis that interruption of the process of cyclic embryogenesis is difficult to accomplish [2, 3, 5, 28, 35, 41, 82, 87, 96, 99, 111, 152, 157, 171, 175, 195, 208, 217, 226, 235]. In *Carum carvi* [5], *Medicago* sps. [115, 152] and *Sapindus trifoliatus* [220] ABA stops proliferation and promotes further development of the embryos; however, in *Apium graveolens* [137] and *Prunus persica* [182] ABA promotes cyclic embryogenesis. ABA also stops proliferation in *Brassica napus* [82, 186], but, without additional treatments, most embryos fail to develop into shoots. In *Brassica napus* [111] and *Camellia japonica* [90] other factors like high cytokinin content (> 10 mg/l BA or kinetin) in the medium or a cold treatment stops proliferation and allows further development of the embryo. In *Juglans* sps. [35, 217] proliferation is interrupted after isolation of embryos from the explants. In this case matured embryos could still be subjected to embryo proliferation. This could be reduced by either a desiccation treatment [35], cold storage [35, 217], liquid culture instead of solid [35] and culture on GA_3 supplemented media [35]. In *Theobroma cacao* new embryos are formed from the cotyledons or the embryogenic axis and proliferation can be interrupted by excision of the cotyledons. Culture of the remaining embryo in liquid medium with a low concentration of GA_3 plus NAA allows the development of the embryo [2] into a plant. Also in *Glycine max* [23], excision of cotyledons speed up the process of shoot formation. In *Camellia reticulata* [157] and *C. japonica* [225] the use of GA_3 and IAA allows shoot development at the expense of embryo proliferation.

MATURATION OF GLOBULAR EMBRYOS INTO TORPEDO SHAPED EMBRYOS

After proliferation of embryos has been stopped, culture regimes have to be directed to further differentiation of the embryos. In some species culture of explants on the inductive medium allows development up to the torpedo stage or even to maturity. In many species, especially those with characteristics of a suspension culture, the embryo development is blocked at the globular stage [2, 12, 37, 89, 96, 171, 177]. In *Glycine max* [23] maturation of embryos can be improved by increasing the sucrose concentration in the medium. The same effect was observed in *Mangifera indica* [38], *Picea mariana* [214], *P. rubens* [214] and *Zea mays* [47]. But in *Euphoria longan*

[108] a medium with lower sucrose concentration improved maturation. In *Picea* sps. [12] less than 5 percent of the globular embryos maturated on growth regulator free medium and 25 percent did when ABA was applied [12]. Also in species like *Carum carvi* [5], *Medicago falcato* [32], *Pennisetum americanum* [223, 224] and *Pinus* sps. [56, 73] normal maturation is allowed by ABA. In *Mangifera indica* [38] ABA has only a slight positive effect, whereas, Gelrite instead of Difco agar has a distinct positive effect on maturation.

A consequence of insufficient maturation of embryos is the development of malformed somatic embryos and/or the formation of fleshy leaves with fasciated stems. This phenomenon has been named precocious germination [7]. In some species normal shoots can be obtained from these abnormal structures, but, for instance, in *Euphoria longan* [108], *Liriodendron tulipifera* [127] and *Pennisetum americanum* [223, 224] this is not the case.

PRECOCIOUS GERMINATION OF SOMATIC EMBRYOS

In precocious germination, the developing embryo tends to skip the normal stages of embryogenesis and acquires the characteristics of a malformed seedling [36, 105]. In the normal differentiation process only a small number of cells become recognizable as the presumptive shoot apex after the cotyledons are differentiated. The number of meristematic cells is small and they do not divide during maturation. For the production of vigorously growing plantlets a sufficient period of embryogenic growth and maturation is required before germination can proceed [129]. During germination the shoot apex of the somatic embryo becomes active [7]. Ammirato [7] argued that in precocious germination the coordination between development of embryogenic apex into a shoot apex and that of the cotyledons is disturbed.

For normal and high frequency development of shoots from somatic embryos, culture regimes should be aimed at mimicking the different physiological stages occurring in zygotic embryogenesis. In a recent review [77] the role of ABA in this respect was discussed. In a large number of plant species, the ABA levels rise during the mid-stages of zygotic embryogenesis which are comparable with globular somatic embryos. This increase is prior to programmed dehydratation of the seeds. It prevents not only zygotic embryos from precocious germination but is apparently also involved in the acquisition of desiccation tolerance by the embryo. During dehydratation ABA again decreases to low levels [77]. It was concluded that the general role of ABA may be to prepare tissue for entry into a new and different physiological state, perhaps to reset the direction of cellular metabolism.

NORMAL GERMINATION AND SHOOT DEVELOPMENT OF EMBRYOS

In *Abies nordmanniana* [144], *Hevea brasiliensis* [131], *Limnanthes alba* [195], *Picea sitchensis* [177], *Pinus strobus* [56] and *Quercus suber* [116] none of the somatic embryos are developed into shoots. In none of these species embryogenesis is

driven by AUX supplemented medium. But, in *Apium graveolens* [137, 138], *Dactylis glomerata* [27], *Daucus carota* [192], *Glycine canescens* [182] and *G. max* [15, 23], all species with AUX induced embryogenesis, mature embryos can develop into shoots at frequencies higher than 90 percent. Most species listed in Table 1 behave between these two extremes. In *Picea* sps. [12] a maximum of 56 percent of the mature embryos germinated and only 29 percent of them developed into shoots. In *Vitis longii* [68] mature embryos could only be developed into shoots after giving them a cold treatment. Cold storage of embryos can stimulate shoot recovery from mature embryos of *Brassica napus* [82], *Fagus sylvatica* [227], *Juglans regia* [217], *J. regia x nigra* [35] and *Querqus* sps. [63, 64] and desiccation frequently enhances shoot recovery, as was in *Arachis hypogaea* [43], *Brassica napus* [82], *Juglans regia x nigra* [53], *Mangifera indica* [38] and *Querqus alba* [63]. In *Glycine max* [23] the process of shoot formation was accelerated by a desiccation treatment but this had no effect in *Querqus bicolor* and *Querqus rubra* [63].

In auxin driven embryogenesis cytokinins are not necessary for shoot development, but they often increase the frequency [70, 87, 205].

SOMACLONAL VARIATION OF SOMATIC EMBRYO DERIVED PLANTS

Detailed studies on the occurrence of somaclonal variation in plants from cyclic embryogenic cultures are scarce. In most cases research was restricted to an evaluation of the morphology of a small number of plants and usually no obvious variation was found [43, 68, 83, 95, 113, 201, 203, 236]. Shoots of fourth cycle embryos of *Picea abies* [133] and of secondary embryos in *Trifolium repens* [119] had the normal ploidy level. No variation was observed in the banding pattern of 16 isoenzymes and the ploidy level of long-term cultured somatic embryos of *Picea glauca x engelmanni* [45]. In *Medicago media* [139], depending on the clone, between 35 and 90 percent of the regenerants had the expected chromosome number, the others were aneuploid or polyploid which was associated with morphological abberations. In embryo derived plants of *Glycine max* [11, 60, 80, 191] morphological abberations could be observed including variation for leaf shape [11, 60, 80, 191], leaf variegation [11, 60, 80, 191], growth habit [60, 191], sterility [11, 191], iso-enzyme patterns [191] and lipid composition of seed [80]. In most cases, it was found that these altered traits were heritable [11, 60, 191]. Contrary to expectation, the number of plants with altered morphology decreased with increasing 2,4-D concentration [191]. This unexpected phenomenon might be associated with a difference in the origin of embryos. It was shown that with low 2,4-D concentrations the embryos originate from single or a few cells, whereas with high concentrations the origin is multicellular [76].

GENERAL CONCLUSIONS

Although in most species somatic embryos can be converted into shoots, the frequencies are mostly low and the process is ac-

companied with malformations. In some species a two step culture is sufficient, whereas in others more steps are necessary to allow proper embryo development. In general, embryos induced by growth regulator free or CYT supplemented media meet more difficulties in shoot development than embryos induced by AUX supplemented media.

In at least some species cyclic embryogenesis can lead to aberrant genotypes.

CHAPTER 3

INDUCTION, GERMINATION AND SHOOT DEVELOPMENT OF PRIMARY SOMATIC EMBRYOS IN CASSAVA

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ABSTRACT. ***Four Indonesian and two Latin-American cassava genotypes (Manihot esculenta Crantz), were evaluated for their ability to develop somatic embryos from young leaf lobes. All genotypes formed somatic embryos but they differed in the frequency of embryos induced. The best genotypes, M.Col22 and Tjurug, produced germinating embryos (GE) on 81 percent (22.1 GE/initial leaf lobe) and 46 percent (4.3 GE/initial leaf lobe) of the cultured leaf lobes, respectively. Up to 57 percent of the germinating embryos of M.Col22 and 12 percent of Tjurug produced either normal or malformed shoots. Most malformed shoots developed into shoots with normal morphology after prolonged culture. All shoots formed roots after transfer to medium without BAP. Roots of all normal and most malformed regenerants had the original ploidy level (2n=36). Regardless of whether the plants were multiplied in vitro (150 plants) or in the greenhouse (30 plants) there were no morphological differences compared to parent plants.***

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is cultivated throughout the lowland tropics of South-East Asia, Africa and South America for its starchy tuberized roots. Although this crop is the fourth most important source of carbohydrates in the tropics [26], it has not received much attention from agricultural research in the past. This has changed recently due to initiatives in both industrialized and developing countries.

To improve cassava by genetic engineering, an essential prerequisite is the development of an efficient regeneration and transformation procedure. Several groups have failed to obtain adventitious shoots of cassava tissue, but somatic embryogenesis has been described [25, 197, 198, 201, 205]. In a two-step procedure, embryos were obtained from seed and clonal leaf tissue of at least 40, mainly Latin-American genotypes, but only a few produced plants and at a low frequency [25, 205].

The aim of this study was to optimize the factors required for the induction of high frequency somatic embryogenesis in cassava and to improve somatic embryo germination and subsequent development into plants.

MATERIALS & METHODS

PLANT MATERIAL

The Latin-American (M.Col22 and M.Ven77) and Indonesian (Tjurug, Gading, Mangi no. 4 and Faroka) cassava genotypes were grown in a greenhouse (temperature throughout the year 20-45 °C, natural light). Shoot tips, containing the youngest unfolded leaves, were sterilized by immersion in a 0.5 % sodium hypochlorite, 0.01 % Triton X-100 solution for 5 min. Shoot tips which depending on the time of the year, consisted of one to four leaves. From these shoot tips between three to 28 leaf lobes were isolated and used as starting explants. The leaf lobes were folded along their midvein with the adaxial surface,

which was assumed to be embryogenically competent [205], inside.

EXPERIMENTAL PROCEDURES

In step one (embryo induction), leaf lobes were incubated on basal medium (BM) with 0.5-8 mg.l^{-1} 2,4-D. In some experiments, BM was supplemented with 2-40 mg.l^{-1} NAA with or without 0.1 mg.l^{-1} BA. BM consisted of Murashige and Skoog [136] salts and vitamins, 20 g.l^{-1} sucrose and 0.5 % agar (Oxoid no. 1, UK). The pH was adjusted to 5.7, before autoclaving (15 min, 120 °C). After 20 days explants were transferred to BM with 0.01 mg.l^{-1} 2,4-D and 0.1 mg.l^{-1} BA for germination of embryos (step two). Embryos were defined as globular structures. After the formation of visible, translucent cotyledon primordia embryos had the characteristics of cotyledonary embryos. Torpedo shaped embryos possessed a distinct, translucent hypocotyl. Germinated embryos (GE's) were defined as structures with distinct green cotyledons. In step three GE were isolated from the explant and cultured separately for shoot development on BM with 0.1 mg.l^{-1} BA. Shoots were defined as structures with a distinct stem with two or more leaves. They were classified as normal if the leaves had the phenotype of in vitro grown cassava leaves (lobed leaves, lanceolate shaped). Shoots that exhibited irregular shaped leaves without leaf lobes were classified as being malformed. Shoots were rooted on BM (step four).

Induction of embryos was studied in more detail with M.Col22 and Tjurug. Leaf lobes with an initial length of 1-3 mm were compared to leaf lobes of 4-6 mm and 7-10 mm by culture on BM with 1 or 4 mg.l^{-1} 2,4-D for 20 days.

Germination of embryos and their development into shoots were studied in more detail on BM with 0.01 mg.l^{-1} 2,4-D supplemented with 0.1-0.7 mg.l^{-1} BA or zeatin (Tjurug) or 0.1-0.4 mg.l^{-1} BA (M.Col22).

Statistically significant differences in the average number of leaf lobes with GE and number of shoots produced per GE were determined by the X^2 test (P=2.5%). The average number of GE per initial leaf lobe and the average number of shoots per initial leaf lobe were determined by the LSD test (P=2.5%) after a logarithmic transformation of the data (ln(1+x)).

The day/night temperature in the growth chamber was 28/24 °C with a photoperiod of 12 hours and an irradiance of 40 μmolm^{-2}s^{-1}. Step one was performed in the dark, steps two, three, and four in the light. Every 3 to 4 weeks explants were transferred to fresh media.

Ploidy level determinations were performed by the method described by Wilkinson [230].

RESULTS

GENERAL ASPECTS OF EMBRYOGENIC RESPONSE

After culture of leaf lobes on medium free from growth regulators no embryogenic tissue was formed and only limited callusing occurred at the cut end of the leaf lobe. Addition of 2 - 40 mg.l^{-1} NAA, with

or without 0.1 mg.l^{-1} BA, gave rise to abundant callus growth (data not shown). Also, on 2,4-D supplemented medium the first sign of activity (after two to four days of culture) was the development of callus, but after six to ten days embryogenic tissue was visible. It consisted of globular shaped somatic embryos which were not associated with veins or wounded tissue and were visible only on the adaxial surface of the leaf lobes.

Table 1: Effect of 2,4-D on percentage leaf lobes with germinating somatic embryos of several cassava genotypes (leaf lobes harvested in September 1989 of greenhouse grown plants; 12-26 leaf lobes cultured treatment.

2,4-D (mgl^{-1})	Tjurug	M.Col22	M. Ven77
0.5	0 b	0 b	0 a
1	29 a	8 b	6 a
2	0 b	15 ab	25 a
4	8 ab	15 ab	25 a
8	0 b	35 a	0 a
	Gading	Mangi 4	Faroka
0.5	0 a	0 a	8 a
1	18 a	9 a	0 a
2	6 a	0 a	0 a
4	12 a	0 a	0 a
8	0 a	0 a	0 a

Means with the same letter in one column are not significantly different by the X^2 test (P = 2.5%)

Leaf lobes of all genotypes formed globular embryos and callus. During normal development, globular embryos passed through cotyledonary and torpedo stages before they germinated (Fig. 1), however, many of the induced embryos remained in the globular stage, were overgrown by callus, or developed into foliose structures. Most GE developed on the proximal end of the leaf. Normally, single GE were obtained. However, sometimes the hypocotyl of two or more GE were fasciated and other malformations were found such as trumpet-like cotyledons and multiple cotyledons. The features described above were seen in all genotypes. The genotypes Tjurug and M.Col22 had the highest number of leaf lobes with GE on media with 1 and 8 mg.l^{-1} 2,4-D respectively (Table 1). Faroka formed only on one of the 60 cultured leaf lobes GE (0.5 mg.l^{-1} 2,4-D).

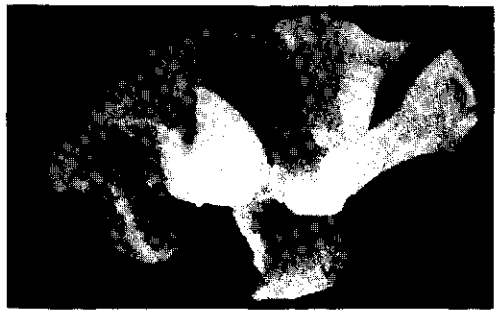

Figure 1: Germination of embryos after 21 days step two.

INFLUENCE OF MEDIUM AND EXPLANT SIZE ON INDUCTION AND GERMINATION OF EMBRYOS

In September, only 8 percent of M.Col22 leaf lobes formed GE on 1 mg.l^{-1} 2,4-D (Table 1) compared to more than 40 percent of leaf lobes cultured in January (Table 2). In both seasons, this percentage was almost doubled by using 4 mg.l^{-1} 2,4-D instead of 1 mg.l^{-1} 2,4-D. Also the num-

Table 2: Effect of 2,4-D and BA on germination and shoot development of somatic embryos from leaf lobes of cassava variety M.Col22 (leaf lobes harvested in january 1990 of greenhouse grown plants).

step 1 2,4-D ($mg.l^{-1}$)	step 2 BA ($mg.l^{-1}$)	number of leaf lobes	percentage leaf lobes with GE [A]	GE per initial leaf lobe [B]	percentage GE with shoots [A]	shoots per initial leaf lobe [B]
1	0.1	21	52 ab	2.3 b	39 ab	0.9 c
1	0.4	15	40 b	1.9 b	57 a	1.1 c
4	0.1	20	75 ab	16.6 a	26 b	4.3 b
4	0.4	16	81 a	22.1 a	46 a	10.2 a

Legend see Table 3

ber of GE per initial leaf lobe (GE/ILL) was significantly enhanced from less than 2.5 to more than 15 (Table 2) when 4 $mg.l^{-1}$ 2,4-D instead of 1 $mg.l^{-1}$ 2,4-D was used. Forty-four percent of the M.Col22 leaf lobes with an initial length of 1 to 3 mm, cultured on 1 or 4 $mg.l^{-1}$ 2,4-D, formed GE (6.3 GE/ILL) compared to 71 percent of the leaf lobes of 4 to 6 mm (12.3 GE/ILL), whereas 67 percent of leaf lobes of 7 to 10 mm formed GE (12.8 GE/ILL). From a single M.Col22 leaf lobe up to 71 GE were isolated.

In September Tjurug gave the best response on 1 $mg.l^{-1}$ 2,4-D (Table 1), whereas in January none of the 148 leaf lobes formed GE (data not shown). However, with 4 $mg.l^{-1}$ 2,4-D between 10 and 46 percent of the leaf lobes formed GE, depending on the cytokinin concentration.

Table 3: Effect of zeatin and BA on germination and shoot development of somatic embryos from leaf lobes of cassava variety Tjurug after culture on step one medium with 4 $mg.l^{-1}$ 2,4-D (leaf lobes harvested in january 1990 of greenhouse grown plants).

step 2 ($mg.l^{-1}$)	number of leaf lobes	percentage leaf lobes with GE [A]	GE per initial leaf lobe [B]	percentage GE with shoots [A]	shoots per initial leaf lobe [B]
0.1 ZEATIN	14	36 ab	1.7 ab	0 a	0 a
0.4 ZEATIN	22	27 ab	2.6 ab	3 a	< 0.1 a
0.7 ZEATIN	23	17 ab	3.0 ab	1 a	< 0.1 a
0.1 BA	28	46 a	4.3 a	3 a	0.1 a
0.4 BA	21	38 ab	1.7 ab	12 a	0.2 a
0.7 BA	21	10 b	0.8 b	8 a	< 0.1 a

means with the same letter in one column are not significantly (P = 2.5%) different by the X^2 test [A] or the LSD test [B]
GE: germinating embryos

There seemed to be a negative correlation between the BA or zeatin concentration and the percentage of responding leaf lobes. However, only the treatments 0.1 and 0.7 mg.l^{-1} BA differed significantly (Table 3).

Thirty-four percent of the Tjurug leaf lobes with an initial length of 1 to 3 mm formed GE (3.4 GE/ILL), compared to 28 percent of the leaf lobes of 4 to 6 mm (2.3 GE/ILL), whereas only 8 percent of leaf lobes of 7 to 10 mm formed GE (< 0.1 GE/ILL). Up to 38 GE were isolated from a single Tjurug leaf lobe.

For both genotypes leaf lobes which had started to unfold (longer than 10 mm) did not initiate embryos. The germination of embryos was asynchronous in both genotypes: during step two, 50 percent of the total number of GE was isolated after 3 weeks, 35 percent after 6 weeks, 14 percent after 9 weeks and 1 percent after 12 weeks.

FACTORS AFFECTING SHOOT DEVELOPMENT

Foliose structures never developed into shoots. Many GE cultured on shoot development medium formed a leaf within a week. There was no apparent correlation between the morphology of the GE and the ability to form either normal or malformed shoots. Shoots were obtained from all genotypes, except Faroka.

For both 2,4-D concentrations, less than 40 percent of the M.Col22 GE developed into shoots on a medium with 0.1 mg.l^{-1} BA. Shoot development was increased by using 0.4 mg.l^{-1} BA, however, the only statistically significant increase was obtained with treatments of 4 mg.l^{-1} 2,4-D (Table 2).

The number of shoots produced per initial leaf lobe was significantly higher in the step one treatments with 4 mg.l^{-1} 2,4-D compared to 1 mg.l^{-1} 2,4-D. In the 4 mg.l^{-1} 2,4-D treatments it was also significantly higher in the step two treatments with 0.4 mg. l^{-1} BA as compared to 0.1 mg.l^{-1} BA. However the two 1 mg.l^{-1} 2,4-D treatments did not vary significantly with respect to number of shoots per initial leaf lobe (Table 2). In the best treatment, 4 mg.l^{-1} 2,4-D, 0.4 mg.l^{-1} BA, on average 10.2 shoots/initial leaf lobe (Table 2) and as many as 31 shoots of a single leaf lobe could be obtained.

The production of shoots from Tjurug GE was also enhanced, but not significantly, by a BA concentration of 0.4 mg.l^{-1} compared to 0.1 mg.l^{-1}. Up to 12 percent of the GE produced shoots. Zeatin (0.1-0.7 mg.l^{-1}) was not effective; not more than 0.1 shoots per initial leaf lobe were formed (Table 3).

PHENOTYPE AND PLOIDY LEVEL OF REGENERATED PLANTS

After one month of culture, 54 percent of the 279 regenerated shoots were phenotypically normal (0.1 mg.l^{-1} BA: 61 percent and 0.4 mg.l^{-1} BA: 43 percent normal). The other 46 percent were malformed (Fig. 2). With prolonged culture most malformed shoots produced normal shaped shoots either at the apex or from an axillary bud. All shoots were branched and did not root on BA-containing medium. This effect disappeared after subculture on BA-free media. The first rooted plants

were transferred to the greenhouse two months after starting the embryogenesis procedure. During the first two weeks they were grown in high humidity and after that they were gradually exposed to normal greenhouse conditions. Ninety percent of the plants survived the transfer. After four months of growth, regenerants

Figure 2. Shoot development of germinating embryos after 30 days step three: upper row malformed shoots and lower row normal shoots.

of M.Col22 and of Tjurug (15 each) formed swollen roots, indicative of tuber formation.

