REGENERATION AND TRANSFORMATION

OF

CASSAVA (*MANIHOT ESCULENTA* CRANTZ.)

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Bibliographic Abstract: This thesis describes different regeneration systems of cassava. In the first system the embryos were highly organized. The use of the auxins NAA and 2,4-D to induce this organized system of somatic embryogenesis were compared in several genotypes. Bombardment of organized tissues did not result in transformed plants and culture of protoplasts isolated from organized cultures did not result in plant regeneration. In the second system, so called friable embryogenic callus, the embryos are less organized. Protoplasts isolated from friable embryogenic callus regenerated into plants. Bombardment of this friable embryogenic callus with DNA of constructs containing the luciferase gene resulted in transformed tissue. Transgenic tissue was selected using luciferase activity. Transformed mature embryos were multiplied by the organized system of embryogenesis before they were allowed to develop into plants. The transformed nature of the plant was confirmed by PCR and Southern Blot Analysis.

Theorems (Stellingen)

1. Friable embryogenic callus is more successful than somatic embryos as a source for protoplasts which regenerate into plants (This thesis).

2. Development and selection of unorganized cells from organized tissue can probably reduce the genotype dependency for transformation.

3. I admire protoplasts and their contents, particularly the nuclei, because they drag my mind to apprehend the beginning of life (This thesis; Lackie and Dow (1995), Dictionary of Cell Biology P:388).

4. There are many opportunities to integrate conventional and biotechnological approaches to achieve the desired end product like resistance to cassava bacterial blight. (This thesis; Jacobsen (1991), Biotechnological Innovations in Crop Improvement, P:38).

5. When a higher production of carbohydrates has to be achieved in marginal soils with minimum tillage by peasant farmers, cassava is the best crop.

6. The lack of knowledge of the factors controlling somatic embryogenesis, the asynchrony of somatic embryo development, and low true-to-type embryonic efficiency are responsible for its reduced commercial application (This thesis; Pedroso and Pais (1995), Plant Cell Tissue and Organ Culture 43:147-154).

7. Substitution of cassava flour by one third for the imported wheat flour can save to the extent of U$ 150 million per year in Indonesia (FAO, 1993).

8. "It is forgotten that change could be for the better or the worse. Even more important than change is the direction of change and our sense of right and wrong, of truth and falsehood, of justice and injustice and a commitment to prefer right, truth and justice on their antithesis" (Azzam, 1992).

9. "After life's fitful fever, he sleeps well" (William Shakespeare).

10. Unlike students, businessmen and extension workers who go abroad, mingle with others and get a "cultural shock", diplomats do not experience this simply because they are insulated from the local community (Adapted from Weaver (1987), The Advising Quarterly, P:6).

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CONTENTS

LIST OF ABBREVIATIONS

2,4-D = 2,4-dichlorophenoxyacetic acid
ABA = abscisic acid
AARD = Agency for Agricultural Research and Development
A.tum = *Agrobacterium tumefaciens*
bar = Basta-resistance gene
BAP = 6,-benzylaminopurine
BM = basal medium
CIAT = Centro Internacional de Agricultura Tropical
CSE = cyclic somatic embryogenesis
Dicamba = 3,6-dicloro-2-methoxybenzoic acid
DNA = Deoxyribose nucleic acid
EDTA = ethylenediamine-tetra-acetic acid.
GD = Gresshoff and Doy
GUS = ß-glucuronidase
FDA = Fluorescein diacetate
FEC = Friable Embryogenic Callus
IAA = [3-indoly]acetic acid
IBA = 4-[3-indoly]butyric acid
IITA = International Institute of Tropical Agriculture
IL = initial leaf explants
LM = leaf mesophyll
LUC = luciferase
ME = mature embryo
NAA = α-napthaleneacetic acid
NPTII = neomycin phosphotransferase II
PCV = Pack Cell Volume
PE = Plating Efficiency
PEG = poly ethylene glycol
Pic = Picloram = 4-amino-3,5,6,-trichloropicolinic acid
PP = protoplast(s)
PSE = primary somatic embryogenesis
SH = Schenk and Hildebrandt
TDZ (thidiazuron) = N-phenyl-N1(1,2,3 thiadiazolyl)urea
Zea= Zeatin = 6-[4-hydroxy-3-methylbut-2-enylamino]purine

CHAPTER 1

GENERAL INTRODUCTION

Cassava in Indonesia

Cassava (*Manihot esculenta* Crantz) is known as 'ubi kayu' or 'singkong' in Indonesia. It is grown particularly in rural areas, either as monocrop or intercrop. It is a low capital crop and easy to grow even in the marginal land areas with minimum culture practices. Farmers will choose cassava as a main crop if they do not have enough capital or if the physical environment is not suitable for cultivating their land with other crops. However, cassava is a crop of ever growing importance to Indonesia. It has an important role in the economy of rural areas in Indonesia. A large proportion of the cassava production is for the starch-based industry and for animal feed, the rest is for fresh human consumption (Damardjati et al., 1991). The use of cassava will increase in Indonesia in the future due to several reasons, such as high demand for animal feed and industrial purposes.