Roots of seven normal and 14 malformed shoots were used for determination of ploidy level. It was obvious that all structures, except one malformed shoot, showed the expected ploidy level (2n=36) [1] since in all cells between 32 and 36 chromosomes were counted. The deviant malformed shoot had one normal (2n=36) and one mixoploid (2n=36 and 2n=54) root.

DISCUSSION

For cassava, 2,4-D has the capacity to induce embryo formation on leaf lobes. The embryogenic process can be improved not only by medium manipulation, but also by manipulating the growth of donor plants. Almost 80 percent of the winter leaf lobes of M.Col22, incubated on 4 $mg.l^{-1}$ 2,4-D formed germinating embryos (Table 2) compared to less than 15 percent of the summer grown leaf lobes (Table 1). Applying exactly the same procedure, Stamp [197] did not obtain GE. These negative results were explained by damaging effects of the sterilization procedure. However, the obstacle could be that the growth conditions for the donor plant were unfavourable for subsequent somatic embryogenesis. Sub-optimal conditions, like low 2,4-D concentration, unfavourable growth of donor plants and too short a duration of the induction step, hampered normal development of GE and enhanced the formation of foliose structures. Although foliose structures resembled the leaf lobes of cassava shoots, they never developed into functional shoots. Stamp and Henshaw [201] suggested that these foliose structures are caused by a partial expression of morphogenetic competence.

Genotypic differences were noted in both the number of developed GE per explant and the percentage of shoot development from GE (57 percent for M.Col 22 and 12 percent for Tjurug). On a medium with 4 $mg.^{-1}$ 2,4-D and 0.4 $mg.l^{-1}$ BA, M.Col22 formed 10.2 shoots per initial leaf lobe.

This is more than ten fold higher than Szabados et al. [205] reported for this variety (1.15 shoots/leaf lobe) on a medium with 0.1 mg.l^{-1} BA with 1 mg.l^{-1} GA. On both media, 60 percent of the GE developed into shoots. It is clear from our results that M.Col22 is superior to the other genotypes tested both for somatic embryogenesis and subsequent ability to develop shoots. Experiments are now ongoing to improve the primary embryogenic response and to use embryogenic cultures for transformation.

CHAPTER 4

CYCLIC SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN CASSAVA

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ABSTRACT. *With a four step procedure, using the cassava clone M.Col22, a cyclic system of somatic embryogenesis was developed. Primary embryos were induced in step 1 (2,4-D dependent) on young leaf lobe explants. Primary embryos were isolated in step two (BA dependent) and were used as starting explant for secondary embryogenesis (steps one and two repeated). In this way, repetitive embryogenic cultures were established.*

It was shown that in all successive cycles embryo explants produced significantly more embryos than leaf lobe explants. According to their shape embryos were classified in two developmental stages; torpedo shaped embryos and germinated embryos.

Germinated embryos produce more than three times as many embryos than torpedo shaped embryos. A third step in the embryogenic process was the development of shoots. The shoot conversion of embryos was increased if benzyl-amino purine (1 mg/l) was added. In that case more than half of the isolated embryos developed into normal shoots. The shoot conversion rate of germinated embryos was higher than that of torpedo shaped embryos. Embryos were kept in culture for 9 months equalling 6 cycles. The production of embryos from the 6^{h} cycle was as efficient as that from the 2nd cycle. The shoot conversion rate of embryos from the 4^{h} cycle was comparable with that from the 2nd cycle. Rooting of the embryo derived shoots (step four of the procedure) occurred on a medium without BA. More than 100 regenerants obtained from 4^{h} cycle embryos were multiplied in vitro. They showed no phenotypical differences as compared to in vitro multiplied parental material.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a tropical crop grown for its thickened tuberized roots in the lowlands of Asia, Africa and South America. It is an important crop for over 500 million people. Cassava roots are used as a rich source of starch for human nutrition, cattle feed and industrial purposes [26].

Problems in the cultivation of cassava are virus diseases, insect pests, low protein content of the roots and the presence of toxic cyanogenic glucosides [26]. The feasibility of overcoming these problems by only classical breeding is hampered because of several problems such as a high degree of heterozygosity, polyploidy, low fertility, poor seed set and low rates of seed germination in certain genotypes [17, 86, 120].

Therefore, emphasis is being placed on the application of genetic manipulation to overcome some of these problems. However, an efficient regeneration and transformation procedure is a prerequisite. Despite some reports on adventitious shoot formation [187, 212], the only good documented regeneration method for cassava is somatic embryogenesis. In general, as starting explants for the induction of embryos young leaves [201, 205, chapter 3], zygotic embryos [196] and somatic embryos [165, 200] can be used. The use of leaf lobes and of zygotic embryos is best described. Earlier experiments showed that the response of leaf lobes depends on the physiological state of the plants grown in a greenhouse [Chapter 3]. Somatic embryo explants give the oppor-

tunity to develop cyclic embryogenic cultures as was described for walnut [217].
In this report a method is described to obtain a continuous proliferating culture of cassava embryos.

MATERIALS & METHODS

PLANT MATERIAL

Cassava (*Manihot esculenta* Crantz) clone M.Col22 was used throughout. Glasshouse grown plant material was brought in-vitro after sterilisation of single bud cuttings for 20 minutes in 1.5 % sodium hypochlorite. The plants were maintained on Basal Medium supplemented with 30 g/l sucrose (BM 30), 0.1 mg/l BA and 0.1 mg/l IBA. BM consisted of Murashige and Skoog [136] mineral salts and vitamins and 0.5 % agar (Oxoid no. 1), pH 5.7.

SOMATIC EMBRYOGENESIS

The culture procedure was subdivided into four steps as described before for leaf lobes of greenhouse grown plants [Chapter 3]. In short (see Fig. 5):
In step one embryos were induced on young leaf lobes (2-6 mm)which germinated in step two. Embryos (torpedo shaped or germinated) were cultured separately in step three for shoot development. Shoots were defined as structures with a distinct stem, cotyledons and two or more leaves. Shoots were classified as normal if the leaves had the phenotype of in vitro grown cassava and as malformed if otherwise. In step four the shoots were rooted.
The day/night temperature in the growth chamber was 28/24 °C and the irradiance 40 $\mu molm^{-2}s^{-1}$. Step one was performed in the dark and steps 2, 3, and 4 in the light (12 hours a day). Explants were incubated in tubes with 10 ml medium. Every three to four weeks, explants were transferred to fresh media.

EXPERIMENTAL PROCEDURES

Primary cycle

Young leaf lobes of 1-6 mm were isolated from in-vitro grown plants and cultured on BM 20 supplemented with 4 or 8 mg/l 2,4-D (step one). After 25 days the explants were transferred to BM 20 supplemented with 0.1 mg/l BA for growth and germination (step two). After three weeks step two embryos of different developmental stages were isolated from the lobes.

Secondary and higher cycles

Torpedo shaped and germinated embryos were cultured for a further cycle as described for the primary cycle.

Shoot development

Torpedo shaped and germinated embryos were isolated from the explants and cultured for shoot development on BM 20 supplemented with 1-8 mg/l BA (step 3). The effect of varying cytokinin concentrations and of the stage of the embryo on shoot development was studied.

RESULTS

INDUCTION OF PRIMARY EMBRYOS

Forty-nine percent of the leaf lobes incubated on 8 mg/l 2,4-D formed embryos compared to 15 percent of the lobes incubated on 4 mg/l 2,4-D (Fig. 1A). On average 4 embryos per responding lobe were isolated for both 2,4-D concentrations (Fig. 1B). After 10 days of culture globular

shaped embryos were visible. Embryos were only formed on the adaxial side of the leaves. Not all the globular shaped embryos passed through the torpedo shaped stage to germination.

Beside embryos, at least two types of callus (creamish-white smooth and brownish friable callus) were formed simultaneously, especially on the part of the explant which was in contact with the medium. Occasionally it seemed that some embryos were developing out of so called smooth callus.

Approximately 20 percent of the cultured lobes formed green foliose structures which with a prolonged step two culture were overgrown by (brownish) callus.

INDUCTION OF SECONDARY AND FURTHER CYCLES EMBRYOS

Foliose structures isolated from leaf lobes and incubated on 4 or 8 mg/l 2,4-D were not embryogenic, they only showed callus formation.

Different types of callus could be isolated from the lobes, which did not regenerate embryos but showed only further callus proliferation.

Only somatic embryos were able to form new embryos. As with primary embryogenesis secondary embryos appeared as soon as after 10 days of culture, mostly on the top part of the cotyledon tissue of the explant. They originated directly from the parental tissue. During further culture more embryos appeared on the lower parts of the cotyledon especially from the meristematic zone. Embryos rarely developed from the hypocotyl of the explant. From 35 isolated hypocotyls only one embryo was isolated whereas all cotyledons formed embryos. The response of separated cotyledon tissue was better than of whole embryos; about 20 percent more embryos could be isolated per explant.

After 15 days step one the first embryos had reached the torpedo shaped stage. In the second step of the process the torpedo shaped embryos developed quickly into germinated embryos (GE). During step two embryos were isolated after 3, 6 and 9 weeks; the production of embryos isolated after 6 and 9 weeks was almost a factor of 9 lower than the production of embryos isolated after 3 weeks. In the results, described in Figs. 1A and B and Table 1, the period between the beginning of step one and the start of a new cycle was 3 weeks. Figure 1A gives the percentage explants (lobes or embryos) from which embryos were isolated; only torpedo shaped and germinated embryos were isolated. Figure 1B gives the number of isolated embryos per responding explant.

The response in the primary cycle was lower than in all succeeding cycles. The response of 6th cycle embryos (after 9 months of culture) was comparable with that of 2nd cycle embryos.

In the primary cycle the 2,4-D concentration clearly affected the percentage of lobes with embryos (Fig. 1A), but the number of isolated embryos per responding explant (Fig. 1B) was the same for both concentrations. In later cycles sometimes 4 and sometimes 8 mg/l 2,4-D gave the highest percentage of lobes with GE. For the number of embryos per responding explant it seemed that 8 mg/l 2,4-D gave some better results than 4 mg/l 2,4-D.

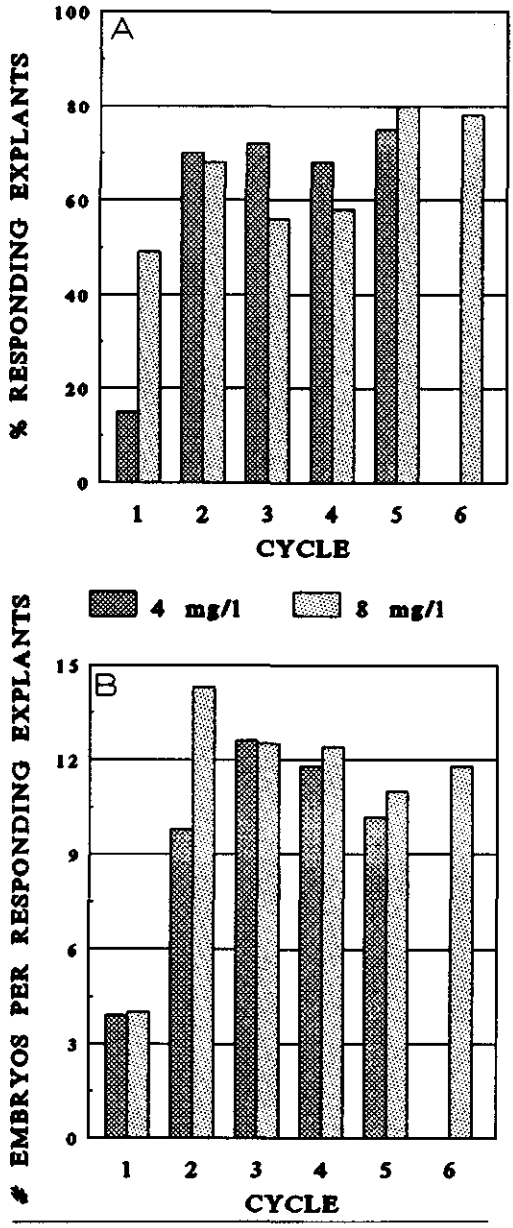

Figure 1. Effect of 2,4-D concentration and cycle on the percentage of explants with embryos (A) and the number of embryos per explant (B) (N= 24-200 torpedo shaped and germinated embryos per measurement).

However, only for the secondary cycle the difference between the two 2,4-D concentrations was significant.

Combining the results of Fig. 1A and 1B gives the average production of embryos per initial explant. In the primary cycle the average production was between 0.5 and 2.0 for respectively 4 and 8 mg/l 2,4-D. In the higher cycles the average production varied between 6.8 (2nd cycle, 4 mg/l 2,4-D) and 9.9 (2nd cycle, 8 mg/l 2,4-D).

The correlation between the numbers of embryos produced from explants of the same lineage, comparing the 3rd with the 4th cycle and 4th with the 5th cycle, was low (respectively $r=0.22$ and $r=0.41$) and not significant. So the isolated embryos of a high yielding explant do not give a better production in the following cycle than the embryos of a low yielding explant.

INFLUENCE OF THE DEVELOPMENTAL STAGE OF THE EMBRYO ON INDUCTION OF EMBRYOS

Embryos can be divided, according to their shape and colour into two different stages (Fig. 2).

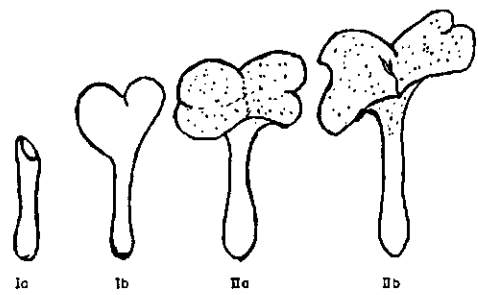

Figure 2. Developmental stages of embryos: Ia and Ib=young and mature torpedo shaped embryos, IIa and IIb=young and mature germinated embryos.

Stage I embryos are torpedo shaped embryos, with a distinct hypocotyl, translucent and less than 2 mm long. The stage I embryos can be subdivided, rather arbitrarily, according to the appearance of visible cotyledon primordia (Ia without and Ib with). Stage II embryos are germinated with distinct cotyledons and with a length of 4-15 mm. Also these embryos can be divided into two subgroups: IIa light green cotyledons and IIb green cotyledon (mature). Some of the stage IIb embryos are developing a primary leaf. Both the percentage explants with embryos and the number of embryos per responding explant are dependent on the developmental stage of the embryo used for the explant. Just over 50 percent of the torpedo shaped embryos (stage Ia and Ib) used as explants initiated embryos (Table 1). Stage Ib embryos (with visible cotyledon primordia) formed more than twice as many embryos as stage Ia (embryos without primordia).

About 75 percent and 87 percent of the germinated embryos of stage IIa and IIb respectively, formed new embryos. Also the number of embryos per responding stage II embryos was higher than that of stage I embryos. It is furthermore obvious from these results that, relatively, a higher number of embryos per responding explant could be obtained from the more developmentally advanced stage II embryos.

Embryos further developed than stage IIb possess already (primary) leaves. They respond as leaves of in vitro grown plants and also leaf lobes of first cycle regenerants behaved like leaf lobes of original parental plants (cf. Fig. 1) showing no improved embryogenic response (results not shown).

Table 1. Effect of the developmental stage of embryos (5th cycle) on the formation of embryos in the 6th cycle (33-67 explants per treatment).

	% responding explants	# GE/responding explant	# GE/initial explant
Ia	54	3.6	1.9
Ib	53	7.6	4.0
IIa	75	10.1	7.5
IIb	87	15.0	13.1

EFFECT OF BA CONCENTRATION ON SHOOT DEVELOPMENT

On a BA free medium one third of the embryos developed into shoots. All shoots were malformed (Table 2). BA enhanced shoot conversion rate. The optimal BA concentration was around 1 mg/l, but even at concentrations as relatively high as 8 mg/l shoots could still be obtained at a frequency comparable with that on 0 mg/l BA. A large percentage of these shoots was normal (see M. & M. for explanation) in contrast to the development of malformed shoots on a medium without BA. A step three medium with 1 mg/l IAA had an effect comparable with 0.1 mg/l BA (results not shown).

After three months of shoot culture normal looking shoots developed out of these malformed structures. This process could be accelerated and enhanced by making cuttings of the malformed structures. By culturing these cuttings 45 percent of the malformed shoots developed within two months into normal looking shoots (data not shown).

BA concentrations above 0.5 mg/l stimulated the formation of green nodular struc-

tures on the cotyledon part of the explant. Sometimes leaf like structures developed out of these nodular structures, but shoot development was never observed.

Table 2. Effect of the BA concentration on the percentage shoot development of 3rd cycle embryos after 90 days of culture.

BA mg.l^{-1}	0	0.1	0.5
number of embryos	24	24	24
percent shoots	33	46	71
├—> malformed	100	28	54
└—> normal	0	72	46
BA mg.l^{-1}	1.0	2.0	8.0
number of embryos	24	24	24
percent shoots	76	50	34
├—> malformed	50	66	38
└—> normal	50	38	62

Higher BA concentrations also stimulated the formation of multiple shoots. Sometimes more than 15 shoots developed from one germinated embryo.

EFFECT OF CYCLE DEVELOPMENTAL STAGE OF EMBRYO ON SHOOTING

Shoots were regenerated from 1st to 4th cycle embryos originating of the 4 mg/l and the 8 mg/l 2,4-D treatment.

Between 54 and 76 percent of the isolated embryos developed into either normal or malformed shoots (Fig. 3). Between 33 and 55 percent of these shoots were normal. Because 2nd and 4th cycle embryos were cultured for 60 days their response was lower than of 1st and 3rd cycle embryos which were cultured for 90 days. Combination of the results of Figs. 1A, 1B and 3 gives the production of normal and malformed shoots per initial explant.

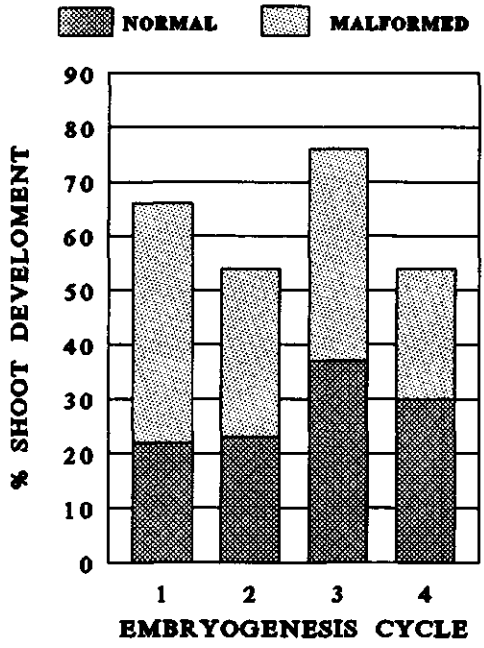

Figure 3. Effect of cycle on shoot development of embryos cultured on BM with 1 mg/l BA after 60 (cycle 2 and 4) or 90 days (cycle 1 and 3) (N = 24-48 per cycle).

In the primary cycle on average between 0.4 (4 mg/l 2,4-D) and 1.3 (8 mg/l 2,4-D) shoots per initial lobe were obtained and in the higher cycles it varied on average between 3.6 (2nd cycle, 4 mg/l 2,4-D) and 6.9 shoots per initial embryo (2nd cycle, 8 mg/l 2,4-D).

Embryos of different stages (Fig. 2) from the fourth cycle were incubated on 1 mg/l BA to study the effect of the developmental stage on the subsequent shoot development (Fig. 4).

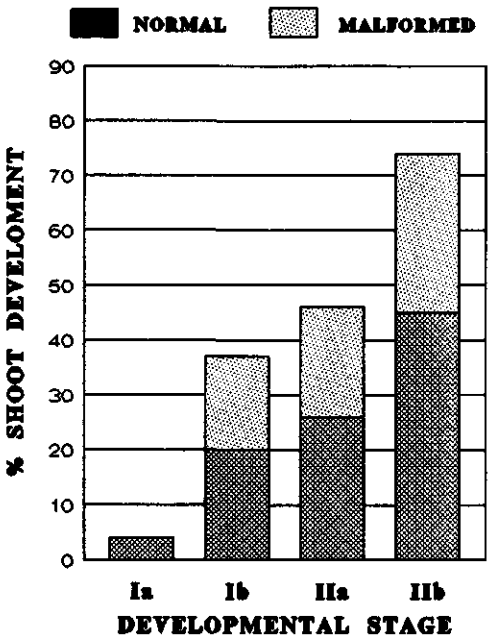

Figure 4. Effect of the developmental stage on shoot development of 4th cycle embryos cultured on BM with 1 mg/l BA for 60 days (N = 24-37 per developmental stage).

Only 4 percent of the stage Ia embryos were able to develop into shoots. Most stage Ia embryos showed no reaction on the step three medium or developed grey-white callus. No nodular structures were seen in this callus. The best shoot development, 74 percent, was obtained with stage IIb germinated embryos; about 60 percent of these shoots were normal. With prolonged culture more of the remaining malformed shoots will revert to normal shoots. Only stage IIa and b embryos formed green nodular structures and the appearance of multiple shoots.

Over one hundred regenerants from the 4th cycle were multiplied in vitro. They all formed easily roots on a medium without BA and single node cuttings could be propagated. They showed no phenotypical differences as compared to normally in vitro propagated plants.

DISCUSSION

The primary somatic embryos used for starting repetitive cultures can be induced on different types of tissue. However, because of the strong heterozygosity and the difficulty to obtain seed from certain genotypes, clonal tissue is desirable for genetic engineering. Therefore only leaf lobes were used to obtain primary somatic embryos. Unfortunately the embryogenic response showed a large variation and was highly dependent on the physiological state of the plants delivering the lobes. Only 15 percent of the lobes of greenhouse plants grown in the summer and incubated on 4 mg/l 2,4-D formed embryos, whereas 80 percent of the lobes isolated from plants grown in the winter formed on average 25 embryos per lobe, so that a production of over 20 embryos per initial lobe was obtained (Chapter 3). Lobes of in vitro grown plants showed less variation, but the production (step one: 4 mg/l 2,4-D) of embryos was a factor 40 lower than the above mentioned "winter" lobes (Figs. 1A and 1B). Despite many efforts it is still not known which factors might be responsible for this difference. This lack of knowledge made it difficult to screen large numbers of clones for embryogenesis.

Embryo explants, however, combine a high response and repeatability, although the

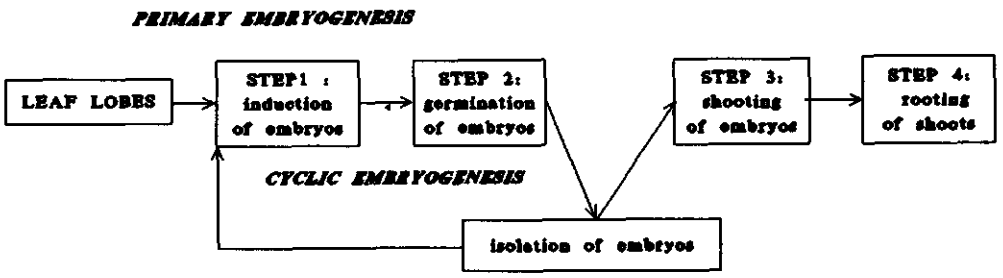

Figure 5. Schematic representation of primary and cyclic somatic embryogenesis in cassava.

production per initial lobe varied between 6.8 and 9.9 which is still more than a factor of two lower than the above mentioned "winter" lobes. The culture of somatic embryos therefore is an easy method to obtain large numbers of somatic embryos of cassava. The elegance of the system is the cyclic development of embryos from embryos (Fig. 5).

Although only M.Col22 was used extensively, preliminary results with the Nigerian clones TMS90853 and TMS4(2)1244 (results not shown) suggested the general application of the described method. The developmental stage of the starting embryo determined the number of new embryos initiated. The two younger stages, characterized by the absence of green cotyledons, were less embryogenic than the two older stages characterized by the presence of green cotyledons. This was not surprising as generally only the cotyledon part of the embryo was capable of initiating new embryos. The embryogenic competence of embryos which had passed the mature germinated stage was highly reduced. The response of leaf lobes of shoots developed out of primary embryos is comparable with that of parental plants. Obviously there had been no selection pressure on physiological or genetical changes towards high embryogenic cells.

The developmental stage of the embryos also influenced the rate of shoot conversion. Again the most mature embryos were optimal for shoot conversion. Also with sweet potato the most mature stage had the best shoot conversion rate. In sweet potato, it turned out that it was better to select for developmental stages than for length of the embryo with regards to shoot conversion [181].

Although BA is not necessary for shoot development it significantly enhanced the shoot conversion rate, irrespective of the embryogenic cycle. The shoot conversion rate of embryos from different cycles was comparable with that of primary embryos.

The repetitive embryogenic culture system is competitive with current large scale propagation methods, but the slow shoot conversion and the stunted morphology of the embryos and derived shoots reduces its application in this field. However, the shoot recovery is high enough to use the repetitive embryogenesis as a method to

recover genetically engineered plants. Another advantage of the system is the possibility to obtain complete transgenic embryos of only partly transformed embryos by prolonged embryogenic culture as was described for walnut [125].

CHAPTER 5

IMPROVEMENTS OF CYCLIC SOMATIC EMBRYOGENESIS IN CASSAVA

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Abstract. *In cassava a cyclic system of somatic embryogenesis was developed. Primary (torpedo shaped or germinated) embryos, originating from leaf lobes, could only be obtained after culture on solid medium. Cyclic embryos, originating from embryos, could be obtained in both liquid and on solid medium. The production of embryos in liquid medium was distinctly higher, faster and more synchronized than on solid medium. Lower densities and fragmentation of starting embryos improved the production significantly. The highest production found was 32.1 embryos per initial embryo. In all treatments the explants initiated multiple embryos. The production of single embryos was achieved by pressing starting embryos through a fine meshed sieve, indicating that embryos can be produced from a piece of tissue with a restricted number of cells. The shoot conversion rate of embryos from liquid medium was comparable with that of embryos from solid medium.*

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) also known as yucca, tapioca and manioc is a perennial shrub of the Euphorbiaceae family, native to Brazil. After rice, maize and sugarcane it is the fourth most important source of energy in the developing countries [26]. Especially in Africa cassava is a staple crop of immense importance. Despite the importance for the tropics it did not get much attention of agricultural research centres.

Problems in the cultivation of cassava are virus diseases, insect pests, low protein content of the roots and the presence of toxic cyanogenic glucosides [26]. The feasibility of overcoming these problems by only classical breeding is hampered because of several problems such as a high degree of heterozygosity, low fertility, poor seed set and low rates of seed germination [17, 86, 120]. Therefore, emphasis is being placed on the application of genetic modification to overcome some of these problems. To be able to use this an efficient regeneration and transformation procedure is a prerequisite. The only routine way of regeneration in cassava is by somatic embryogenesis since direct or indirect adventitious shoot formation is seldomly observed [187, 212]. In a number of articles the possibility of somatic embryogenesis of cassava was demonstrated [165, 200, 201, 205]. The common procedure is induction of primary somatic embryos on leaf lobes or on seed derived cotyledons on 2,4-D containing medium. Somatic embryos can be used as starting explants for a new cycle. In a previous report a method of cyclic embryogenesis on solidified medium was described [Chapter 4]. To ensure that such a regeneration system becomes more universal and reproducible, the process should be optimalized. Different factors such as embryo density, fragmentation of embryos and type of medium were varied to study their influence on somatic embryo production for three successive cycles.

MATERIALS & METHODS

PLANT MATERIAL

The Columbian cassava (*Manihot esculenta* Crantz) clone M.Col22 was maintained over 10 years in the greenhouse and brought in vitro two years ago. Plants were maintained on solidified (7 g/l Daichin agar) Basal Medium (BM). BM consisted of Murashige and Skoog [136] salts and vitamins and 20 g/l saccharose. The temperature in the growth chamber was 30 °C and the irradiance 40 $\mu molm^{-2}s^{-1}$. All steps were performed in the light (12 hours a day). Explants were placed in 300 ml flasks (liquid culture) or Petri dishes (solid culture). The flasks were put on an orbital shaker (LAB-line instruments inc., model 3519) at 120 rpm.

PRIMARY EMBRYOGENESIS

Forty-eight leaf lobes of 1-6 mm, isolated of in vitro grown plants, were cultured for induction of embryos either in liquid (8 leaf lobes per 50 ml) or on solid step 1 medium (BM with 4 mg/l 2,4-D). The liquid medium was refreshed after 10 days. After 20 days the explants were transferred to step 2 medium (BM with 0.1 mg/l BA) to allow germination of embryos. Since not enough developed primary embryos could be isolated from leaf lobes, for starting the different comparisons, the primary embryos were multiplied by cyclic embryogenesis on solid medium [Chapter 4].

CYCLIC EMBRYOGENESIS

The experiment was set up as a complete randomized design. Every treatment consisted of 4 replications (per replication 10 embryos). The factor, degree of fragmentation, had two levels. Fragmentation was applied by cutting the embryos with a scalpel into small pieces (4-25 mm^2) versus intact embryos. The density-factor had three levels; 1, 2.5 and 5 ml of medium volume per initial embryo (MIE). A solid control was added which was exposed to fragmentation. The effect of the different treatments on the production of embryos was studied for three successive cycles.
The liquid step 1 medium (BM with 4 mg/l 2,4-D) was refreshed after 10 days step 1. After 20 days step 1 the content of each flask was divided in two equal parts. One part, cultured in liquid step 2 medium (BM with 0.1 mg/l BA), was used to start a new cycle after 7 days and the other part, cultured on solid step 2 medium, was used to determine the production of embryos after 21 days. A schematic presentation of the flow of material is given in Figure 1.

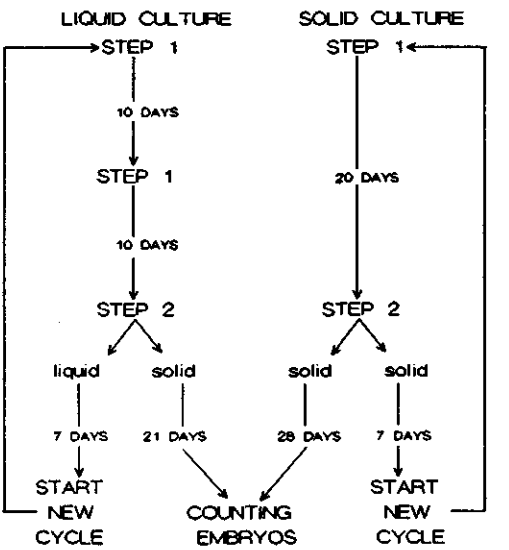

Figure 1. Schematic representation of the culture flow during the experiments on cyclic embryogenesis.

After 20 days step 1, without refreshing the medium, the content of each Petri-dish was divided in two equal parts and cultured on solid step 2 medium. One part was used to start a new cycle after 7 days and the other part to determine the production of embryos after 28 days. The production was measured as number of newly formed embryos per initial embryo (E/IE). Only stage I and II embryos were counted [Chapter 4]. Stage I embryos are torpedo shaped, translucent and possess a distinct hypocotyl. Torpedo shaped embryos develop into germinated embryos (stage II) which are characterized by the appearance of green cotyledon tissue.

In the second and third cycle the experiment was extended with a third fragmentation treatment. Embryos were pressed through a fine meshed sieve (0.5 or 0.1 mm^2). A statistical analysis was performed using the Manova option of Statistical Package for the Social Sciences (SPSS). Statistically significant mean differences were determined with the LSD test.

SHOOT DEVELOPMENT OF EMBRYOS

Stage II embryos of all treatments of the second and third cycle were cultured on solid BM with 1 mg/l BA (step 3) for shoot development of embryos. In step 4 shoots were rooted on BM without growth regulators.

RESULTS

INFLUENCE OF MEDIUM TYPE ON PRIMARY EMBRYOGENESIS

Leaf lobes of 1-6 mm, cultured in liquid step 1 medium, formed no embryos or embryo-like structures, only callus proliferation occurred. On solid medium almost all leaves formed globular shaped embryos but only 30 percent of the lobes developed torpedo shaped or germinated embryos. The production was 0.78 embryos per initial leaf lobe.

INFLUENCE OF CYCLE ON CYCLIC EMBRYOGENESIS

The production of somatic embryos was significantly different ($p = 1\%$) between the three cycles tested (Table 1). Averaged over all treatments, the production in the first cycle was 7.5 embryos per initial embryo (E/IE). In the second and third cycle it was more than doubled to respectively 18.6 and 20.8 E/IE.

The production of embryos in all liquid treatments of the first cycle (Fig. 2A), except fragmented embryos in 2.5 MIE, was significantly lower ($p = 10\%$) than the corresponding treatments of the second (Fig. 2B) and third cycle (Fig. 2C). The production of fragmented embryos in 2.5 MIE differed significantly ($p = 10\%$) from only the corresponding treatment of the third and not the second cycle. The two solid treatments of the first cycle had no significantly different production compared to the second cycle, and compared to the third cycle only the production of intact embryos was significantly ($p = 10\%$) lower. This differential response of liquid and solid cultured embryos between the cycles was responsible for the significant interaction (Table 1; $p = 10\%$) between cycle and medium type.

The production of all second cycle treatments, except intact embryos on solid

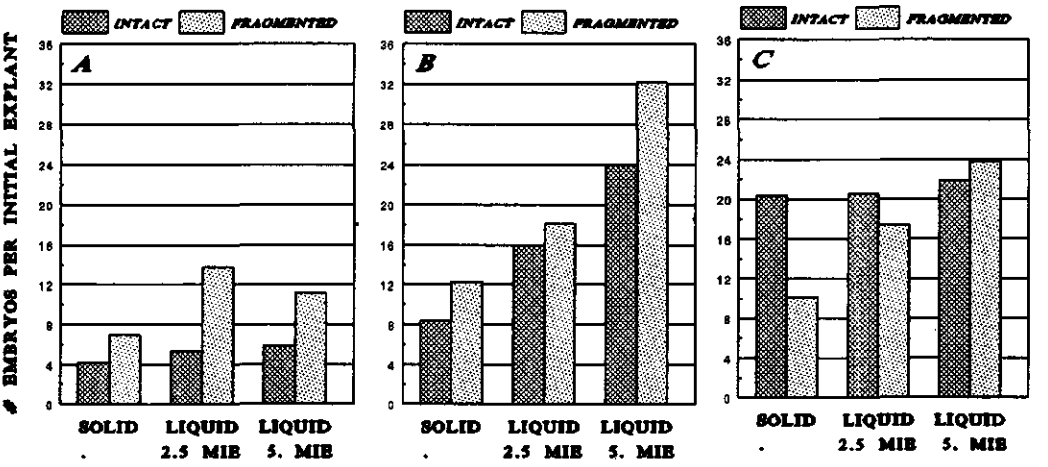

Figure 2. Influence of fragmentation of initial embryos, medium type and medium volume per initial embryo on the production of embryos in the first (A), second (B) and third (C) cycle (means with the same letter are not significantly different by LSD at 10% level).

medium, was not significantly different from the corresponding third cycle treatments.

INFLUENCE OF MEDIUM TYPE ON CYCLIC EMBRYOGENESIS

After 3-5 days step 1 culture of explants turned from green to creamish-white and started to form callus. After 10 days of culture on solid medium most of the explants were covered with callus, whereas in liquid medium only minor amounts of callus were present on the explants, but instead the flasks were filled with a cell suspension. The first torpedo shaped embryos were visible after 10 to 15 days. Almost all torpedo shaped embryos developed cotyledon primordia. The stage I embryos on solid medium had a distinct hypocotyl whereas those in liquid medium had not. The first stage II embryos (germinated embryos) appeared at the end of step 1 especially in the liquid medium, but the majority of the embryos germinated after transfer to step 2. Most of the torpedo shaped embryos developed into germinated embryos. The development of embryos in liquid medium was faster and more synchronized than on solid medium. Another advantage was the ease in which embryos could be isolated of liquid cultured explants compared to solid cultured explants. Averaged over all cycles the production in liquid medium was significantly higher (Table 1; $p = 10\%$) than on solid medium, respectively 18.1 versus 10.7 E/IE.

INFLUENCE OF MEDIUM VOLUME ON CYCLIC EMBRYOGENESIS

In the first and second cycle all flasks with 1.0 ml medium per initial embryo (1 MIE) did not produce embryos. In the third cycle

the 1 MIE treatment was adjusted by using 20 ml, instead of 10 ml, and the double amount of embryos, so the density level remained unaltered. The production in the adjusted 1 MIE of intact embryos and fragmented embryos was respectively 15.7 and 12.5 E/IE which was significantly lower than the production in the corresponding 2.5 and 5 MIE treatments (Fig. 2C). Averaged over all three cycles 5 MIE produced significantly more embryos (Table 1; p = 1%) than 2.5 MIE, respectively 23.7 and 17.2 E/IE. But analysis of variance of cycles separately showed that only in the second cycle 5 MIE produced significantly more embryos.

Table 1. Multivariate analysis of variance of fragmentation, medium type and medium volume per initial embryo on the production of embryos in the 1st, to 3rd cycle.

Cycle	1	2	3	1-3
Fragmentation (F)	#	#	ns	#
Medium type (M)	ns	##	#	#
medium volume (MIE)	ns	##	ns	##
Cycle (C)	-	-	-	##
F * M	ns	ns	#	#
F * MIE	ns	ns	ns	ns
F * C	-	-	-	ns
C * MIE	-	-	-	#
M * C	-	-	-	#

#, ##:significant at respectively 10 % and 1 %
ns:not significant

This explained the significance (Table 1; p = 10%) of the interaction between cycle and medium per initial embryo.

INFLUENCE OF FRAGMENTATION ON CYCLIC EMBRYOGENESIS

Averaged over all cycles fragmentation enhanced the production significantly (Table 1; p = 10%) from 14.1 to 17.5 E/IE. In only one of the nine combinations presented in Figs. 2A, B and C intact embryos produced more than fragmented embryos (third cycle, solid medium, Fig. 2C).

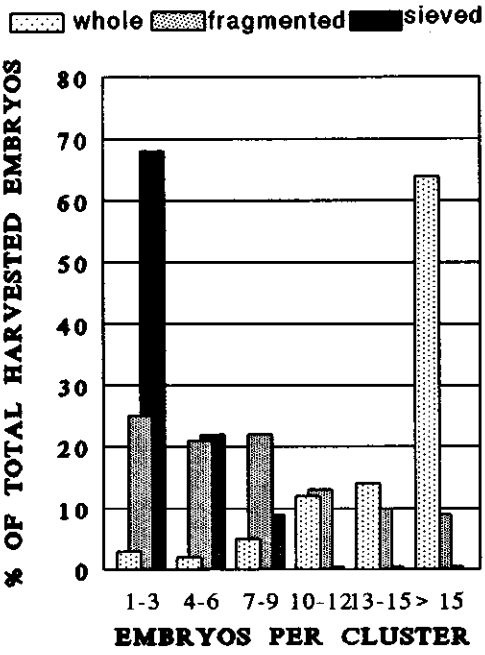

Figure 3. Influence of different degrees of damaging on the formation of clustered embryos.

In this treatment the production of embryos was inexplicable high whereas that of fragmented ones was comparable to those obtained in the second cycle. The unusual high production of intact embryos was responsible for the significance (Table 1; p = 10%) of the interaction between fragmentation and medium type. in the second and third cycle embryos were pressed through a fine meshed sieve (0.5 mm^2) and cultured in liquid medium. A

piece of tissue consisted of approximately 200 cells. In the second cycle the production of these sieved embryos (14.2 E/IE) was lower than in all liquid, but higher than in all solid treatments (Fig 2B). In the third cycle the production of sieved embryos, 3.4 E/IE, was lower than in all other treatments. Even embryos fragmented through a sieve with a diameter of 0.1 mm^2 gave new embryos, but at a very low frequency (0.1 E/IE). The morphology of the embryos produced by the sieved material was different from that of the embryos produced by fragmented and intact embryos. The hypocotyl was taller and larger than normal, the cotyledons were smaller and the embryo was of a stronger consistency. Figure 3 gives the distribution of clustered embryos among the different damaging treatments. Almost 70 percent of the total amount of embryos out of the sieved (0.5 mm^2) material were produced as single, paired or triple embryos. The clusters of embryos on intact embryos had the widest distribution. It varied from 1 to 44 embryos per cluster. The fragmented embryos had the second widest distribution which varied from 1 to 18.

SHOOT DEVELOPMENT OF EMBRYOS

Embryos of the developmental stage II, from second and third cycle, were cultured for shoot development in step 3 medium. Shoots were characterized by the presence of a distinct stem with, besides the cotyledons, at least one leaf. Shoots were classified as normal if the leaves had the normal cassava phenotype (lanceolated shaped lobes) and as malformed if otherwise. With prolonged culture most of the deformed shoots reverted to normal. In Table 2 the results are summarized according to the type of culture medium used for the production of embryos.

There seemed to be a positive effect of using liquid medium during step 1 and 2 on the shoot conversion rate (Table 2), however not statistically significant.

Table 2. Influence of medium type on the development of shoots of second and third cycle embryos.

Step 1 Step 2	liquid solid	liquid liquid	solid solid
number of embryos	47	60	48
percentage shoots	57	60	50
├——>normal	85	80	83
└——>malformed	15	20	17

In all combinations more than 80 percent of the obtained shoots had the normal cassava in vitro phenotype. Not only of all medium types, but also of all damaging treatments, including sieving, shoots were obtained.

About 100 normal regenerants, originating from liquid cycles were examined for their growth behaviour in vitro. They showed no phenotypical differences with in vitro multiplied plants. Fifty of the 100 regenerants were multiplied in vitro. During multiplication one regenerant, appeared to have a chimeric nature. Two of the four cuttings of the embryo derived shoot produced normal shoots and the two other cuttings shoots with variegated leaves. During prol-

onged multiplications most of these cuttings expressed the variegated phenotype. Some reverted to normal green and others to albino shoots. All shoots even the albino ones formed roots on a medium without growth regulators.

DISCUSSION

Szabados et al. [205] and Stamp and Henshaw [200] mentioned the possibility, of somatic embryogenesis of cassava in liquid medium without showing data. Here we present results showing that in liquid medium, even after three successive cycles, more embryos were produced than on solid medium. Solid medium has some disadvantages compared to liquid medium. On solid medium the explants only make basal contact with the medium, the nutrient and growth regulator uptake is limited and gradients are formed. This promotes variation and reduced growth which is less obvious in liquid medium. The more homogeneous conditions of liquid medium might also explain the better synchronization of development of embryos in liquid medium. An important advantage of liquid medium, specific for the embryogenic culture of cassava, where the formation of callus suppresses the development of embryos [Chapters 3 and 4], is the mechanical removal of callus developed on the explant due to shaking.

Although not significantly better in all cycles, a density of 1 embryo per 5 ml of medium (5 MIE) gave a higher production than a density of 1 embryo per 2.5 ml medium (2.5 MIE). But irrespective of the density, also the amount of medium used, influenced the production. In the 1 MIE treatment with 10 ml medium no embryos were formed whereas in the adjusted 1 MIE treatment (20 embryos in 20 ml) embryo production was established, but lower than at 2.5 or 5.0 MIE. In 10 ml medium the explants were not completely covered with medium during shaking which might be negative for development.

In eight of the ten combinations tested fragmentation of starting embryos enhanced the production compared to intact embryos. Also in soybean fragmentation of starting explants enhanced the production of embryos [105]. It is likely that on intact embryos, newly induced embryos have to compete for space. By fragmentation of embryos more surface area is created so less newly induced embryos will be suppressed. More surface area also enhances the contact between explants and medium. The embryo production out of sieved material was not as abundant compared to fragmented or intact embryos, probably because of excessive damaging of cells. However, sieving provides a relatively large amount of single embryos which might be of importance in specific areas such as cryopreservation, synthetic seed development or automated plant tissue culture.

It was previously shown [Chapter 4] that the production of new embryos depended on the developmental stage of the initial embryos. The production was also influenced by the time period between the transfer of explants to step 2 medium and the start of a new cycle. This is the reason for the lower production of embryos in the first cycle (Fig. 2A), because this cycle

was started after 21 days of step 2 with a production of 8.0 E/IE. In the second and third cycle, started after 7 days of step 2, the production was more than doubled.
The shoot conversion rate of embryos originating from liquid medium was comparable with those from solid medium. They both were higher as those described previously for the first to fifth cycle on solid medium [Chapter 4].

In this report a method is described to obtain routinely embryos of liquid cultures. This type of culture has many advantages compared to a solid culture. The production is higher, the procedure is less time consuming and the development is faster and more synchronized thus providing ideal starting material for transformation experiments.