A general problem of cassava in Indonesia is its low production. Susceptibility to major pests and diseases is a main reason. In wet regions like Western Java and Sumatra the problem of Cassava Bacterial Blight is often very serious, while in dry areas red mite infections are a large problem (Soenarjo et al., 1987). Two types of superior cassava cultivars are required. For industrial purposes a plant is needed with high starch and dry matter content. A non-branching growth habit is preferred for mechanized harvest. For fresh human consumption a low cyanide content is favourable. Other common agronomical traits such as early harvestability, good root shape and broad adaptation to various soils and climatic conditions have also become a major concern in several breeding programmes (Damardjati, 1991; Soenarjo et al., 1987). To solve the above mentioned problems breeding programmes, at the Central Research Institute for Food Crops-Agency for Agricultural Research and Development, in Bogor, Indonesia were initiated. Selection and crossing of advanced clones from a germplasm collection is the first possibility to achieve these goals. A trend of significant changes of cassava utilization in Asia, due to the international market development and domestic demand, will press the breeder to supply the best cultivar/clone in a short time. To fulfil this goal, breeding programmes should not only rely on conventional techniques, but they also need support from other techniques such as mass clonal propagation and genetic modification. In this thesis the main interest was to develop a genetic

modification system.

The availability of a genetic modification system in cassava will have tremendous implications for increasing the economic value of cassava products. To improve the quality of cassava, particularly for the starch industry, genetic transformation is a prerequisite. Modification of starch content and starch composition will also be beneficial for food industries, sweeteners in drinks and for non-food applications like chemical industry (e.g. production of ethanol and fructose), paper-board, textiles, cosmetics and pharmaceutical industry (Visser and Jacobsen, 1993). In recent years, in Indonesia food factories such as bakeries which utilize wheat flour in large quantities have increased. If the ratio of amylose to amylopectin of cassava starch can be manipulated then the wheat flour for bread-making can be partly substituted with cassava flour. This will lead to reduced wheat import and to increased income of the cassava farmer in Indonesia.

Requirements for the development of genetic modification techniques

Three key components are required for developing a reliable genetic modification system. These are: (1) the delivery of DNA into plant cells in such a way that cell damage is minimized to enhance stable transgene integration into the recipient genome and to permit cell proliferation leading to transformation events; (2) the availability of appropriate selectable markers, or reporter genes which have no detrimental effects on metabolism and which are suitable to multiply and isolate individual transformation events (for review see Wilmink and Dons, 1993); (3) the development of a culture system where (transformed) cells are efficiently converted to plants (DeBlock, 1993; McElroy and Brettell, 1994; Songstad et al., 1995). Over the past years several transfer techniques of DNA to plant cells have been developed such as silicon fibers (Kaeppler et al., 1990), intact tissue electroporation (DeKeyser et al., 1990), microinjection (DeLaat and Blaas, 1987) and electrophoresis (Griesbach and Hammond, 1993). The most commonly used and potentially-applicable ones are *Agrobacterium*-mediated gene delivery, microprojectile/particle bombardment and protoplast electroporation (see Table 1 for some examples).

Table 1. Selected examples of successful transformation systems in some plant species.

Species	Explants	Methods[a]	Outcome[b]	Reporter gene	Reference
Carthamus tinctorius	cotyledons	A.tum	st + r	nptII + gus	Orlikowska et al., 1995
Carrica papaya	somatic embryos	Pb	st + r	nptII + gus	Fitch et al., 1994
Cucumis sativus	protoplasts	Peg + El	tr + c	cat + gus	Wieczorek & Sanfacon, 1995
Nicotiana tabacum	protoplasts	Peg + El	st + r	nptII + gus	Spörlein and Koop, 1991
Solanum tuberosum	stems	A. tum	st + r	nptII	Visser et al., 1989
Vitis vinifera	somatic and zygotic embryos	Pb	st + r	nptII	Scorza et al., 1995
Oryza sativa	protoplasts	El	st + r	gus	Shimamoto et al., 1993
	immature embryos	Pb	st + r	gus	Christou and Ford, 1995
	protoplasts	Peg	st + r	gus + bar	Cornejo et al., 1993
Triticum aestivum	suspensions	El	st + r	gus + bar	He et al., 1994
Zea mays	suspensions	Pb	st + r	nptII + bar	Register et al., 1994

a: A.tum=A.tumefaciens, El=electroporation, Pb=particle bombardment, Peg=polyethylene glycol. b: c=callus, r=regenerated, st=stably transformed, tr=transient.