CHAPTER 6

A GENERAL METHOD TO OBTAIN HIGH YIELDING EMBRYOGENIC CULTURES IN CASSAVA

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SUBMITTED FOR PUBLICATION TO EUPHYTICA

ABSTRACT. *Depending on the time of the year, between 0 and 80 per cent of the leaf explants from greenhouse grown M.Col22 plants formed germinated embryos after culture on induction medium supplemented with 4 mg/l 2,4-D. The highest production found was 22.1 germinated embryos per initial leaf explant (GE/IE). The response of in vitro derived leaf explants showed less variation; 5 to 22 per cent of the leaf explants formed germinated embryos. But the number of GE/IE was almost 30 times lower than the highest production obtained with greenhouse derived leaf explants. The production from in vitro derived leaf explants was increased to a maximum of 3.2 GE/IE by using 8 mg/l 2,4-D. Picloram and Dicamba proved to induce embryos, but not better than 2,4-D whereas NAA did not. The production of germinated embryos was further enhanced (6.6 Ge/IE) by growing in vitro donor plants at low irradiance, whereas growth at different day lengths had no influence. The highest production of germinated embryos was obtained when the donor plants were grown in liquid medium and pretreated with 2,4-D two days before harvest of leaf explants (9.4 GE/IE). In vitro grown Nigerian clones which were non-embryogenic in the standard procedure, became so after a pretreatment of the donor plants. Although the production of primary germinated embryos was extremely low for some clones (< 0.1 GE/IE), high yielding embryogenic cultures could be obtained by cyclic culturing of somatic embryos (>5 GE/IE). The embryogenic capacity of several Nigerian clones was maintained for more than one year by regular subculture of the germinated embryos.*

INTRODUCTION

Genetic modification of crop plants is dependent on the availability of efficient regeneration and transformation procedures. The prerequisite of a regeneration system should be such that it is applicable to a wide range of genotypes. Most systems make use of shoot organogenesis from callus induced on stem or leaf explants (see for recent reviews [67, 232]. Only in two reports [187, 212] successful shoot organogenesis from callus in cassava (*Manihot esculenta* Crantz) has been described. These results could not be repeated by others. Thus, shoot formation, the most common route of plant regeneration, is not yet available in cassava. Another way of plant regeneration is somatic embryogenesis which has been described for cassava by several investigators [196, 200, 205]. Recently a cyclic system was developed in which repetitively embryos were formed on somatic embryos [Chapters 4 and 5]. Nevertheless, it is still difficult to obtain sufficiently large numbers of primary embryos for many genotypes. Primary embryos can be induced on leaf explants from in vitro [205] or greenhouse grown plants [201, Chapter 3] and on zygotic embryos [196]. Because of the vegetative propagation of cassava, leaf lobes of existing varieties are preferred as source material for genetic engineering. In this report several aspects of primary somatic embryogenesis, using in vitro derived leaf explants, leading to an improvement of the general applicability are described.

MATERIALS & METHODS

SOMATIC EMBRYOGENESIS IN CASSAVA

In a two step procedure leaf explants were cultured for primary somatic embryogenesis and germinated embryos (GE) were cultured as explants for cyclic embryogenesis. In step 1, explants were cultured for the induction of somatic embryos on basal medium (BM) supplemented with different auxins (see "Experimental procedures"). BM consisted of Murashige and Skoog [136] salts and vitamins and 20 g/l sucrose and 7 g/l Daichin agar. After 20 days step 1, explants were transferred to step 2 medium (BM supplemented with 0.1 mg/l BA) to allow further development and germination of embryos. After 3 weeks step 2, the medium was refreshed. Germinated embryos were counted after 3 and 6 weeks step 2.

In all steps the explants were cultured in light and in tubes with 10 ml medium.

The standard growth condition in the culture room was: a temperature of 30 °C, a day length of 12 hours and an irradiance of 40 $\mu molm^{-2}s^{-1}$. Deviations from the standard conditions are mentioned below.

EXPERIMENTAL PROCEDURES

Primary embryogenesis from leaf explants of greenhouse grown M.Col22 plants.

The Columbian cassava clone M.Col22 (CIAT, Columbia) was maintained over 10 years in the greenhouse. Shoot tips were sterilized as described earlier [Chapter 3]. Leaves of 1 to 10 mm were isolated from the shoot tip and subdivided into leaf lobes. Single leaf lobes were used as explants for primary embryogenesis. On average, more than 10 leaf explants were obtained from one shoot tip. Leaf explants were cultured for the induction of primary embryogenesis on BM with 4 mg/l 2,4-D (step 1).

Primary embryogenesis from leaf explants of in vitro grown M.Col22 plants.

Nodes with one bud, isolated from greenhouse grown M.Col22 plants, were sterilized in a 1.5 % sodium hypochlorite and 0.01 % Triton X-100 solution for 20 min, washed in sterile water and brought in vitro. The plants were maintained on solid BM medium. The youngest, unfolded leaves (0.5-5.0 mm) were isolated from the shoot tips after a growth period of 7 to 300 days. Because leaves from in vitro plants are smaller and more vulnerable than leaves from greenhouse grown plants and to avoid excessive damage, not all the leaves were subdivided into individual lobes. About 30 to 50 percent of the cultured leaf explants were individual lobes, the others were complete leaves. These two different types of explants were divided equally over the treatments of one experiment. On average, 1.7 leaf explants were isolated from one in vitro shoot tip. Leaf explants were cultured for primary embryogenesis on BM supplemented with 4 or 8 mg/l 2,4-D (step 1).

Primary embryogenesis from leaf explants of M.Col22 plants grown in vitro at different light regimes.

Plants were grown on solid BM at day lengths of 8, 12 16 or 24 hours (irradiance 40 $\mu molm^{-2}s^{-1}$). Furthermore, to study the effect of irradiance, plants were grown at 40, 28 and 8 $\mu molm^{-2}s^{-1}$ (12 hours day

length). The standard irradiance of 40 $\mu molm^{-2}s^{-1}$ was reduced by covering the tubes with dark foil. Leaf explants were isolated after 14 days of growth and cultured for primary embryogenesis on BM with 8 mg/l 2,4-D (step 1).

Leaf explants which had been cultured for 17 days on step 1 medium, were morphologically investigated by scanning electron microscopy (SEM). For this explants were fixed in aqueous FAA solution, postfixed in saturated $KMnO_4$ for 1 min, rinsed thoroughly and dehydratated through an ethanol series. Explants were prepared for scanning electron microscopy by critical point drying in CO_2 and coated with gold/palladium in a sputter coater. The explants were examined and photographed in a Jeol-JSM-5200 scanning electron microscope (SEM) at 15 kV.

Primary embryogenesis from leaf explants in vitro M.Col22 plants pretreated with 2,4-D.

To study the influence of a 2,4-D pretreatment on primary embryogenesis, 2,4-D was added (final concentration 8 mg/l 2,4-D) after 12 days of growth to liquid and agar grown donor plants. In liquid medium plants were kept in rockwool plugs. Control plants were pretreated with water with the same pH as the 2,4-D solution. Two days later the leaf explants were isolated from the donor plants and cultured for primary embryogenesis on BM with 8 mg/l 2,4-D.

Influence of different auxins and $CuSo_4$ on primary and cyclic embryogenesis of M.Col22

To study the effect of different auxins on somatic embryogenesis, leaf (in vitro) and GE explants were cultured for respectively primary and cyclic embryogenesis on BM with 4 or 8 mg/l Dicamba, Picloram NAA or 2,4-D (step 1). Mathews et al. [122] have reported that the additions of 2 μm $CuSO_4$ to the step 1 medium improved somatic embryogenesis. Therefore, half of the material was cultured on medium with 2 μm $CuSO_4$ and the other half without.

Somatic embryogenesis of Nigerian clones

The clones TMS30555, TMS 4(2)1244, TMS50395, TMS60506, TMS90059, TMS 30211, TMS 60444, TMS30395, TMS 90853, TMS30001 and TMS30572 (IITA, Nigeria) were grown on solid BM medium at an irradiance of 40 or 8 $\mu molm^{-2}s^{-1}$ and in liquid medium at 40 $\mu molm^{-2}s^{-1}$. Liquid grown plants were pretreated with 2,4D after 12 days of growth. Leaf explants were isolated from solid and liquid grown plants after 14 days of growth and cultured on BM with 8 mg/l 2,4-D.

Primary embryos were cultured for secondary embryogenesis on BM with 8 mg/l 2,4-D. Proliferating somatic embryos of several clones were maintained by subculture of somatic embryos (cyclic somatic embryogenesis). Germinated embryos of several clones were cultured for shoot development on BM with 1 mg/l BA or on BM with 4 mg/l NAA and 2 μM $CuSO_4$.

STATISTICAL ANALYSIS

Significant differences in the percentage explants with GE were determined with the chi-square test ($p=0.1$) and in the number of GE per responding explant and per initial explant with the LSD test.

RESULTS

PRIMARY EMBRYOGENESIS FROM LEAF EXPLANTS OF GREENHOUSE GROWN M.COL22 PLANTS

The results of 11 experiments are summarized in Fig. 1. In all experiments callus began to develop on the leaf explant after 3 days of culture on step 1 medium and 7 days later embryos were visible as nodular structures. When normal development occurred these nodular structures were converted into torpedo shaped embryos. The torpedo shaped embryos germinated after transfer of the explants to step 2 medium. Germinated embryos (GE) were defined as structures with a hypocotyl and large green cotyledons. As can be seen in Fig. 1 the percentage of leaf explants with GE was dependent on the time of the year.

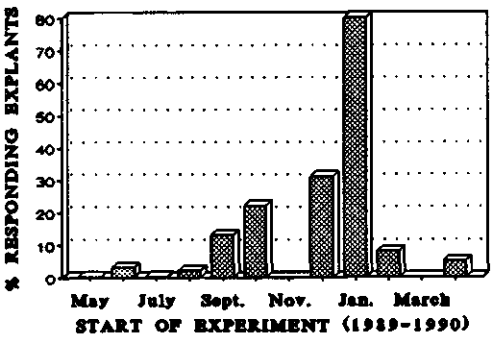

Figure 1. Primary embryogenesis of leaf explants from greenhouse grown M.Col22 plants (24-48 explants per experiment).

From May to August, 1989, between 0 and 10 percent of the leaf explants formed GE (Fig. 1). In this period less than 0.5 GE per initial leaf explant (GE/IE) were formed. From August onward the percentage of responding leaf explants gradually increased to a maximum of 80 percent in January, 1990. In this experiments 22.1 GE/IE were formed. After January 1990 the percentage of responding leaf explants declined to less than 10 percent and the production dropped to less than 0.5 GE/IE. In April, 1990 the response of explants was investigated with scanning electron microscopy (Fig. 2). Almost 50 percent of the explants had formed nodular embryos after 10 days step 1 culture. Most of these nodular structures were overgrown by callus. After 17 days of step 1 explants on which still nodular embryos were visible were investigated with SEM. The nodular embryos developed directly from the explant. In most cases the nodular structures were very irregular shaped and had no cotyledon primordia (Fig. 2a) as was observed in normal development at this stage (Fig. 2b). Nodular embryos isolated of the explant and cultured on step 1 or step 2 medium developed into a smooth callus without formation of somatic embryos. The nodular embryos themselves became disrupted by callus formation, showing that not only the embryogenic tissue was overgrown by callus formation from other parts of the leaf explants but that the embryogenic tissue itself was subjected to callus formation.

PRIMARY EMBRYOGENESIS FROM LEAF EXPLANTS OF IN VITRO GROWN M.COL 22 PLANTS

As it was impossible to control the growth

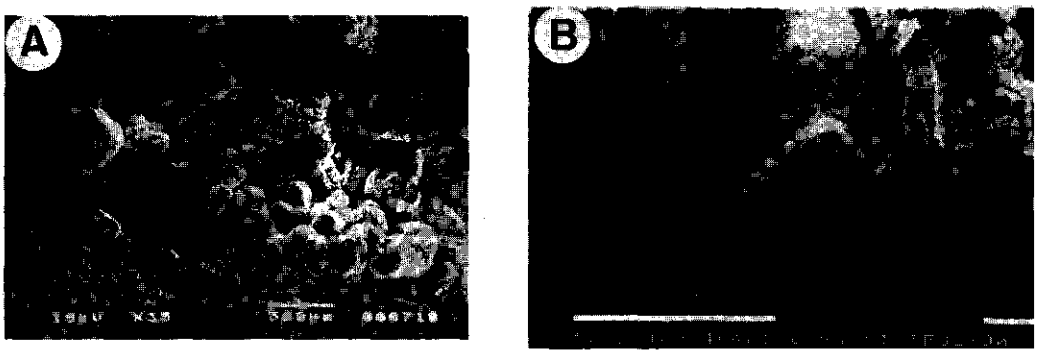

Figure 2: Reaction of leaf explants of greenhouse grown plants after 17 days of induction culture. **A)** formation of nodular structures, note callus formation and **B)** formation of normal torpedo shaped embryo.

of cassava plants in the greenhouse, in vitro grown plants were used as source of leaf explants for primary embryogenesis. The results of these experiments are summarized in Fig. 3.

On the same step 1 medium (4 mg/l 2,4-D) as used for greenhouse derived leaf explants, a maximum of 22 per cent of the leaf explants formed GE (Fig. 3) and the highest production was 0.8 GE/IE (Fig. 3). Although not always significant, doubling of the 2,4-D concentration to 8 mg/l had a positive effect on the number of responding leaf explants (Fig. 3). Up to 50 percent of the leaf explants formed GE, and the highest production found was 3.2 GE/IE. In four of the six experiments the production was lower than 1.5 GE/IE.

Significant differences between the experiments with the same 2,4-D concentration were observed (Fig. 3). Higher 2,4-D concentrations (16 and 32 mg/l) did not further improve the embryogenic capacity of explants. Furthermore, there was no distinct effect of the age of the donor plants on the embryogenic capacity of isolated leaf explants (data not shown).

Individual leaf lobes used as explants produced less GE than whole leaves. In a typical experiment 23 percent of the leaf lobes and 39 percent of the leaves formed GE and the production was respectively 0.9 and 1.5 GE/IE.

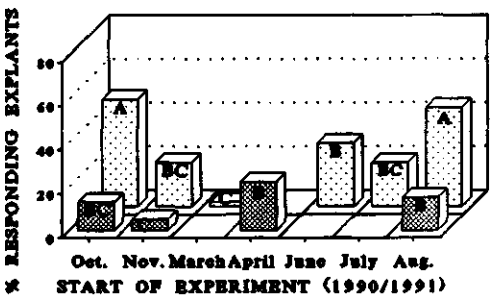

Figure 3. Primary embryogenesis of leaf explants from in vitro grown M.Col22 plants (24 leaf explants per experiment).

INFLUENCE OF LIGHT CONDITIONS DURING GROWTH M.COL22 DONOR PLANTS ON PRIMARY EMBRYOGENESIS

The results obtained using greenhouse derived source material (Fig. 1) show that the embryogenic capacity of the leaf explants depended on the time of the year in which donor plants were grown. Therefore, the effect of different light regimes on the embryogenic capacity during the growth of donor plants in vitro was studied. Different day lengths (8, 12, 16 or 24 hours) had no influence on the embryogenic capacity of the corresponding leaf explants. The response was in the range of the results presented in Fig. 3. However, a reduction of the irradiance (day length 12 hours) had a clear positive effect (Table 1).

Thirty-eight per cent of the leaf explants from donor plants grown in a standard irradiance of 40 $\mu molm^{-2}s^{-1}$ formed GE and the production was 1.7 GE/IE.

Table 1. Influence of irradiance ($\mu molm^{-2}s^{-1}$) during growth of M.Col22 plants on primary embryogenesis of isolated leaf explants (48 explants per treatment).

irradiance	% responding explants [A]	# GE/responding explant [B]	# GE/initial explant [B]
40	38 b	4.6 b	1.7 b
28	54 ab	9.0 a	4.9 ab
8	64 a	10.3 a	6.6 a

means with the same letter are not significantly (p = 10%) different by Chi-square [A] or LSD test [B]

Both the percentage of responding leaf explants and the number of GE per responding leaf explant was increased after growing donor plants in an irradiance of 28 $\mu molm^{-2}s^{-1}$. Consequently the production per initial leaf explant was higher, but not significant (Table 1). When donor plants were grown at 8 $\mu molm^{-2}s^{-1}$, both the percentage of responding leaf explants and the number of isolated GE per responding leaf explant had clearly increased, leading to a significantly higher production of GE (Table 1).

INFLUENCE OF DIFFERENT AUXINS ON PRIMARY AND CYCLIC EMBRYOGENESIS OF M.COL22

Leaf explants (primary embryogenesis) and GE explants (cyclic embryogenesis) were cultured on different auxins for the induction of somatic embryos. Leaf and GE explants cultured on NAA supplemented medium did not form somatic embryos. Picloram and Dicamba induced embryos in the same way as 2,4-D. As reported previously [Chapter 3], embryos were only formed on the adaxial side of leaf explants. However, they were formed on both sides of the GE explants. In a separate experiment either the abaxial or the adaxial side of cotyledons of GE's were culture on the agar. Independent of the orientation more than 80 percent of the explants formed GE's. They were formed predominantly on the side opposite of the agar. GE explants gave a significantly higher percentage of responding explants and a higher number of isolated GE per responding explant and consequently a higher production than leaf material (Table 2).

For both types of explants, Picloram gave a slightly higher production of GE than 2,4-D and 2,4-D in turn gave a slightly higher production than Dicamba. The differences in production of GE between Dicamba and Picloram were significant in all

combinations, except leaf explants cultured on 4 mg/l (Table 2).

Table 2. Influence of Dicamba, Picloram and 2,4-D on primary and cyclic somatic embryogenesis of M.Col22 (24 explants per treatment).

auxin ($mg.l^{-1}$)	% responding explants [A]	# GE/responding explant	# GE/initial explant [B]
PRIMARY EMBRYOGENESIS			
4 mg/l 2,4-D	12 ab	4.3	0.5 ab
8 mg/l 2,4-D	17 a	3.4	0.6 ab
4 mg/l Dica.	0 b	0.0	0.0 b
8 mg/l Dica.	8 b	1.5	0.1 b
4 mg/l Picl.	25 a	5.5	1.4 a
8 mg/l Picl.	29 a	4.2	1.2 ab
CYCLIC EMBRYOGENESIS			
4 mg/l 2,4-D	88 ab	14.7	12.9 abc
8 mg/l 2,4-D	83 b	13.9	11.5 abc
4 mg/l Dica.	88 ab	10.9	9.6 c
8 mg/l Dica.	92 ab	11.9	11.0 bc
4 mg/l Picl.	100 a	15.2	15.2 ab
8 mg/l Picl.	96 ab	17.2	16.5 a

means with the same letter in one column are not significantly different (P=0%) by Chi-quare [A] or LSD test [B].

Picloram and 2,4-D induced GE's had a similar morphology, whereas, Dicamba induced GE's were not as green, larger in size and more irregularly shaped and the formation of the hypocotyl was less pronounced (data not shown).

There were no significant differences between the 4 and 8 mg/l of the auxins Picloram, Dicamba and 2,4-D.

Addition of 2 μm $CuSO_4$ to step 1 media with 2,4-D, Picloram and Dicamba had no effect on the production of GE's (data not shown). On NAA medium without $CuSO_4$ the GE explants only developed callus. When $CuSO_4$ was added together with NAA the GE explants developed into shoots within 21 days of culture. These shoots were not malformed as was reported for shoot conversion on BA supplemented medium [Chapter 4 and 5].

PRIMARY EMBRYOGENESIS OF 2,4-D PRETREATED PLANTS OF M.COL22

In order to enhance the embryogenic potential of leaf explants, 2,4-D was added during the growth of donor plants. Culture of cuttings directly on 2,4-D supplemented medium resulted in the formation of callus and shoot growth ceased. Therefore, plants were first allowed to grow for 12 days before 2,4-D was supplied. A 2,4-D pretreatment on agar grown plants gave a production of GE which was in the range of the experiments summarized in Fig. 3. Alternatively plants were grown in liquid medium. When leaf explants were isolated two days after 2,4-D pretreatment and cultured on embryo induction medium devoid of growth regulators, they did not form embryos. However, when these explants were cultured on step 1 medium (8 mg/l 2,4-D) 71 percent of the leaf explants formed GE and the production was 9.4 GE/IE (Table 3).

Table 3. Influence of 2,4-D pretreatment during growth of M.Col22 plants on primary embryogenesis (24 explants per treatment).

pretreatment	% responding explants [A]	# GE/responding explant [B]	# GE/initial explant [B]
H_2O	48 b	7.6 b	3.5 b
2,4-D	71 a	13.2 a	9.4 a

means with the same letter are not significantly (p=10%) different by Chi-square [A] or LSD test [B]

In the control 48 percent of the leaf ex-

plants formed GE and the production was 3.5 GE/IE.

In another experiment liquid grown plants were pretreated after 21 days of growth. The embryogenic response of leaf explants isolated 2 or 4 days after the 2,4-D pretreated was slightly, but not significantly, higher than that of leaf explants of control plants. However, if explants were isolated 6 days after the 2,4-D pretreatment 58 percent of the explants formed GE and the production was 5.8 GE/IE. In the control this was significantly lower, respectively 24 percent and 1.6 GE/IE.

PRIMARY AND CYCLIC EMBRYOGENESIS OF NIGERIAN CLONES

Eleven Nigerian clones were investigated for induction and outgrowth of primary embryos from leaf explants of in vitro grown donor plants.

Nodular structures were formed on leaf explants of 5 clones, isolated of agar grown donor plants at an irradiance of 40 μmolm^{-2}s^{-1} (Table 4). Further development of the nodular structures into GE was only observed in a low frequency (< 0.1 GE/IE) in TMS90853 and TMS4(2)1244. The clones were considered to be recalcitrant for primary embryogenesis. The general applicability of the primary embryogenesis enhancing treatments "growth of donor plants at reduced irradiance" and" pretreatment of donor plants" was studied on these clones.

Growth of donor plants at an irradiance of 8 μmolm^{-2}s^{-1} had no positive effect. Not one single GE was formed.

After 2,4-D pretreatment of donor plants at and irradiance of 40 μmolm^{-2}s^{-1} leaf explants of all clones, except TMS30572, formed nodular embryos. This was observed in more than 50 percent of the leaf explants of TMS4(2)1244, TMS60444, TMS30395 and TMS90853. In seven clones a low frequency of the nodular embryos matured into primary GE (Table 4). TMS60444 gave the highest production (1.1 GE/IE) and all other clones produced less than 1 GE/IE. The primary GE of eight clones except TMS60506 were used successfully for secondary embryogenesis. The production of tertiary embryos, using secondary GE, is shown in Table 4.

Table 4. Influence of 2,4-D pretreatment on primary embryogenesis of leaf explants followed by the production of GE in cyclic embryogenesis.

embryogenesis:	primary				cyclic
pretreatment:	H_2O		2,4-D		
	NE	GE/IE	NE	GE/IE	GE/IE
TMS30555	+	0	+	0.7	6.2
TMS50395	+	0	+	< 0.1	5.3
TMS60506	+	0	+	< 0.1	0
TMS90059	-	0	+	< 0.1	7.2
TMS30211	-	0	+	0	nd
TMS60444	-	0	+	1.1	9.9
TMS30395	-	0	+	0.1	6.7
TMS90853	+	< 0.1	+	0.2	8.2
TMS4(2)1244	+	< 0.1	+	0	5.4
TMS30001	-	0	+	0	nd
TMS30572	-	0	-	0	nd

NE = nodular embryos: visible (+) or not (-) after 10 days step 1, GE/IE = # of germinated embryo per initial explant (48-74 leaf explants for primary and 24-48 GE explants for cyclic embryogenesis per clone), nd = not determined

Sixty to 100 percent of the GE formed new GE nd the production varied between 5.3 GE/IE for TMS50395 and 9.9 GE/IE for TMS60444. GE's of the 7 clones which had formed secondary embryos were maintained for more than one year by regular subculture of GE on 2,4-D supplemen-

ted medium. As was previously reported for the clone M.Col22 [Chapter 5], the production of new GE's was increased by using liquid instead of solid step 1 and 2 media and by fragmenting the GE for the start of a new cycle.GE's of the clones TMS30395 and TMS 90853 were cultured for shoot development on BA supplemented (1 mg/l) medium. Seventy-five percent of the GE'S of TMS90853 developed into normal looking and 15 percent in malformed looking shoots. For the clone TMS30395 this figure was respectively 1 and 17 percent. GE's of all other clones formed shoots after culture on medium supplemented with 4 mg/l NAA plus 2 μM $CuSO_4$.

DISCUSSION

In cassava the embryogenic capacity of leaf explants greatly depends on the growth conditions of donor plants. For leaf explants from greenhouse grown plants it varied between 0 and 80 percent, depending on the time of the year. This kind of variation has also been reported in crops like *Zea mays* [180], *Triticum aestivum* [18], *Hordeum vulgare* [112] and *Helianthus annuus* L. [84]. All these observations state the importance of the internal physiological state of the embryogenic competent cells for embryogenesis.

To obtain a more reproducible system of somatic embryogenesis, in vitro grown plants were used as source for leaf explants. These leaf explants showed, in contrast to greenhouse derived leaf explants, less variation in embryo production. However, the production of GE was more than a factor 30 lower than the highest production of GE obtained with greenhouse derived leaf explants.

Several factors acting during the in vitro growth of donor plants or during the embryogenic culture of leaf explants were identified to improve production of GE. One of them was growth of donor plants at low irradiance (8 $\mu molm^{-2}s^{-1}$). Low irradiance might provide conditions which make cells embryogenically more competent or sensitive to 2,4-D. Also, with greenhouse derived leaf explants, the highest production was found when donor plants were grown under natural low irradiance. The highest production of GE from leaf explants of M.Col22 was found when liquid grown donor plants were pretreated with 2,4-D.

Growth of donor plants of Nigerian clones at low irradiance had no effect on primary embryogenesis. Several Nigerian clones which were recalcitrant in the standard procedure, became embryogenic after a 2,4-D pretreatment. A 2,4-D pretreatment might provide more embryogenically competent cells with 2,4-D. Also in *Zea mays* [42], a non-embryogenic line became embryogenic after a 2,4-D pretreatment of greenhouse grown plants. The use of liquid media makes it possible to supply plants in vitro with high doses of growth regulators, which become immediately available to the plant, without disrupting the growth of plants by making cuttings.

In this study all tested auxins, except NAA gave somatic embryogenesis in cassava. The differences between Picloram, Dicamba and 2,4-D were small, but Picloram

was significantly better than 2,4-D.

Evans et al., [50] noted that 2,4-D was used for embryo induction in more than 50 percent of the successfully cultured crop plants. They did not describe examples of Picloram or Dicamba mediated somatic embryogenesis. During the past ten years this has changed. Picloram gave better results than 2,4-D in *Arachis hypogaea* L. for direct embryogenesis [147] and in *Allium cepa* [156] for indirect embryogenesis. Dicamba gave better results than 2,4-D in *Glycine max* [23] for both direct and indirect embryogenesis and in *Triticum aestivum* L. [18] for indirect embryogenesis.

The embryogenic capacity of leaf explants (primary embryogenesis) of all tested clones was considerably lower than that of GE (cyclic embryogenesis). In practice a few primary GE are needed as source material in order to induce cyclic embryogenic cultures. Millions of GE are delivered in one year when they are cultured in liquid medium and when the starting explants are fragmented [Chapter 5]. Also in crops like *Aesculus hippocastanum* [93], *Arachis hypogaea* [10, 43], *Juglans regia* [217] and *Picea abies* [133], where a cyclic or repetitive system of somatic embryogenesis has been developed, the production of primary embryos was much lower. The factors which are responsible for this difference are however unclear.

In screening tests of genotypes for somatic embryogenesis, the production of primary somatic embryos is a bottleneck which can be overcome by pretreatment of donor plants with 2,4-D. The development of a cyclic system which is based on primary somatic embryos is much more independent of the genotypic factor and needs only a few embryos as starting material.

CHAPTER 7

MORPHOGENESIS AND HISTOGENESIS OF CYCLIC SOMATIC EMBRYOGENESIS IN CASSAVA

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ABSTRACT. *Somatic embryos of cassava form new somatic embryos after culture on induction medium (i.e. 2,4-D supplemented nutrient medium). This process of cyclic embryogenesis was investigated morphologically by light and scanning electron microscopy. In general, cell division was initiated within 2 days of culture and cells of the explanted somatic embryo turned into callus cells or became meristematic. First divisions occurred in cells near or within the vascular tissue of the cotyledons of the explanted somatic embryo, independent of the orientation of the explant on the induction medium. Depending on the developmental stage of the explanted embryo, a strand or groups of meristematic cells appeared when explanted embryos were cultured for 4 days. Cell divisions led either to somatic embryos, often organized in a broad band, or to a less differentiated meristematic mass from which individual embryos were formed later on. A part of the meristematic cells reverted to callus cells, a differentiation to which they were irreversibly committed.*

Embryos originated from the meristematic tissue by budding after 8 days of culture and had a multicellular origin. Because of their endogenous origin the newly formed embryos developed their own epidermis. Sometimes however, the epidermis of the original explant took part in the formation of embryos, too.

Both the endogenous development and the multicellular origin of the somatic embryos are disadvantages for plant transformation. From the data obtained it is expected that transformation of globular embryos arising of the surface of explants will be most profitable.

INTRODUCTION

Somatic embryogenesis has been reported in at least 200 species [50, 213, 218, 231]. Analogues to zygotic embryogenesis, somatic embryogenesis is defined as the process in which a bipolar structure [189, 207] arises through a series of stages characteristic for zygotic embryo development [74, 189, 229] from one somatic cell [74, 189, 229] and having no vascular connection with the parent tissue [6, 74]. The strict single cell origin of embryos has been debated since also a multicellular origin has been observed [213, 231]. Two types of embryogenesis, direct and indirect, are distinguished [50, 189]. The first type of embryogenesis proceeds directly from cells of the initial explant without callus interphase. These cells are determined to be embryogenic. In indirect embryogenesis, cells require redifferentiation before they express embryogenic competence. In such cases, callus formation from the initial explant precedes the formation of embryos.

Somatic embryogenesis in cassava was published for the first time in 1982 [196]. Somatic embryos were induced in the cotyledons of zygotic embryos after culture on 2,4-D supplemented medium, called induction medium. Later it was shown that also leaf explants had that capacity [201, 205, Chapter 3]. In both types of explants the origin of primary embryos appeared to be multicellular and directly from the explant [199]. The first embryogenic divisions occurred in the adaxial mesophyll

tissue and were only observed if the abaxial sides of the leaves were in contact with the induction medium.

Primary somatic embryos, derived from leaves or from cotyledons of zygotic embryos, produced secondary embryos after subculture on induction medium and embryogenicity was maintained (cyclic embryogenesis) for prolonged periods by repeated subculture of somatic embryos on induction medium [200, 205, Chapter 4 and 5]. Subculture is an elegant system to be used in conjunction with plant transformation. For transformation it is imperative important to know how somatic embryos do originate and develop. Therefore the ontogeny of cyclic embryogenesis in cassava is analyzed morphologically in this report. The utility of cyclic embryogenesis for the transformation of cassava will be discussed.

MATERIALS & METHODS

PLANT MATERIAL AND REGENERATION PROCEDURE

Primary somatic embryos of cassava (*Manihot esculenta* Crantz) were initiated from cultured immature leaves [Chapter 3] and used to initiate cyclic embryogenic cultures [Chapter 4]. In short: torpedo shaped and germinated somatic embryos were incubated on Basal Medium (BM) supplemented with 4 mg/l 2,4-D to induce somatic embryogenesis. The BM consisted of Murashige and Skoog [136] salts and vitamins, 7 g/l agar and 20 g/l sucrose. The pH was adjusted to 5.7 before autoclaving (20 minutes, 120 °C). After 20 days of culture, the explants were transferred to BM with 0.1 mg/l BA or growth regulator free medium to induce the germination of somatic embryos. The temperature in the growth chamber was 30°C, the photoperiod of 12 hours and the irradiance 40 $\mu Em^{-2}s^{-1}$.

LIGHT MICROSCOPY

After T=0, 4, 8 and 20 days of induction culture, somatic embryo explants were fixed in aqueous FAA solution (4 % formalin, 5 % acetic acid and 50 % ethanol), dehydratated through an ethanol series of 70, 80, 90 and 100 %. Hereafter, the explants were embedded in Technovit 7100. Sections of 4-6 μm were made with a Reichert-Jung 2050 Supercut microtome and stained with 1 % toluidine blue in an aqueous solution of 1 % Na-tetraborate.

SCANNING ELECTRON MICROSCOPY

After 20 days of induction culture, the somatic embryo explants were fixed in aqueous FAA solution, postfixed in saturated $KMnO_4$ for 1 min, rinsed thoroughly and dehydratated through an ethanol series. Explants were prepared for scanning electron microscopy by critical point drying in CO_2 and coated with gold/palladium in a sputter coater. The explants were examined and photographed in a Jeol-JSM-5200 scanning electron microscope (SEM) at 15 kV.

RESULTS

MORPHOLOGY OF CYCLIC EMBRYOGENESIS

Somatic embryos arose at the surface of the cotyledons of the explanted somatic

embryos from 13 days of culture onwards. The site of formation was independent of the orientation of the explanted somatic embryo on the agar, and the newly formed somatic embryos always developed with their shoot apices away from the agar. Figure 1a shows a SEM picture of explants with developing embryos at the end of the induction culture (20 days). At several places the epidermis of the cotyledon of the explant was disrupted by callus proliferation. Newly formed embryos were observed in various stages of development. They formed cotyledon primordia within 17 days of culture and were then called torpedo shaped embryos (Figs. 1a and b). One of the embryos shown in Fig. 1a had already two large, green, cotyledons and a well developed shoot meristem.

This mature stage of development is called "germinated embryo". The size of embryos ranged from 0.5 to 2.0 cm (cf Figs. 1a and 1b).

Not all the embryos are directly connected with the cotyledon of the explanted somatic embryo. Figure 1b shows torpedo shaped somatic embryos which are attached to a tissue which itself had developed on the cotyledon of the explant. This particular tissue is called an meristematic mass

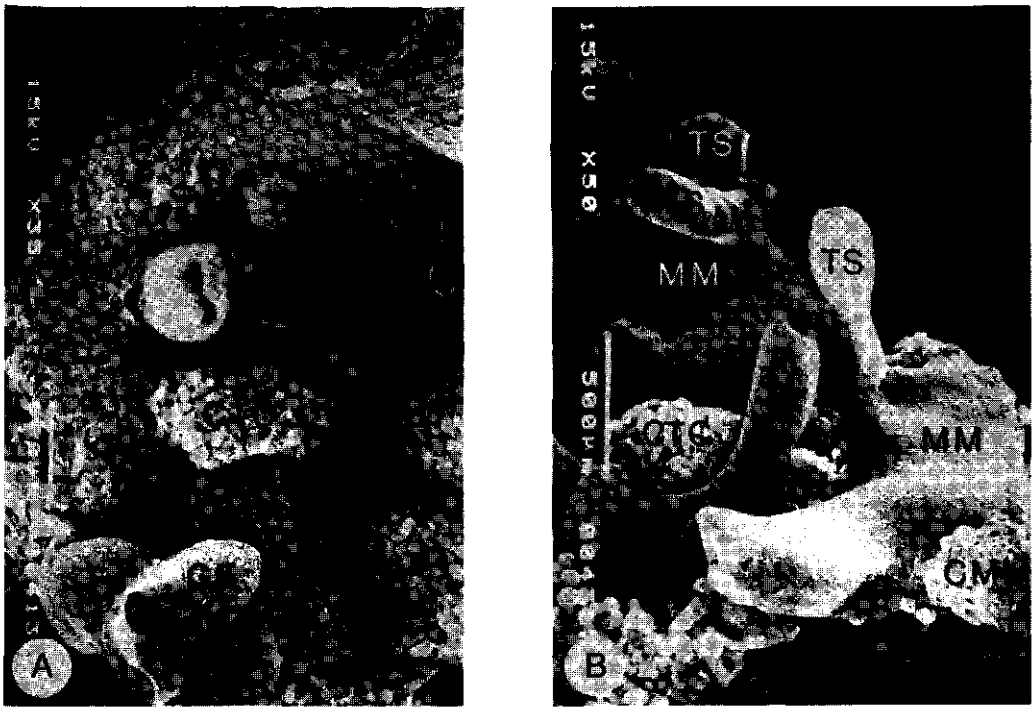

Figure 1: Scanning electron micrograph of newly formed somatic embryos of cassava after 20 days of culture on induction medium. **A)** Embryo formation directly from cotyledon, and **B)** on an meristematic mass. Note callus formation from meristematic mass.

For abbreviations see p. 70.

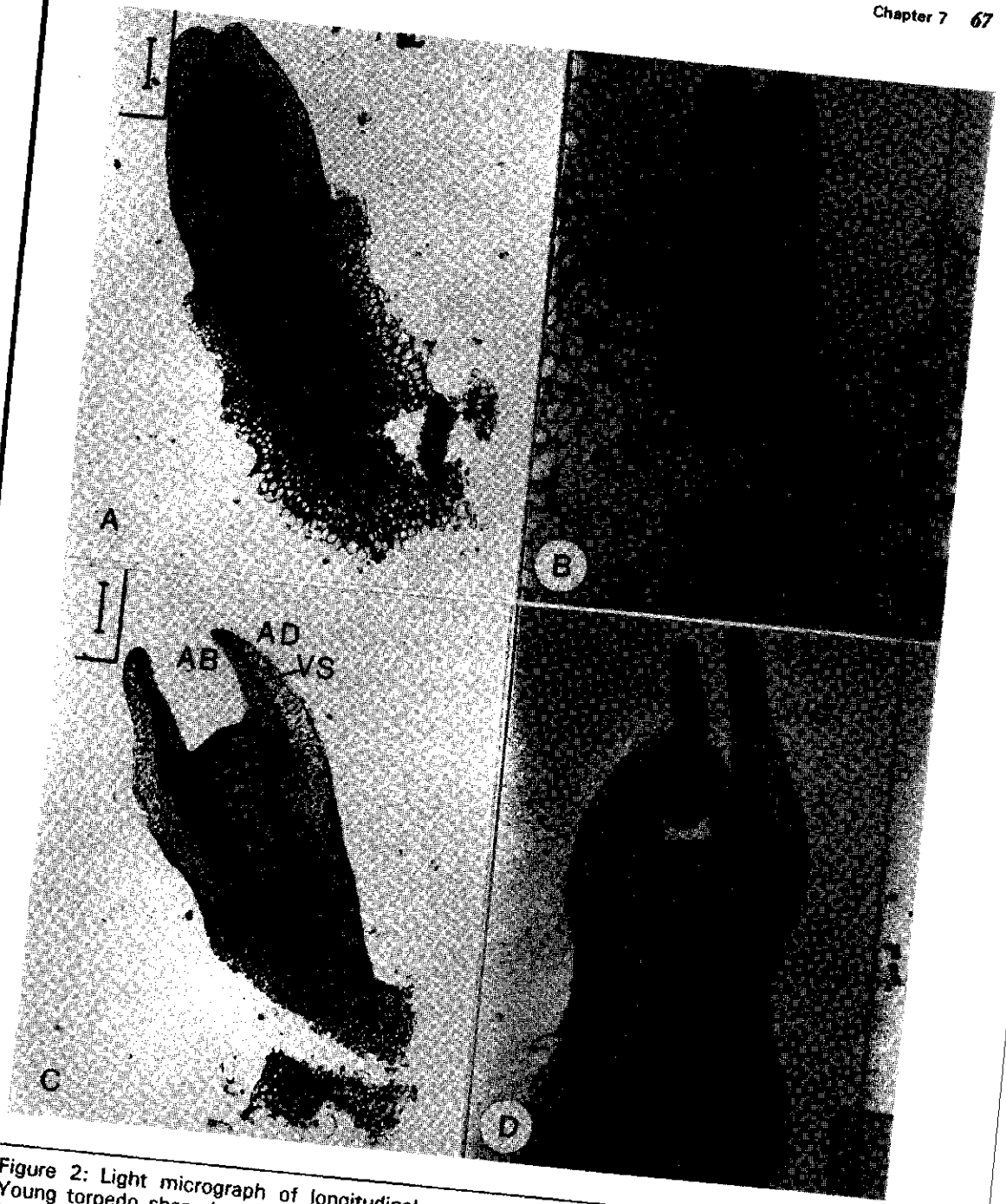

Figure 2: Light micrograph of longitudinal sections of somatic embryos of cassava. **A)** Young torpedo shaped embryo (bar = 95 μm). **B)** further developed torpedo shaped embryo (bar = 190 μm) and **C)** "germinated" embryo (bar = 95 μm). **D)** idem as C, note small, newly formed embryo on cotyledon (bar = 190 μm). For abbreviations see p. 70

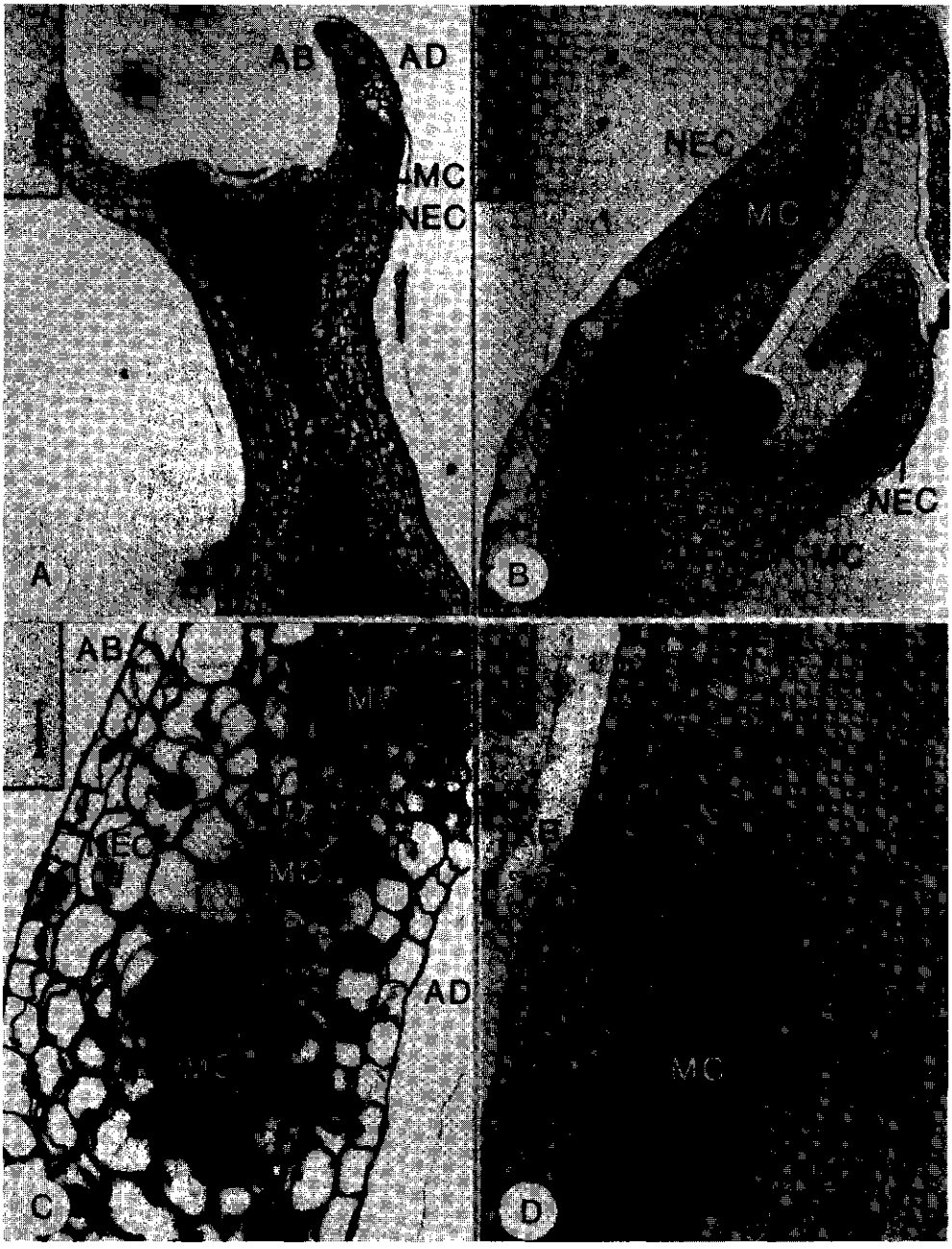

Figure 3: Histology of explanted somatic embryo after 4 days of induction culture. **A)** Explanted embryo was torpedo shaped at T=0 days and formed only some groups of dividing cells (bar= 190 μm), **B)** Explanted embryo was mature or "germinated" at T=0 days and formed a broad band of meristematic cells in the mesophyll (bar=190 μm). **C)** Detail of clusters of meristematic cells in and near the meristele of a cotyledon (bar=24 μm). **D)** idem as C but showing tracheids near the meristematic cells clusters (bar=24 μm). For abbreviations see p. 70