Gene transfer techniques

Agrobacterium-mediated transformation

The *Agrobacterium tumefaciens* DNA delivery system is the most commonly used technique. It probably relates to the first invention of DNA delivery in plants by this method. Initially it was limited to Kalanchoe and *Solanaceae*, particularly tobacco. Nowadays, the use of *Agrobacterium*-mediated transformation has changed dramatically, it is possible to transform a wide range of plants with a limitation in monocots (reviewed by Wordragen and Dons, 1992). Although cassava is a host for *Agrobacterium* it has proven to be not highly amenable to it (Table 2).

Protoplast and electroporation-mediated transformation

In principle protoplasts are the most ideal explants for DNA delivery. They can be cultured as single cells that produce multicellular colonies from which plants develop. Plants derived from protoplasts are generally clonal in origin. This provides a useful tool for any transformation system, because it will eliminate chimerism in transgenic plants. The use of protoplasts is, however, hampered by the regeneration system which is highly species dependent. For

transformation, protoplasts can be used in conjunction with PEG to alter the plasma membrane which causes reversible permeabilization that enables the DNA to enter the cytoplasm as was demonstrated, for example, in *Lolium multiform* (Potrykus et al., 1985) and *Triticum monococcum* (Lörz et al., 1985). Another technique to increase the permeability of plasma membranes and even cell walls to DNA is by electroporation (for review see Jones et al., 1987). In this method electrical pulses enable the DNA to enter the cells. Rice was the first crop in which fertile transgenic plants resulted from protoplast electroporation (Shimamoto et al., 1989). Electroporation, like particle bombardment has the advantage that also intact tissues can be used as target cells (Abdul-Baki et al., 1990; Dekeyser et al., 1990; McCabe et al., 1988). This reduces the problem associated with regeneration to a minimum and provides the technology applicable to a wider range of species. In cassava electroporation of tissue has so far not resulted in stably transformed plants (Luong et al., 1995) (Table 2). A real bottle neck is regeneration of protoplasts via callus into plants.

Table 2. Cassava transformation and its results (1985-1995)

Explants	Methods[a]	Outcome[b]	Reporter gene	References
Leaf-discs	Pb	tr	gus	Franche et al., 1991
Stem-discs of greenhouse various tissues of in vitro plants and somatic embryos	A.tum+Pb	partial trf	gus	Fauquet et al., 1993
Somatic embryos	A.tum	partial trf	gus	Raemakers et al., 1993
Somatic embryos	A.tum+Pb	trf	gus+nptII	Sarria et al., 1995
Somatic embryos	A.tum	tr	gus+nptII+bar	Chavarriaga et al.,1993
Protoplasts	El	tr	gus	Cabral et al., 1993
Somatic embryos	A.tum	partial trf	gus+bar	Sarria et al., 1995
Somatic embryos	A.tum	tr	gus	Arias et al., 1995
Axillary nodal buds	Pb	tr	gus+nptII	Konan et al., 1995
Somatic embryos	El	tr	gus	Luong et al., 1995
Embryogenic suspensions	Pb	tr	gus+nptII	Schöpke et al., 1995

a: A.tum=A.tumefaciens, el=electroporation, pb=particle bombardment; b: trf=transformed no information of regeneration; tr=transient; partial trf= transgenic plants are not obtained.

Microprojectile/particle bombardment-mediated transformation

The use of particle bombardment or biolistics to deliver foreign DNA provides an alternative method in cassava transformation. Particle bombardment is the only procedure capable of delivering DNA into cells almost in any tissue. Until a certain level this method will eliminate genotype/species dependency and regeneration problems like they occur in protoplast-mediated transformation. The first transgenic plant obtained by using this method was in tobacco (Klein et al., 1989). Following this successful transformation method, particle bombardment is widely used in plants which are less amenable to *Agrobacterium* infection, particularly monocots. Improvement of several DNA delivery devices to accelerate the particle (microprojectile) has resulted in the most recent model the Biolistic ™ PDS-1000 (Bio-Rad Laboratories, Richmond, Ca). Those devices are available commercially, however the price is relatively high at present. Tungsten or gold particles, coated with DNA, are commonly used as microprojectiles to deliver DNA into the target tissue (recently reviewed by Songstad et al., 1995).