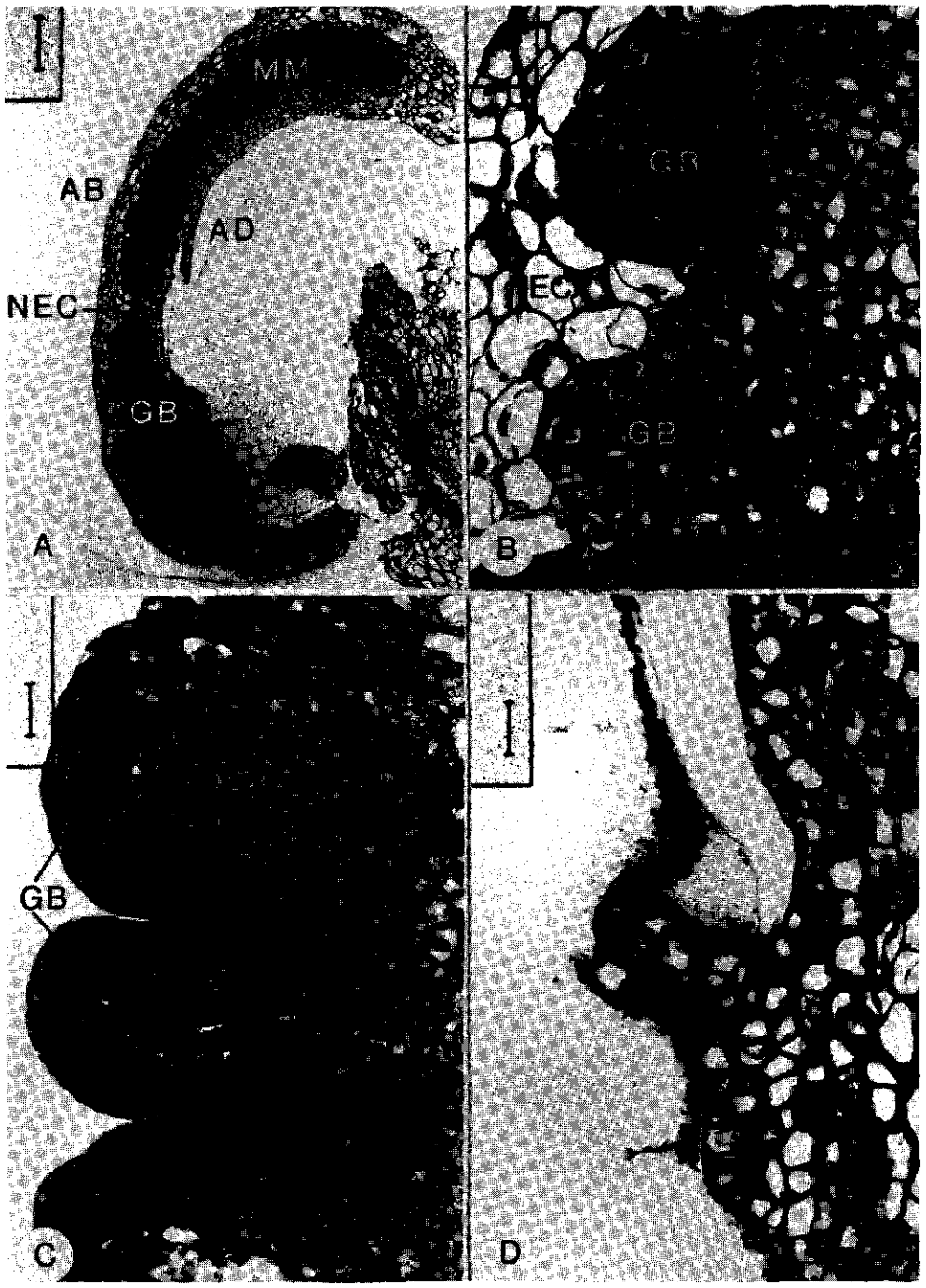

Figure 4: Histology of somatic embryos, explanted at the germinated stage, and cultured for 8 days. **A)** Distinct globular embryos and a meristematic mass (bar = 190 μm). **B)** Detail of globular embryos of A showing endogenous development of somatic embryos (bar = 24 μm). **C)** Embryo formation from internal as well as epidermal cell layers (bar = 24 μm). **D)** Development of somatic embryo with oligocellular origin (bar = 24 μm). For abbreviations see p. 70

Figure 5. Histology of somatic embryos, explanted at the germinated stage, and cultured for 20 days. **A)** Formation of embryos direct from the cotyledon. Note the well developed suspensor like structure (bar = 190 μm). **B)** As A but not accompanied by abundant callus formation. Note the fusion of the basal parts of two embryos (bar = 190 μm). **C)** Formation of somatic embryo connected with a procambium like strand to the meristematic mass (bar = 190 μm). **D)** Formation of somatic embryos from a meristematic mass (bar = 190 μm). For abbreviations see p. 70

because embryos developed on it. Locally the meristematic mass proliferated into callus. Sometimes embryos were connected to each other with their basal parts (Fig. 1b). Polycotyledony, fasciation of embryos, and the formation of trumpet like cotyledons were frequently observed. All these embryos, however, initiated new embryos after culture on induction medium (data not shown).

HISTOLOGY OF SOMATIC EMBRYO EXPLANTS AT THE ONSET OF INDUCTION CULTURE (T = 0 d)

Somatic embryos, isolated from proliferating cultures and cultured on growth regulator free or BA supplemented medium, developed further without the induction of new embryos. On growth regulator free medium they formed a primary root indicating the true embryo nature. Globular embryos and older germinated embryos, which had already formed cotyledons and a first true leaf, did not initiate new embryos after culture on induction medium. Only torpedo-shaped embryos (Figs. 2a and 2b) and germinated embryos without a first true leaf (Figs. 2c and 2d) had that capacity.

In young torpedo-shaped embryos no vascular tissue was observed and the cotyledons were still primordial (Fig. 2a). In a more developed stage, procambium strands were observed in the hypocotyl of the embryo, but not yet in the cotyledons (Fig. 2b). Germinated embryos had thickened and their cotyledons had enlarged. Cotyledons of germinated embryos were 9 to 25 cell layers thick and showed a continuous vascular system (Fig. 2c and 2d). The mesophyll was parenchymatic. Usually the vascular tissue was located at the adaxial side. Incidentally, newly formed embryos developed asynchronously on their cotyledons (Fig. 2d). The vascular tissue of this embryo was not connected with the vascular tissue of the parental embryo.

HISTOLOGICAL CHANGES IN SOMATIC EMBRYOS DURING INDUCTION CULTURE (T = 4, 8 and 20 d)

First cell divisions in the cotyledons of explanted embryos were already observed after 2 days of culture. In Figs. 3a and 3b cotyledons of explanted somatic embryos are shown after 4 days of induction culture. Usually the cotyledon which was in contact with the agar had increased more in size than the other one (Fig. 3b). The number of dividing cells depended on the developmental stage of the explanted embryos at the onset of induction culture. When torpedo shaped embryos (see e.g. Figs. 2a and 2b) were explanted, only small groups of dividing cells developed (Fig. 3a).

LEGEND TO THE FIGURES 1 to 5.

AB: abaxial side, **AD:** adaxial side, **CMM:** callus proliferation from meristematic mass, **CTC:** callus proliferation from cotyledon of explanted embryo, **GB:** globular embryo, **GE:** germinated embryo, **MC:** meristematic or embryogenic cells, **MM:** meristematic or embryogenic mass, **NEC:** non embryogenic cells, **PS:** procambium strands, **S:** suspensor, **T:** tracheid, **TS:** torpedo shaped embryo, **VC:** vascular tissue

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When germinated embryos were cultured (see e.g. Fig. 2c and 2d), they responded with the formation of a broad strand of dividing cells in or near the vascular tissue (Fig. 3b) and at the place of the presumed shoot meristem. The dividing cells were small, had dense cytoplasm, and a large nucleus with prominent nucleoli (Figs. 3c and 3d). They are considered to be meristematic and potentially embryogenic. In some cases tracheids were observed adjacent to the meristematic cells, showing again that divisions started from cells near or in the vascular tissue (Fig. 3d). Other cells were larger, stained poor with toluidine blue and their nuclei and nucleoli were less frequently observed. They were not embryogenic.

Continued division of the meristematic cells led either to distinct globular embryos, often organized in a broad band throughout the cotyledon (Figs. 4a, **GB** and 4b), or to a less differentiated meristematic mass (Fig. 4a, **MM**) from which the embryos arose by budding. These developments were observed from 8 days of induction culture onwards. Embryo development was often restricted to parts of the mesophyll (Figs. 4a and 4b) but embryos were also formed from mesophyll and epidermal tissue opposite to the agar (Fig. 4c).

In some cases embryos seemed to develop from a few cells (Fig. 4d). At the base, these embryos were two cells thick, indicating an oligocellular origin.

After 20 days of induction culture well developed embryos were observed (Fig. 5). They were formed either directly connected with the explant (Figs. 5a, b, c) or with the meristematic mass (Fig. 5d). Most non-embryogenic cells had turned into callus cells (Figs. 5a, c). During culture also embryogenic cells turned into callus (Figs. 5a, **CMM**), especially those cells which were in or near the agar. Because of this callusing reaction, embryos could be embedded in a callus layer and a root meristem was not distinguished.

Embryogenesis did not proceed in a fixed pattern. Sometimes globular embryos were sustained by multicellular suspensors (Fig. 5a). Other times suspensor-like structures were absent. Embryos might be formed near the surface (Figs. 5a and b) or they were formed endogenously from the meristematic mass (Fig. 5d). They could be connected with the underlying tissue by procambial like strands (Fig. 5c) or they were completely devoid of such connections (Figs. 5a, b). However, all these different patterns led to the formation of mature germinated embryos.

DISCUSSION

Primary somatic embryos were initiated directly on cotyledons of zygotic embryos [196] and on immature leaves [201, 205, Chapter 3]. When such primary somatic embryos were explanted on the same medium as used for the first cycle, it allowed the development of a new cycle of somatic embryogenesis. Primary and cyclic embryogenesis do differ. Primary embryogenesis occurred only when the abaxial side of the explant was in contact with the agar [196, 201] whereas cyclic embryogenesis took place independently of the

orientation of the explants.

Cyclic embryogenesis has been reported in at least 30 species [231, Chapter 2]. In cassava cyclic embryos were only initiated on the cotyledons of somatic embryos. This was also observed in *Trifolium repens* [117], *Feijo sellowiana* [29] and *Apium graveolens* [92]. In other species somatic embryos were initiated on hypocotyls [90, 92, 158], roots [116] or combinations of organs [110, 159, 225].

In both primary [199] and cyclic embryogenesis of cassava the origin of the embryos was multicellular, although there is evidence for oligocellular origin, too. However in these cases the embryos were composed of highly vacuolated cells and therefore, it is unknown if they will develop into mature embryos (Fig. 4d). Multiple cell origin was also reported in cyclic embryogenesis of *Camellia reticulata* [158] and *Trifolium repens* [117]. Single cell origin of cyclic embryos has been reported for *Brassica napus* [209] and *Juglans regia* [159] and both single cell and multiple cells origin for *Feijo sellowiana* [29], *Glycine max* [110] and *Theobroma cacao* [153]. This indicates that not in all species embryos originate from single cells necessarily.

Cyclic embryos not only originated from specific organs but from specific cells as well. In cassava two types of origin were distinguished. Either the embryos originated completely from internal cells (see Fig. 4a) or from internal and epidermal cells (see Fig. 4c). When embryos arose from internal meristematic cell layers, they had to form their own epidermis. In the other case the embryo epidermis was derived from the epidermis of the explanted embryo.

Also in *Dactylis glomerata* [216], *Solanum aviculare* [4] and *Trifolium rubens* [30] embryos originated from internal cell layers; either from mesophyll cells (*Dactylis glomerata*) or vascular bundle sheet cells (*Solanum aviculare* and *Trifolium rubens*). In none of these three species the embryos originated from cells in the vascular bundle. In cassava the origin of embryos could not be exactly determined. In *Brassica napus* [209], *Camellia reticulata* [158], *Feijo sellowiana* [29], *Glycine max* [110], *Juglans regia* [159], *Trifolium repens* [117] and *Theobroma cacao* [153] embryos originated from epidermal and subepidermal cells.

In cyclic embryogenesis in cassava the first embryogenic divisions led either to direct somatic embryogenesis or to the formation of an meristematic mass on which several somatic embryos developed later (Fig. 6). In germinated embryos more cells became embryogenic than in torpedo shaped embryos (Figs. 2a versus 2b). This explains the observation that germinated embryos produced about four times more new somatic embryos than torpedo shaped embryos [Chapter 4]. Also, in primary embryogenesis of cassava [199] and in cyclic embryogenesis in other species, the formation of an meristematic mass preceded the formation of embryos [29, 70, 117, 158]. Such a developmental pattern has characteristics of both direct and indirect embryogenesis. It can be regarded as direct because the embryogenic tissue is

an integral part of the original explant and it can be regarded as indirect because cells have to divide first before they can express the ability to form embryos.

In most species where transformation has been accomplished, plants were regenerated by indirect organogenesis or indirect embryogenesis [129]. The callus phase was used as a stage to sort out transformed from untransformed cells and multiply the former ones. After this stage, calli were allowed to regenerate plants.

In direct regeneration, a callus phase is absent. In *Glycine max*, a species with direct embryogenesis, only chimeric, transformed plants were obtained [150]. The problem of chimerism was solved by culturing the chimeric transformed embryos for a new cycle of embryogenesis [57]. The same strategy was applied successfully in *Brassica napus* [204] and *Juglans regia* [124, 125]. In these three species cyclic embryos originated from (sub)epidermal cells and in at least some cases from single cells. Cassava is amenable for transformation and transformed cells are able to divide [166]. The majority of the transformed cells developed into non-regenerable callus and only a fraction into embryogenic competent cells which gave rise to somatic embryos. Completely transformed embryos were, however, not found. The results presented in this paper show that embryos originate from mesophyll cells which are subepidermal and thus not easy to be penetrated by *Agrobacterium*. Additionally, because of their multicellular origin, embryos could be chimeric at the most, which was shown recently [166]. This paper demonstrates that transformation experiments might be most profitable when globular embryos become within reach at the surface of the explants i.e. after 8 days of culture, experiments which are currently done.

CHAPTER 8

EVALUATION OF SOMATIC EMBRYO-DERIVED PLANTS OF CASSAVA

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ABSTRACT. *In cassava, somatic embryos obtained from leaf explants can be cultured for the mass production of new somatic embryos. After 2½ years of culture, with 17 successive cycles of embryogenesis, the production of new embryos and shoot conversion seemed not to be changed. These long-term cultured embryos still had the normal ploidy level. Nearly 3000 plants of 485 independent regenerants of different cycles were evaluated in vitro. Only one regenerant, originating from a seventh cycle embryo, showed an different leaf morphology (variegated leaves).*

In addition almost 700 plants of 105 independent first, third and seventh cycle regenerants were evaluated in the greenhouse for 41 quantitative and qualitative shoot, leaf, root and flower characters and compared with control plants. Virus-like symptoms, which were less severe in regenerants than in control plants, explained most of the variation observed between control plants and regenerants. Form and texture of the roots were more variable in regenerants than in control plants. Since not all plants of one regenerant deviated for these root characters, it can be expected that the differences between regenerants and control plants will disappear during further multiplication. It can be concluded that in the regenerants variation did occur, but that this was not accompanied with clear negative plant development characteristics.

INTRODUCTION

Cassava is one of the most important food crops in the lowland tropics [26]. It faces several severe problems which cannot be solved by classical breeding techniques [Chapter 1]. Biotechnology could be a means to overcome these problems. Therefore, efforts are made to implement molecular- and cellular-genetic techniques in cassava breeding programs. Such techniques require efficient, genotype independent regeneration procedures. One of the side-effects of regeneration is the occurrence of phenotypic variation, usually referred to as somaclonal variation [51, 104]. Somaclonal variation of genetic origin has been recognized by breeders as a novel source of genetic variation [104], but in most cases somaclonal variation has turned out to be negative. If regeneration is used for multiplication purposes or in conjunction with genetic engineering, the culture procedures should be aimed at minimizing somaclonal variation.

In cassava the only routinely available route of plant regeneration is somatic embryogenesis. Primary somatic embryos can be initiated from zygotic embryos [196] or from leaves [201, 205, Chapter 3]. Likewise, somatic embryos can be used as starting material for cyclic embryogenesis [200, 205, Chapters 4 and 5]. Thus somatic embryos undergo repetitive cycles of embryogenesis. However, such long-term culture duration is generally accompanied with enhanced genetic instability [145].

Different methods are used to assess the incidence of somaclonal variation [33]. A straightforward method, operating at the whole plant or population level, is the comparison of the morphology of regenerants with that of the original genotype. Biochemical methods have been applied

for instance in *Picea glauca engelmanni* complex [45] and *Trifolium repens* [119]. Several methods have been developed to assess alterations at the chromosomal level, ranging from changes in ploidy level to that in individual nucleotides [103, 160].

In this report the morphology of 485 in vitro grown regenerants, originating from embryos which had passed through up to 17 cycles of embryogenesis, was compared with that of control plants. More than 100 independent regenerants of first, third and seventh cycle embryos were transferred to and multiplied in the greenhouse. These plants were evaluated for several qualitative and quantitative leaf, shoot, root and flower characters according to the cassava descriptor list issued by the IBPGR [71]. The occurrence of somaclonal variation was investigated and the nature of the observed variation is presented and discussed.

MATERIALS & METHODS

INITIATION AND MAINTENANCE OF EMBRYOGENIC CULTURES

The culture media consisted of Murashige and Skoog [136] mineral salts and vitamins, 20 g/l sucrose and 7 g/l Daichin agar (BM). The pH was adjusted to 5.7 before autoclaving (20 min, 120 °C). The temperature in the culture room was 30 °C, the day length 12 hours and the irradiance 40 $\mu molm^{-2}s^{-1}$.

Primary or first-cycle embryos were initiated on leaf explants isolated from one greenhouse grown M.Col22 plant (Fig. 1). Secondary somatic embryos were initiated on primary somatic embryos cultured on the same media as used for leaf explants. In this way embryogenicity was maintained by repeated subcuture of somatic embryos. An embryogenic cycle consisted of two steps: step 1 for induction of embryos (BM supplemented with 4 mg/l 2,4-D) and step 2 for germination of embryos (BM supplemented with 0.1 mg/l BA). In the first to fourth cycle, embryos were cultured on solid step 1 and 2 media [Chapter 4] and in the fifth to seventh either on solid or in liquid step 1 and 2 media [Chapter 5] and thereafter only in liquid step 1 and 2 media.

In step 3 of the process, germinated embryos developed into shoots. Germinated embryos of the first and second cycle were cultured for shoot development on BM supplemented with 0.1 mg/l BA, third cycle embryos on BM supplemented with 0.1 or 1 mg/l BA and after this germinated embryos were cultured on only BM supplemented with 1 mg/l BA. Germinated embryos which had passed through up to 17 cycles were used (Fig. 1). Shoots were rooted (step 4) and maintained on BM.

The original M.Col22 plant that provided the leaf explants for first cycle embryogenesis was brought in vitro. The resulting plants were maintained on BM and were used as control. The in vitro growth characters of these control plants and of 485 regenerants of different cycles (Table 1) were evaluated.

MULTIPLICATION OF REGENERANTS AND CONTROL PLANTS

In May 1991 control plants and 109 randomly selected first, third and seventh

Table 1. Number of regenerants and plants per regenerant evaluated in vitro and in the greenhouse.

	embryo-derived plants (number of cycle)						con-trol
	1	2	3	4	7	17	
IN VITRO							
# of regenerants	150	23	74	124	64	50	-
# of plants per regenerant	3-50	8	8	3	8	3	-
total number of plants	1000	184	592	372	512	150	400
GREENHOUSE							
# of regenerants	8		45		56		-
# of motherplants per regenerant	48		3		3		-
total # of transferred motherplants	384		135		168		72
total # of survived motherplants	134		113		111		37
total # of cuttings made from motherplants	300		176		222		63

cycle regenerants (Table 1) were cultured for two multiplication rounds, of four weeks each, on BM supplemented with 0.1 mg/l BA. Cuttings were then taken and rooted in vitro on BM.

In a two-week period, in August 1991, the 72 control plants, 48 (cycle 1) and 3 (cycle 3 and 7) plants of each regenerant were transferred to 12 cm ϕ pots and covered with translucent cups for one week for acclimatization to greenhouse conditions (Table 1).

Fifty two percent of a total of 759 plants survived the transfer to the greenhouse,

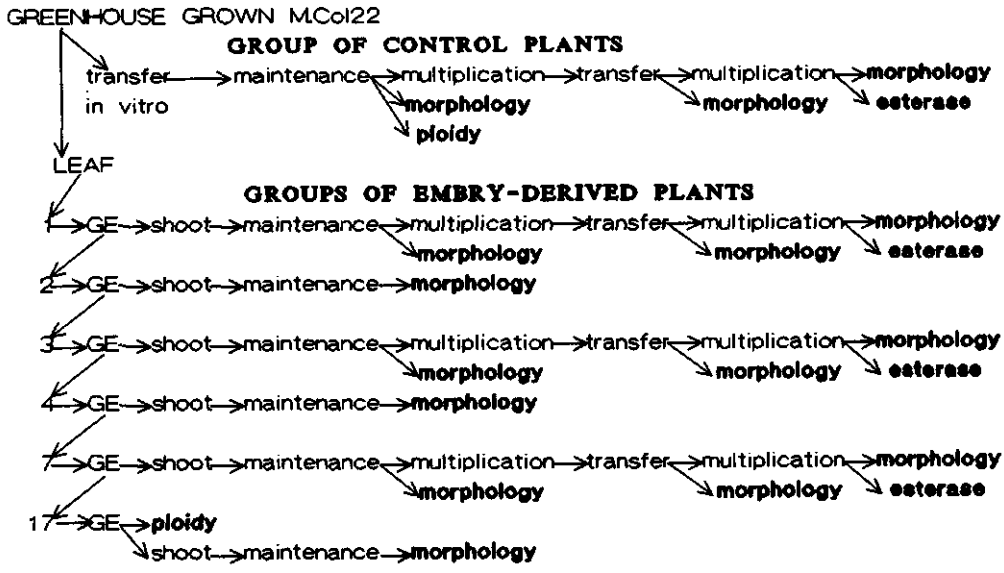

Figure 1. Procedure followed to assess the amount of somaclonal variation.

the rest died from a fungal disease or wilting. For the regenerants this figure varied from 35 and 84 percent (Table 1). These differences were caused by climatic differences during the period of transfer. From 105 regenerants at least one plant survived the transfer to the greenhouse. In the text these plants will be described with mother plants.

The mother plants (358 plants of 105 regenerants and 37 control plants) were multiplied in the greenhouse after 7 months of growth to minimize possible epigenetic effects of the in vitro culture on their performance in the greenhouse. Cuttings, taken 2 cm below the apex consisted of 2-4 nodes and were 3-10 cm long and 0.5-1.5 cm thick. The distal end was immersed in Rhizopon A powder (indolbutyric acid) for stimulation of rooting. To avoid excessive loss of moisture, both ends of the cuttings were covered with paraplast. Cuttings were placed in 44 cm ϕ pots. During the first month air humidity was kept at 80 percent, and hereafter at 70 percent. Ninety-two percent of the in total 766 cuttings sprouted, the rest shrivelled. The plants were fertilized once with Nutrifol (2:1:1) after twelve months of growth. In the section results these plants are described as cuttings from mother plants.

EVALUATED CHARACTERS AND STATISTICAL ANALYSIS

According to the descriptor list issued by IBPGR for cassava [71], all control plants and plants of regenerants were evaluated for 41 quantitative and qualitative morphological characters (Table 2).

Table 2. List of 41 evaluated qualitative (normal script) and quantitative (italic) characters of greenhouse (1) and in vitro (2) grown plants.

leaf characters	
colour of unexpanded leaf	1, 2
colour first expanded leaves	1, 2
shape of leaf lobe	1, 2
leaf below apical branching	1
place of highest width	1
anthocyanin pigmentation petiole	1, 2
stipulae	1
hairs	1
length of leaf lobes	1
width of leaf lobes	1
length/width ratio of leaf lobes	1
number of leaf lobes per leaf	1, 2
number of leaves	1
shoot characters	
virus symptoms	1, 2
shape of stem	1, 2
growth habit of stem (zig-zag)	1, 2
basal branching (side shoots)	1, 2
esterase enzyme pattern	1
length of shoot	1
internode length	1
flower characters	
first level apical branches	1, 2
second level apical branches	1
abortion of flowers	1
colour of sepals	1
length of first apical branch	1
levels of flowers	1
number of 1st level branches	1
root characters	
form	1
constrictions	1
position	1
surface texture	1
length	1
diameter	1
ease of periderm removal	1
ease of cortex removal	1
colour of outer surface cortex	1
colour of inner surface cortex	1
number of roots	1
total fresh weight of roots	1
fresh weight per root	1
dry matter content	1

Most leaf and shoot characters were evaluated twice; 7 months after transfer of in vitro plants to the greenhouse (mother plants) and after 3 months of growth of cuttings derived from the mother plants. Root and flower characters were evaluated once: after 6 months of growth of the cuttings.

The data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS). Significant differences between means of quantitative characters were determined using the Duncan Multiple Range test. Significant differences for qualitative characters were determined using Pearson Chi-square test.

PLOIDY LEVEL DETERMINATION

DNA content was determined in different explants. To isolate nuclei, somatic embryos, non-embryogenic callus, leaves of regenerants and control plants were chopped with a razor blade in a solution containing 15 mM hepes, 1 mM EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 300 mm sucrose, 0.2 % triton X-100, 15 mM dithiothreitol, and 2 mg/l DAPI. The nuclei were filtered through miracloth (40 μm). The flow cytometer ICP22 (Ortho Diagnostic Systems, B-2340 Beerse, Belgium) was used. The fluorescence of individual nuclei was assessed and the distribution was represented by a histogram of DNA content in arbitrary units. For each individual sample at least 2000 nuclei were analyzed. The analysis was executed by Plant Cytometry Services (P.O. Box 299, 5480 AG Schijndel).

ISO-ENZYME ELECTROPHORESIS

Young stem and leaf material of greenhouse grown control and embryo-derived plants were analyzed for the enzyme esterase after maceration in 5 % mercaptoethanol. The extracts were centrifuged (13000g) for 5 min at 4 °C and 1 μL of the samples was applied on a continuous polyacrylamide gel system. After running the gels in an automated system (Phast System, Pharmacia LKB) they were stained for esterase activity.

RESULTS

PLOIDY LEVEL OF LONG-TERM EMBRYOGENIC CULTURES

In cassava the embryos originate directly from the leaf or embryo explant. Apart from newly initiated embryos also callus is formed on the explants. Seventeenth cycle embryos and callus (15 samples each) of independent origin were used for DNA determinations by flow cytometry. The youngest leaves of in vitro maintained plants were taken as a reference. The results of three different explants are given in Fig.2. All samples gave one major (2C) and one minor peak (4C) of fluoresceine, as presented in Fig. 2, which is a strong indication that the phenomenon of chromosome doubling is absent, even in the callus phase.

MORPHOLOGICAL EVALUATION OF REGENERANTS IN VITRO

As was reported previously [Chapters 3 to 5], the shoots initially derived from somatic embryos were frequently malformed. They had swollen, thickened and irregularly shaped leaves and stems. However, the

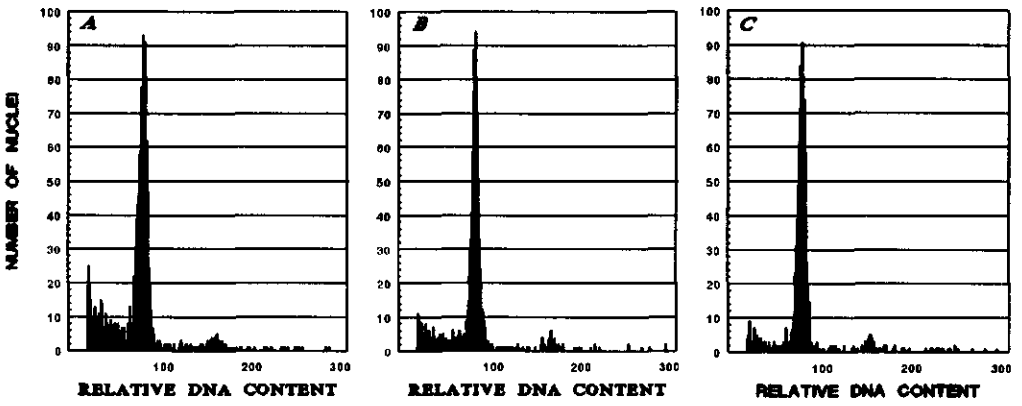

Figure 2. Histogram of DNA-content of control plant (A), seventeenth cycle embryos (B) and callus (C).

malformations disappeared after prolonged culture of these shoots. Circa 3000 in vitro plants of 485 independent regenerants, except one, did not differ from control plants for the 10 evaluated characters (Table 2). Apical branching, which is characteristic for the Euphorbiacea family and which is associated with flowering, did not occur in vitro. Also swelling of the roots which is indicative for tuber formation was not observed in these in vitro plants. One seventh cycle regenerant clearly deviated from control plants. Initially its leaves had a normal green colour, but after subculture two of the four cuttings produced plants with variegated leaves while the other two still had normal green leaves. The green plants remained green after further subculture, whereas the variegated plants gave rise either to plants with normal green, variegated or albino leaves (Fig. 3a). After several subcultures sometimes green spots appeared on the leaves of albino plants (Fig. 3b). The albino plants, as expected, grew more slowly than the green plants and did form roots.

MORPHOLOGICAL EVALUATION OF REGENERANTS IN THE GREENHOUSE

Virus-like symptoms

In the winter of 1991 it became obvious that the originally greenhouse grown M.Col22 plant of which the embryogenic procedure was started, was virus infected. Its leaves became chlorotic and had necrotic spots. Three months after transfer of the in vitro plants to the greenhouse the same virus-like symptoms were observed in the mother plants (Fig. 3c). A significantly ($p = 1\%$) higher percentage of plants of the control group (66 percent) had shown these symptoms than plants of the three groups of regenerants (3-11 percent).

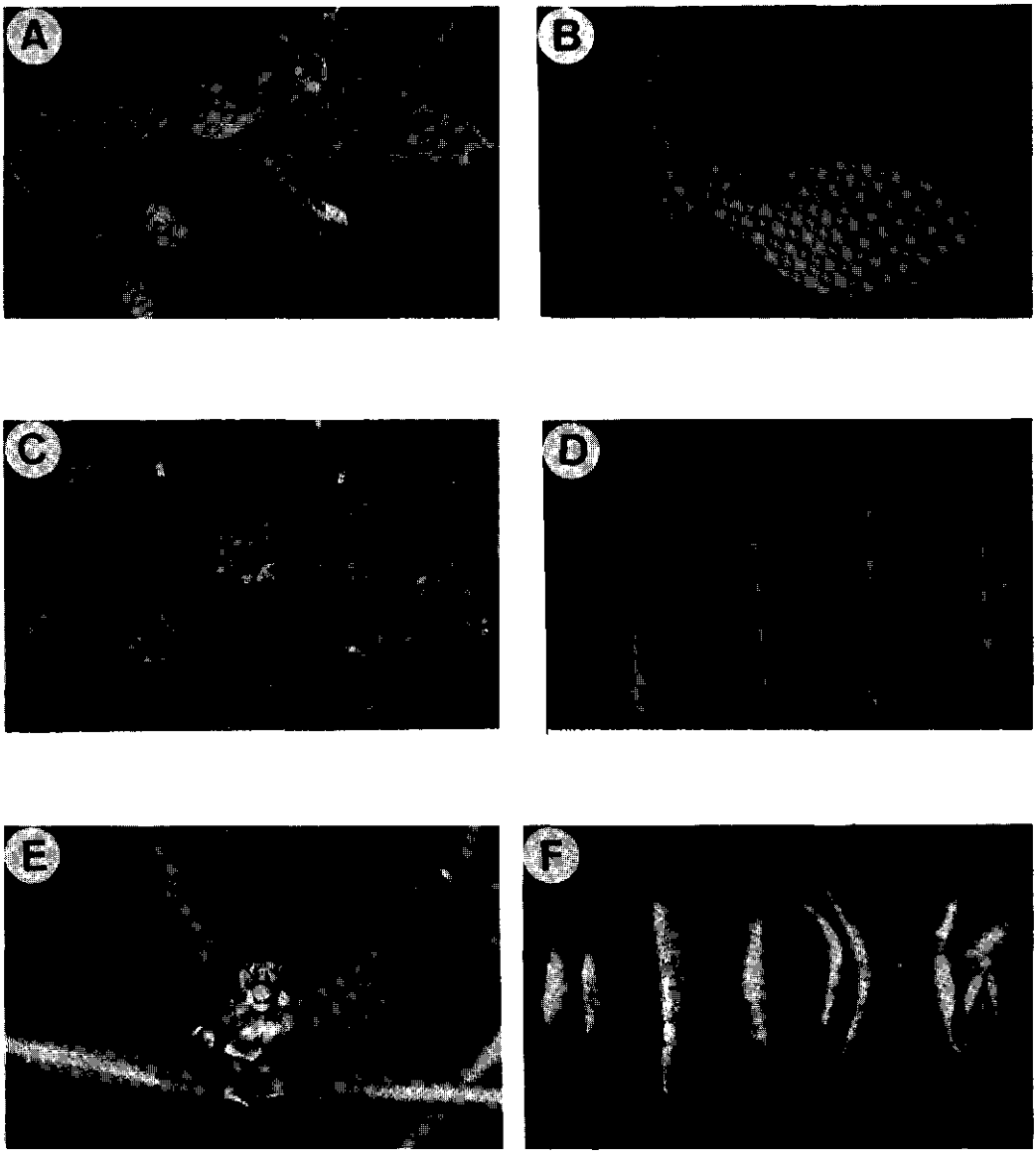

Figure 3. Morphology of embryo-derived plants. **A)** variegated (top) and albino-like plant (bottom). **B)** Detail of leaf of albino-like plant. **C)** Motherplants 3 months after transfer of in vitro to the greenhouse. **D)** different leaf shapes observed after 3 months of growth (from left to right: oblanceolate, elliptic, pandurate, lanceolate. **E)** Development of flowers in the branch point. **F)** Different root forms observed (from left to right: conical, conical-cylindrical, fusiform, irregular)

Four months later, just before cuttings were made from the mother plants, more than 90 percent of the mother plants of the control group and the three groups of regenerants showed symptoms (Fig.4). One month after growth of the cuttings, 84 percent of the control group and between 14 (seventh cycle) and 46 (first cycle) percent of the regenerants had symptoms. Two months later this was the case with more than 95 percent of the plants. However, in the control group these symptoms were significantly ($p = 1\%$) more severe than in the three groups of regenerants (Fig. 4).

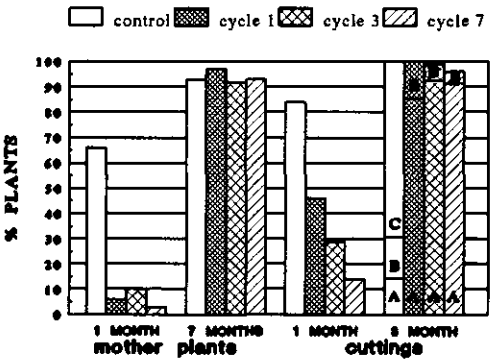

Figure 4. Percentage of plants of control group and the three groups of regenerants with virus-like symptoms (A = chlorosis youngest leaf, B = chlorosis whole plant, C = chlorosis and necrosis).

Shoot and leaf characters

Mother plants of the control group had a length of 11.4 cm after 7 months of growth. Those of the three groups of regenerants were significantly ($p = 1\%$) longer, between 28 and 42 percent (Table 3). With time the differences between the control and the regenerants became less pronounced. The cuttings derived from the mother plants of the control group had a length of 45 cm after 3 months of growth. Only the shoots of third and seventh cycle regenerants were significantly ($p = 1\%$) larger (Table 3). After 6 months of growth of the cuttings no significant differences between the control group (length of 94 cm) and the three groups of regenerants were observed (data not shown).

During the period of observation, the length of the central leaf lobe of the first expanded leaf was doubled or even more whereas the width remained constant. This is reflected in the shape of the leaf lobes. Thirty eight percent of the mother plants of the control group (after 7 months of growth) had elliptic, 53 percent pandurate and the remaining 9 percent oblanceolate or lanceolate shaped leaves (Fig. 3d). The shape of leaves of regenerants of the first ($p = 1\%$) and seventh cycle ($p = 10\%$) differed significantly from the control group. As the plants grown from the cuttings became older the leaves became more uniform. After 3 months of growth of the cuttings derived of the mother plants, 98 percent of the control plants had pandurate and only 2 percent elliptic shaped leaves. There were no significant differences with the regenerants (Table 3).

The mother plants were evaluated for 12 leaf and shoot characters. Significant differences in 7 of the 12 characters were observed between the control and at least one of the three groups of regenerants

Table 3. Level of significance of differences in qualitative and quantitive (italics) leaf and shoot characters between control and embryo-derived greenhouse grown plants (for significant differences between regenerants and control for quantitative characters the procentual difference is given in bold).

	mother plants (7 months after transfer to the greenhouse)			cuttings (3 months old)		
	cycle 1	cycle 3	cycle 7	cycle 1	cycle 3	cycle 7
leaf characters						
colour unexpanded leaves	ns	ns	ns	ns	ns	ns
colour of first expanded leaf	##	##	##	ns	ns	ns
shape of leaf lobe	##	ns	#	ns	ns	ns
leaf below apical branching	-	-	-	ns	ns	ns
place of highest width	##	ns	ns	ns	ns	ns
anthocyanin pigmentation petiole	-	-	-	ns	ns	ns
stipulae	-	-	-	ns	ns	ns
hairs	-	-	-	ns	ns	ns
length of leaf lobes	**##+14**	**##+15**	**#+8**	ns	ns	ns
width leaf lobe	ns	**##+11**	**#+8**	**##-10**	**##-6**	**##-6**
length/width leaf lobe	**#+12**	ns	ns	ns	ns	ns
number of leaf lobes per leaf	ns	ns	ns	ns	ns	ns
number of leaves	-	-	-	ns	ns	ns
shoot characters						
virus symptoms						
shape of stem	ns	ns	ns	ns	ns	ns
growth habit of stem (zig-zag)	ns	ns	ns	ns	ns	ns
basal branching (side shoots)	ns	ns	ns	ns	ns	ns
length of shoot	**##+28**	**##+38**	**##+42**	ns	**##+18**	**##+17**
internode length	-	-	-	ns	**#+12**	**#+7**

ns: not significant, #, ##: significant at 10 and 1% level respectively (qualitative characters Chi square and quantitative characters Duncan multiple range test)

(Table 3). Three months later when the cuttings derived from the mother plants were evaluated, only two characters differed significantly (plant length, and leaf width). At the same moment plants were evaluated for 6 more characters, of which only one (internode length) differed significantly (p = 10%) between the control group and the three groups of regenerants (Table 3).

Flowering characters

Only the cuttings derived from the mother plants were evaluated for flowering characters. The control group differed significantly with one or more of the three groups of regenerants for 2 of the 7 characters (Table 4). Eighty nine percent of the plants of the control group had formed either two (85 percent) or three (15 percent) apical branches, which in cassava is indicative for flower induction. The length of the stem below the first apical branch in the control group was 79 cm, which was significantly (p = 10%) smaller than the groups of third and seventh cycle regenerants (Table 4). All plants with apical branching formed flowers, but on most plants they aborted in an early stage of

development (Fig. 3e). In only 24 of the 700 plants flowers developed sepals. Capsules were formed on only 4 plants. In 43 percent of the plants, the first level apical branches, branched again (second level), sometimes up to 4 levels. The mean level of apical branching was significantly higher (p = 10%) in first cycle and significantly lower in third and seventh cycle regenerants than in the control group.

Table 4. Significance level of differences in qualitative and quantitative (italics) flowering and root characters between control and embryo-derived plants after 6 months of growth of cuttings (for significant differences between regenerants and control for quantitative characters the procentual difference is given in bold).

	cycle 1	cycle 3	cycle 7
flower characters			
1st level apical branching	ns	ns	ns
2nd level apical branching	ns	ns	ns
abortion	ns	ns	ns
colour of sepals	ns	ns	ns
levels of flowering	**##+20**	**##-11**	**##+9**
number of 1st level branches	ns	ns	ns
length to 1st apical branches	ns	**#+11**	**#+11**
root characters			
form	ns	**#**	**#**
constrictions	ns	ns	ns
position	ns	ns	**#**
surface texture	**#**	**##**	**##**
length	ns	**#**	ns
diameter	ns	ns	ns
ease of periderm removal	ns	ns	ns
ease of cortex removal	ns	ns	ns
colour outer surface cortex	ns	ns	ns
colour of inner surface cortex	ns	ns	ns
number of tuberized roots	**#+9**	**#+22**	**#+20**
fresh weight roots	**##+33**	**##+22**	**##+22**
fresh weight per root	**#+21**	ns	ns
dry matter content	ns	**#-11**	ns

ns: not significant ##, #: significant at 1 and 10% respectively in Chi square test (qualitative characters) and Duncan multiple range (quantitative characters).

Root characters

Only the cuttings derived from the mother plants were evaluated for root characters. The control group differed significantly with at least one group of the regenerants for 7 of the 14 root characters (Table 4). The three groups of regenerants had a significantly (p = 10%) higher number of tuberized roots and a higher (p = 1%) total fresh root weight than control plants, but dry matter contents was similar for first and seventh cycle and significantly (p = 1%) lower for third cycle regenerants (Table 4).

In ninety two percent of the control plants the root form was conical to cylindrical and 8 percent had fusiform roots. In the three groups of regenerants, between 51 and 77 percent of the plants had conical to cylindrical, 19 to 26 percent fusiform roots and 4 to 23 percent had irregular formed roots (Fig. 3f).

Ninety two percent of the control plants had roots which tended to grow horizontally, compared to 54 to 83 percent of the regenerants. Only the difference between control and seventh cycle regenerants was significant (p = 10%). For both the root characters, form and growth direction, the difference between the control group and the regenerants was highest for the seventh and lowest for the first cycle regenerants. For example only 4 percent of the plants of first cycle regenerants and 23 percent of the plants of seventh cycle regenerants had irregularly formed roots. Irregularity of root form was not exclusive for all plants of one regenerant. Plants with normal shaped roots were also found,

indicating a non-genetic origin of the variation.

Esterase iso enzyme patterns

Because stem material gave more reproducible bands than leaf material, the stem material from only one plant of each regenerant was analyzed. No differences were found in the numbers and relative mobility of bands between embryo-derived and control plants (data not shown).

INFLUENCE OF EMBRYOGENIC CULTURE PROCEDURE ON MORPHOLOGY OF REGENERANTS

The third cycle regenerants were obtained from embryos which had been cultured on shoot development medium supplemented with 0.1 or 1 mg/l BA. The BA concentration had no significant influence on 40 of the 41 qualitative and quantitative leaf, shoot, flower and most root characters (results not shown). Only, the character root form differed significantly ($p = 10\%$). The percentage plants with irregular or fusiform roots increased from 24 to 50 percent by using 1 mg/l BA instead of 0.1 mg/l BA.

The seventh cycle regenerants were converted from embryos which had been cultured on solid or liquid step 1 and 2 medium for three cycles. These two different culture regimes were not accompanied by significant differences for 39 of the 41 qualitative and quantitative root, shoot and leaf characters. Only the root characters, constrictions and diameter, differed significantly ($p = 10\%$) between the two treatments.

DISCUSSION

The results indicate that the process of direct embryogenesis in cassava is accompanied by somaclonal variation (SV). SV can be either genetic or epigenetic [104]. A clear example of genetic SV is variegation of leaves, which was observed in one of the 485 regenerants that were evaluated. Variegation of leaves is observed in numerous species and in some ornamental crops and this is exploited commercially by breeders [211]. The variegated cassava phenotype was not observed in leaves from the primary but in those of the secondary meristem, indicating that the embryo was chimeric for this trait. Whether the variegation originated as a mutation in the nuclear or in the plastid genome can only be elucidated by analyzing the progeny of reciprocal test crosses.

Despite the high 2,4-D concentration used to maintain embryogenicity, the ploidy level of long-term cultured embryos stayed normal. Even the callus, which is an undesired side-effect in cassava, because it is non-embryogenic and inhibits the development of embryos [Chapter 3], had the normal ploidy level.

One hundred and five randomly selected regenerants were grown in the greenhouse for 13 months during which they were evaluated for 41 leaf, shoot, root and flower characters. Differences from the control were observed in the majority of the leaf and shoots characters, but most of them disappeared with age. Although only a few plants of the regenerants appeared to be free from virus-like symp-

toms, it was clear that these were more severe in control plants than in regenerants. Fewer virus-like symptoms are probably associated with most of the differences found for leaf and shoot characters. The number of plants without symptoms might be increased if special care is taken during the in vitro culture to avoid cross infection and if, instead of leaves, meristems are used as starting explant. In *Euphorbia pulcherrima*, virus free plants were obtained from meristems of highly virus infected plants [162] cultured for somatic embryogenesis and the same results were obtained from virus infected nucellus tissue in *Citrus* spp [16].

Despite the fact that almost all plants, controls and regenerants, had formed apical branches indicative for flower induction, only a few plants developed fertile flowers and set seed, independent of the origin. This might be due to the climatic conditions in the greenhouse or it might be intrinsic to the clone used. In Colombia, under more optimal conditions, flowering and seed set of regenerants was comparable with control plants [205].

Regenerants had a higher number of tuberized roots with a higher total fresh weight. The position, shape and texture of the tuber roots were less uniform than in control plants. The fact that plants of one regenerant showed variation for these characters, suggests that the origin is epigenetic and caused by physiological changes during the regeneration procedure. As soon as plants return to their normal physiological state, epigenetically induced SV will disappear. So, despite the fact that cuttings were used, still physiologically induced SV was found. Meins [126] found that two sexual generations were needed before epigenetic variation fully disappeared.

In cassava, embryogenic capacity can be maintained in vitro by cyclic culture of somatic embryos. This kind of culture system has been described in *Aesculus hippocastanum* L. [93], *Arachis hypogaea* L. [10, 43], *Glycine max* [11, 60, 191], *Juglans regia* L. [217], *Medicago sativa* [114, 115], *Picea abies* [133], *Picea glauca engelmanni* [45] and *Trifolium repens* L. [119]. In *Trifolium repens* L. [119] no differences were observed between greenhouse evaluated primary and secondary regenerants in morphological markers, chromosome number, breeding behaviour, iso-enzyme banding patterns and ploidy level [119]. In *Picea abies* L. [133] the ploidy level of fourth cycle regenerants was normal and in *P. glauca engelmanni* [45] complex no differences were observed in the banding pattern of 16 isoenzymes of long-term cultured embryos. However, in *Glycine max* [11, 60, 191] somaclonal variation was evident and heritable.

To our knowledge this is the first report describing extensively the morphology of plants of long-term cultured somatic embryos in cassava. The plants were not affected greatly by the procedure, not even after numerous cycles of embryogenesis. In using the cyclic culture of embryos in cassava for transformation purposes, no laborious procedures are needed to select true-to-type plants.

CHAPTER 9

GENERAL DISCUSSION: SOMATIC EMBRYOGENESIS IN CASSAVA AND PROSPECTS FOR PLANT TRANSFORMATION

PRIMARY EMBRYOGENESIS

Somatic embryogenesis in cassava is a typical example of auxin induced embryogenesis [Chapter 2]). The auxins 2,4-D, Picloram and Dicamba were able to induce primary embryos [Chapter 6], but other auxins as NAA and IAA not [Chapter 3 and 4]. Only a fraction of the induced globular embryos developed into torpedo shaped embryos. Most torpedo shaped embryos germinated (defined the formation of structures as structures with a distinct hypocotyl and large green cotyledons). It was possible to enhance the frequency of germinated embryos by selection of leaf explants at the proper developmental stage [Chapter 3], manipulating the growth conditions of donor plants [Chapters 3 and 6], treating the donor plants in vitro with 2,4-D [Chapter 3] and the use of the appropriate auxin concentrations [Chapters 3 and 4]. In total, 16 clones were tested for primary embryogenesis [Chapter 3 and 6]. All clones, except one, initiated globular embryos; however, in only 12 of them did they develope into germinated embryos indicating that this phenomenon was genotype dependent Chapter 6] as has been shown in other species [2, 88, 147, 168, 236]. Recently, in other research, it was shown that culture of globular embryos of cassava on charcoal supplemented medium stimulated maturation into germinated embryos [122]. In *Glycine max*, *Mangifera indica*, *Picea* sps and *Zea mays* culture of globular embryos on ABA containing medium [12, 37] or on a medium with a higher sugar concentration [23, 37, 47] increased maturation. These additional treatments might also have a positive effect in cassava.

CYCLIC EMBRYOGENESIS

Primary embryos formed large numbers of new embryos on 2,4-D, Picloram or Dicamba supplemented medium, independent of the genotype [Chapter 6]. Cyclic embryogenic cultures of several clones were maintained in vitro for a considerable period of time without loss of embryogenicity, which offers the possibility of large scale propagation. In many species, embryogenesis can be initiated by culturing in liquid medium which reduces labour input. It was estimated that 1 l. of suspension culture of *Daucus carota* contained more than 1 million embryos [129]. In suspension culture young stages of embryos are subjected to a new cycle of embryogenesis and the globules, induced on young embryos, break away easily. Although cassava embryos can be cultured in liquid medium, the results have none of the characteristics of a suspension culture. In cassava, new embryos developed only from torpedo or germinated embryos and they stayed attached to the explant [Chapter 5], most probably because the bulk of them originate from internal cell layers and not from (sub)epidermal ones [Chapter 7]. Specific media adjustments might give cassava cyclic embryogenesis characteristics of a suspension culture.

SHOOT CONVERSION

Auxin driven embryogenesis has in general

the advantage that the developmental processes of shoot formation and induction require different growth regulators and, therefore, are easy to control. In cytokinin and growth regulator free driven embryogenesis it is often difficult to separate these two processes. In the cassava clone M.Col22 embryos developed into shoots at frequencies higher than 50 percent after culture on BA supplemented media [Chapters 2 to 5]. Also the ability of cassava embryos to develop into shoots was genotype dependent [Chapter 3] as was reported for other species [15, 44]. The shoots were at first instance far from normal and characterized by broad, thick and fleshy stems and leaves. This phenomenon is observed in many other species and usually described as precocious germination [5, 8, 56, 223, 224, Chapter 2]. NAA supplemented medium allows a morphologically more normal development of embryos [Chapter 6]. In the cassava clone M.Col1505, desiccation enhanced shoot conversion of somatic embryos [122]. ABA has shown to prevent precocious germination in several other species [5, 12, 45, 223]. Such medium compositions might also increase shoot conversion in cassava clones.

For the formation of roots, shoots had to be transferred to growth regulator free medium. On this medium all shoots, independent of the genotype, developed roots.

CYCLIC EMBRYOGENESIS AND PLANT TRANSFORMATION

CONDITIONS NEEDED FOR PLANT TRANSFORMATION

In plant transformation two conditions should be met. First of all, large numbers of cells should be capable of entering a regenerative pathway and secondly, these cells must be competent to transformation. In practice, genetically modified cells have first to be selected and multiplied before they are stimulated to plant regeneration in order to avoid chimerism. As outlined in Chapter 1 this is one of the key problems of direct embryogenesis. In other species with direct embryogenesis [57,61, 124, 125, 183, 204] this problem was solved by culturing chimeric transformed embryos in a new cycle of embryogenesis [Chapter 1]. In cassava and other species with direct embryogenesis the same strategy could be applied.

THE SITUATION IN CASSAVA

Cyclic culture of embryos in cassava differed in some respects from the species where it was successfully used to isolate transgenic plants. In these species, the cycle duration was relatively short since a new cycle of embryogenesis was started from globular embryos. The embryos originated from (sub)epidermal cells and, at least a portion, developed from single cells [89, 110, 159, 192, 209, 222]. In cassava the cycle duration is relatively long as the new cycle of embryogenesis has to be started from torpedo or mature germinated embryos instead of globular embryos [Chapter 4] and the embryos develop from internal cells and have a multicellular origin [Chapter 7].

In cassava, transformation can be execu-

ted in different steps of the embryogenic procedure. Transformation of germinated embryos during embryo induction (step 1) with either *Agrobacterium tumefaciens* [166] or particle bombardment [59, 166] resulted in a reasonable number of dividing transformed cells. Unfortunately, only very few new embryos were formed, and when they did, there were very few transformed cells. However, subculture of these embryos with small transformed sectors led to the recovery of largely transformed embryos [166]. The majority of the transformed cells did not enter the embryogenic pathway, but instead became callus. Either the embryogenic competent cells had not been transformed or they were not able to express embryogenic competence after transformation.

OVERCOMING THE PROBLEMS IN CASSAVA

An approach to overcome the low embryogenic capacity of transformed cells might be the use of torpedo shaped instead of germinated embryos. In cassava germinated embryos are the optimal stage for the induction of embryos; however, in torpedo shaped embryos, the fraction of embryogenic competent cells might be higher and so could increase the transformation frequency. Furthermore, the embryogenic competent cells of torpedo shaped embryos are not so deeply embedded in cotyledon tissue and, therefore, more easy for *Agrobacterium tumefaciens* to penetrate.

A more gentle wounding treatment which allows the release of *Agrobacterium* virulence inducing compounds but which ensures tissue integrity might be beneficial for the ability of transformed cells to express their embryogenic competence. In *Nicotinia tabaccum* and *Helianthus annuus* [14] Wounding by particle bombardment in combination with transformation with *Agrobacterium tumefaciens* was an effective approach.

Transformation in other steps of the embryogenic procedure might further improve the recovery of transgenic embryogenic competent cells. In step 2 of the embryogenic procedure torpedo shaped embryos develop into germinated embryos. This transition is characterized by a vast increase in the number of cells [Chapter 7]. This process might be used to multiply previously transformed cells. Torpedo shaped embryos with primordial cotyledons should be grown on a medium which allows further development of the cotyledons after transformation. The mature cotyledons can be cultured for a new cycle of embryogenesis.

In step 3 of the procedure germinated embryos develop into shoots. Also this process might be used to combine regeneration and transformation of cells. Both embryos and plant meristems can be cultured on BA supplemented medium to produce multiple shoots [193]. Recent results suggest that the cytokinin TDZ has even a stronger effect than BA on multiple shoot formation in cassava [48]. This approach has been used successfully for plant transformation in *Pisum sativum* [31].

Summarizing, can be said that cassava tissue is amenable for plant transformation and the transformed cells have the capacity to divide. Unfortunately, most of the

transformed cells develop into non-regenerable callus. Culture regimes should be aimed at increasing the recovery of regenerable embryogenic tissue from transformed cells. Most promising is probably the transformation of torpedo shaped embryos and culture of these embryos for cotyledon development. The use of the luciferase-reporter-gene [132] allows a rapid, non-destructive selection of cotyledons with transformed sectors. These sectors will produce solid transformants after culture for a new cycle of embryogenesis. Because of the genotype-independence of cyclic embryogenesis, such a transformation procedure will be applicable to a whole range of agronomically important genotypes.

SUMMARY

SUMMARY

Cassava is one of the major food crops in the tropics. Several of the major problems in cassava can probably only be solved by breeding with cellular and molecular techniques, e.g., the introduction of specific genes (virus resistance, protein content, quality aspects and so on). These genes can be directly applied in existing varieties of vegetatively propagated crops like cassava. Genetic modification requires efficient, genotype-independent regeneration methods. Plant regeneration can be accomplished by two different pathways: organogenesis and somatic embryogenesis. In both organogenesis and embryogenesis, the regenerated structures either originate directly from the explant or indirectly from callus induced from the explant. In most species transformed plants are obtained by indirect regeneration, either by organogenesis or somatic embryogenesis. The callus phase is used to select and multiply transformed cells. Because organogenesis for cassava appeared to be not repeatable, somatic embryogenesis was further investigated. Somatic embryogenesis is defined as the process in which a bipolar embryo is formed which has no vascular connection with the parental tissue. It has been described in more than 200 species [Chapter 1]. It was shown by others that in cassava (primary) embryos originated directly from young leaves or zygotic embryos. Direct embryogenesis has been used successfully in a some species for plant transformation. In these species primary somatic embryos themselves were an excellent source to start a new cycle of (secondary) embryogenesis. Repeated subculture of somatic embryos allowed the development of continuously proliferating embryogenic cultures (cyclic embryogenesis). The phase of embryo proliferation was used to select and multiply transformed cells. An overview of culture regimes which allows continuous proliferation of somatic embryos is given in Chapter 2.

In initial experiments first cycle or primary embryos were formed from young cassava leaf explants derived from greenhouse grown plants. After 10 days of culture nodular or globular embryos were visible. Globular embryos developed into torpedo shaped embryos which germinated after tranfer to the a medium without auxins. Germinated embryos (GE) are defined as structures with a distinct hypocotyl and large green cotyledons. Five of the six tested South American and Indonesian clones formed germinated embryos. The number of germinated embryos produced, was strongly influenced by the genotype and by hardly controlled growing conditions of the donor plants in the greenhouse. The production of the Colombian clone M.Col22 varied between 0 and 22.1 GE per initial leaf explant (GE/IE). The other clones were considerably lower in their response [Chapter 3]. Therefore, M.Col22 was chosen as a model plant.
To create uniform growing conditions in vitro grown donor plants were used as

source for leaf explants. Using the same culture conditions as applied for greenhouse derived leaf explants, this approach gave less variation in germinated embryos but also a much lower production (< 1 GE/IE). Doubling of the 2,4-D concentration in the embryo induction medium increased the production to a maximum of 3.5 GE/IE. The embryogenic capacity of M.Col 22 could be further increased to 6.6 GE/IE by growing donor plants at reduced irradiance. The highest production (9.9 GE/IE) was obtained by a pretreatment of donor plants with 2,4-D, a few days before the isolation of leaf explants.
Another advantage of the 2,4-D pretreatment of donor plants was studied in Nigerian clones. Only 5 out of 11 in vitro grown clones formed globular embryos and only in 2 some of the globular embryos developed into germinated embryos. After 2,4-D pretreatment of the donor plants, 10 out of the 11 clones formed globular embryos and in 8 of them germinated embryos were formed [Chapter 6].

Only torpedo shaped and germinated embryos initiate a new cycle of embryogenesis after reculture on induction medium. Germinated embryos were the best starting material to initiate cyclic cultures [Chapter 4]. Independent of the genotype, germinated embryos formed new germinated embryos at a high rate and the embryogenicity seemed not to be changed after one year of culture [Chapters 4 and 6]. The production of cyclic germinated embryos for M.Col22 varied between 6.8 and 9.9 GE/IE. The production of germinated embryos in liquid medium was significantly higher than on solid medium. Also fragmentation of the initial germinated embryos, before starting a new cycle of embryogenesis, enhanced the production. With both improvements, the production of M.Col22 increased to about 30 GE/IE [Chapter 5].

Culture of torpedo shaped and germinated embryos on BA supplemented medium allowed their development into shoots. As for the induction of new embryos, germinated embryos were also the best material to be cultured for shoot development [Chapter 4]. The frequency of shoot development appeared to be genotype dependent [Chapters 3 and 6]. In the clone M.Col22 more than 50 percent and in the clone Tjurug only 10 percent of the germinated embryos developed into shoots. All shoots, independent of the genotype, formed roots on growth regulator-free medium [Chapters 3, 4 and 5].
Cyclic embryos originated directly from the cotyledons of the somatic embryo by a budding process. The origin appeared to be multicellular. The first embryogenic divisions started with cells in or near the vascular strands. These initial divisions led either directly to a somatic embryo or to meristematic tissue, of which later embryos were formed [Chapter 7].

Almost 500 regenerants of up to the seventeenth cycle embryos were evaluated in vitro for somaclonal variation. Only one regenerant had a visible deviation from control plants (variegated leaves) which

was assumed to be of genetic origin [Chapter 8]. About 110 regenerants were transferred to the greenhouse and evaluated for more than 1 year. Plants of the regenerants showed fewer virus-like symptoms than control plants. The root tubers of control plants were more uniform than that of regenerated plants. Some plants of the regenerants had irregularly shaped roots which were not observed in control plants. Not all plants of a particular regenerant had a abnormal root tuber phenotype and this is a clear indication that the cause of this variation is most probably epigenetic (physiological) and, therefore, is expected to disappear with prolonged multiplications.

Cassava has proven to be amenable for *Agrobacterium*-mediated transformation and the transformed cells are able to divide. Unfortunately, the majority of them developed into callus cells and only a few into embryogenic competent cells. Culture procedures which increase the recovery of embryogenic competent cells from transformed cells together with an efficient non-destructive selection procedure should allow the development of an efficient, genotype-independent transformation procedure. This is of importance for breeding of this vegetatively propagated crop, cassava.

SAMENVATTING

SAMENVATTING

Cassave is een belangrijk voedselgewas in de tropen. De teelt van dit gewas kent een aantal grote problemen die mogelijk alleen maar kunnen worden opgelost met de introductie van specifieke genen met behulp van transformatietechnieken (genetische modificatie). In cassave kan hierbij gedacht worden aan genen voor virusresistentie, opslageiwitten en kwaliteitsaspecten. Deze genen kunnen direct gebruikt worden in bestaande variëteiten van vegetatief vermeerderde gewassen zoals cassave.

Genetische modificatie vereist efficiënte en genotype-onafhankelijke regeneratiemethoden. Regeneratie van planten kan verlopen via twee verschillende wegen: somatische embryogenese en scheut-organogenese. Bij zowel organogenese als embryogenese ontstaan de geregenereerde structuren direct uit het explantaat of indirect uit callus dat daarop gevormd wordt. In de meeste plantesoorten wordt de indirecte regeneratie gebruikt voor het verkrijgen van getransformeerde planten. Hierbij wordt de callusfase gebruikt voor de selectie en de vermeerdering van getransformeerde cellen. Bij enkele plantesoorten wordt directe embryogenese gebruikt voor het verkrijgen van transgene planten. In deze plantesoorten vormen de primaire somatische embryo's wanneer ze uitgelegd worden op een specifiek medium, secundaire embryo's. Door herhaalde subcultuur van somatische embryo's ontstaat een continu prolifererende embryogene cultuur (cyclische embryogenese). De fase van embryo-proliferatie wordt gebruikt voor de selectie en vermeerdering van getransformeerde cellen [Hoofdstuk 1]. Omdat in cassave scheut-organogenese niet herhaalbaar bleek, werd somatische embryogenese verder onderzocht. Andere onderzoekers hebben laten zien dat in cassave de primaire embryo's direct ontstaan uit bladexplantaten of zygotische embryo's. In dit proefschrift wordt een methode beschreven waarmee bij cassave continu prolifererende cultures worden verkregen. In Hoofdstuk 2 wordt een literatuuroverzicht gegeven van het proces van cyclische embryogenese in andere plantesoorten.

In de eerste experimenten werden jonge bladeren van in de kas opgekweekte cassave planten voor de vorming van primaire embryo's gebruikt. Na 10 dagen cultuur op een medium met 2,4-D ontstonden nodulaire of globulaire embryo's. Globulaire embryo's ontwikkelden zich via het torpedo-vormige stadium tot zogenaamde gekiemde embryo's. Deze laatste stap vond plaats op het kiemingsmedium, een medium zonder 2,4-D. Een gekiemd embryo (GE) werd gedefinieerd als een structuur met een duidelijk hypocotyl en grote groene cotylen. Vijf van de zes geteste klonen uit Zuid-Amerika en Indonesië vormden gekiemde embryo's. Het aantal gekiemde embryo's per uitgelegd blad was sterk afhankelijk van het genotype. Omdat M.Col22 de hoogste opbrengst gaf, werd deze kloon gebruikt als een modelplant [Hoofdstuk 3]. Afhankelijk van de omstan-

digheden waaronder de donorplanten werden opgekweekt varieerde de opbrengst van 0 tot 22.2 gekiemde embryo's per initieel explantaat (GE/IE).
Om de groeiomstandigheden van de donorplanten constant te houden, werden M.Col22 planten in vitro opgekweekt. De variatie in opbrengst van embryo's tussen experimenten met bladexplantaten van in vitro planten was lager dan met bladexplantaten van kasplanten. De opbrengst was echter aanzienlijk lager (minder dan 1 GE/IE). Een verdubbeling van de 2,4-D concentratie (tot 8 mg/l) verhoogde de opbrengst tot een maximum van 3.5 GE/IE. De embryogene opbrengst kon verder verhoogd worden door manipulatie van de groeicondities van de donorplanten. Wanneer de donorplanten werden opgekweekt bij een lage lichtintensiteit werden 6.6 GE/IE gevormd. De hoogste produktie werd bereikt als de donorplanten enkele dagen voor isolatie van de bladexplantaten werden voorbehandeld met 2,4-D. Door deze behandeling steeg de opbrengst tot 9.9 GE/IE. De 2,4-D voorbehandeling bleek ook effectief te zijn voor andere klonen. In de standaardbehandeling (zonder 2,4-D voorbehandeling) vormden maar 5 van de 11 Nigeriaanse klonen globulaire embryo's. Als de donorplanten werden voorbehandeld met 2,4-D, dan, vormden 10 van de 11 klonen globulaire embryo's en kiemden de embryo's van 8 klonen [Hoofdstuk 6].

Alleen torpedo-vormige en gekiemde embryo's gaven nieuwe embryo's als ze werden uitgelegd op inductiemedium. Gekiemde embryo's gaven hierbij een hogere opbrengst dan torpedo-vormige [Hoofdstuk 4]. Onafhankelijk van het genotype werden met een hoge frequentie en in grote aantallen nieuwe embryo's gevormd. De embryogeniteit bleef zeker gedurende een jaar op het zelfde niveau [Hoofdstukken 4 en 6]. Voor M.Col22 varieerde de opbrengst tussen 6.8 tot 9.9 GE/IE. De opbrengst kon verder verhoogd worden door vloeibaar in plaats van vast medium te gebruiken en door het fragmenteren van de embryo's voor het begin van een nieuwe cyclus. Met deze beide verbeteringen steeg de produktie van M.Col22 tot ruim 30 GE/IE [Hoofdstuk 5].

Torpedo-vormige en gekiemde embryo's groeiden uit tot scheuten als ze werden uitgelegd op medium met BA. Gekiemde embryo's, gaven evenals bij de inductie van nieuwe embryo's, de beste resultaten [Hoofdstuk 4]. De frequentie van scheutconversie bleek afhankelijk te zijn van het genotype [Hoofdstukken 3 en 6]. Meer dan 50 procent van de gekiemde embryo's van M.Col22 en maar 10 procent van die van Tjurug groeiden uit tot scheuten. Alle scheuten, onafhankelijk van het genotype, vormden wortels als ze vermeerderd werden op medium zonder groeiregulatoren [Hoofdstukken 3, 4 en 5].

Cyclische embryo's ontstaan direct uit de cotylen van het somatische embryo. De ontstaanswijze lijkt multicellulair. De eerste embryogene delingen vinden plaats in cellen die in of vlakbij het vasculair weefsel liggen. Deze eerste delingen leiden of direct tot somatische embryo's of tot een

meristematische massa waaruit later embryo's kunnen ontstaan [Hoofdstuk 7].

Bijna 500 regeneranten die 1 tot 17 embryogene cycli (2 ½ jaar in cultuur) hadden doorlopen, werden in vitro geëvalueerd op het optreden van somaclonale variatie. Eén regenerant, afkomstig van een embryo uit de zevende cyclus, had een duidelijk zichtbare afwijking (gevlekt blad) met een genetische oorsprong. Verder werden in de kas 105 regeneranten uitgeplant en gedurende een periode van 13 maanden geëvalueerd. De regeneranten lieten minder op virusaantasting gelijkende symptomen zien dan de controleplanten. De verdikte wortels van de regeneranten waren minder uniform dan die van de controleplanten. Enkele planten van de regeneranten hadden onregelmatig gevormde wortels. Omdat niet alle planten van een bepaalde regenerant deze onregelmatigheden vertoonden, werd aangenomen dat dit verschijnsel werd veroorzaakt door epigenetische factoren. Waarschijnlijk zal deze afwijking verdwijnen na herhaalde vegetatieve vermeerdering.

Cassave kan getransformeerd worden met *Agrobacterium* en de getransformeerde cellen kunnen zich delen. Helaas ontwikkelden de meeste van de getransformeerde cellen zich tot callus en slechts een kleine aantal tot embryogeen weefsel [Hoofdstuk 9]. Opkweekmethodes die de uitgroei van getransformeerde cellen tot embryogeen weefsel bevorderen, zullen, samen met efficiënte non-destructieve selectiemethoden, het mogelijk maken om te komen tot een genotype-onafhankelijke transformatieprocedure. Dit is van groot belang voor de moderne veredeling van cassave.

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CURRICULUM VITAE

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De studie werd in augustus 1988 afgerond. In juli 1989 begon hij te werken bij de vakgroepen Tropische Plantenteelt en Plantenveredeling. Onder begeleiding van professor Jacobsen, Professor Wessel en dr. Visser heeft hij celbiologisch onderzoek gedaan aan het gewas cassave.
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