Selection and reporter genes used in genetic modifications

To be able to benefit transformed cells, the gene of interest is coupled to a selectable marker gene. This marker gene is necessary to allow selective growth of transformed cells. Transformed cells are benefited through selection procedures involving selectable-markers. Until recently they were restricted to the expression of genes encoding resistance to antibiotics. Nowadays also genes conferring resistance to herbicides are used (Thompson et al., 1987; Gordon-Kamm et al., 1990).

A number of antibiotics and herbicides has been used as selective agent in plant transformation. In cereals resistance to the herbicide phosphinothricin (PPT) was chosen for the selection of transgenic plants (Cao et al., 1990). In *Carica papaya* (Fitch et al., 1994), *Vitis vinifera* (Nakano et al., 1994; Scorza et al., 1995), maize (Rhodes et al., 1988) and rice (Chen et al., 1987) the neomycin phosphotransferase (NPTII) gene, which confers resistance to kanamycin and related antibiotics (Fraley et al., 1986), was used as a selectable marker.

Reporter genes are useful tools for the analysis of gene expression after a transformation event. The most commonly used reporter genes to analyze transient and stable transformation are the genes encoding ß-glucuronidase (GUS)(Janssen and Gardner, 1990), luciferase (Ow et al., 1986) and anthocyanin (Ludwig et al. 1990). Every type of reporter gene has its own characteristics. GUS is a reporter gene of which the expression is detected by destructing the tissue. The other two reporter genes can be visualized without destroying the tissue.

Regeneration of cassava

True seeds of cassava have hardly been used for plant propagation by farmers because of its high level of heterogeneity which is a result of genetic segregation. Conventional in vitro culture of cassava is almost always accomplished by vegetative propagation of stem and bud cuttings. This is categorized as non-adventitious shoot regeneration where existing meristems are allowed to regenerate into plants.

For genetic modification techniques in cassava the availability of an adventitious regeneration system (somatic embryogenesis, organogenesis) would be of great use. The first successful attempts of organogenesis in cassava were reported by Tilquin (1979) and Shahin and Shepard (1980). However, they were not repeatable by others. Plant regeneration by somatic embryogenesis was first reported by Stamp and Henshaw (1982) and this has been repeated for many cultivars by others as well (Raemakers et al., 1993a; Mathews et al., 1993; Szabados et al., 1987; see also Table 2). Organogenesis is the process by which cells and tissues are forced to undergo changes which lead to the production of unipolar structures, namely shoots or roots, where the vascular system is often connected to the parental tissue (Thorpe, 1990). In contrast somatic embryogenesis leads to the production of bipolar structures containing a root and shoot axis and they develop completely separated from the maternal tissue (Emons, 1994). Somatic embryogenesis may start spontaneously from one somatic cell (Hacius, 1978; Sharp et al., 1980; Wann, 1988), although also a multicellular origin has been described (for review see Raemakers et al., 1995).

Somatic embryogenesis

Somatic embryogenesis is the development of embryos from somatic cells via a systematic series of characteristic morphological stages. The structure of somatic embryos resembles that of zygotic embryos. (see for reviews: Ammirato, 1983; Zimmerman, 1993 and Raemakers et al., 1995). Somatic embryogenesis proceeds either through direct or indirect induction of regeneration (Carman, 1990; Sharp et al., 1980; Wann, 1988; William and Maheswaran, 1986). In direct somatic embryogenesis, the embryos form without an intervening phase of callus growth, while in indirect embryogenesis a callus phase precedes the formation of embryos (Ammirato, 1983). The somatic cells that give rise to embryogenesis are called embryogenic cells. In principle every living cell has totipotency though only a limited number of cells from explants, regenerating protoplasts, or suspensions eventually give structures that exhibit embryogenesis. The frequency of cells that actually give rise to somatic embryos in carrot suspension cultures was not more than 2 % (De Jong et al., 1993). The expression of cells competent to regeneration, rely on the tissue culture environment, such as hormone balance, sugars, amino acids, salt concentrations and physical environment (William and Maheswaran 1986; Nuti Ronchi, 1981; Vasil and Vasil, 1986; Franklin and Dixon, 1994). The methods to find the appropriate conditions for somatic embryogenesis are still the main concern in regeneration research.

In the first report on successful regeneration of cassava plants by somatic embryogenesis a two-step procedure was used (Stamp and Henshaw, 1982; Stamp and Henshaw, 1987). In the first step (induction medium of embryos) leaves or zygotic embryos were cultured on medium with a high concentration of 2,4D enabling direct embryogenesis. In step 2 a low concentration of 2,4D was needed for further development of the embryo. Recent improvements of somatic embryogenesis included the use of different types of auxins like Dicamba and Picloram (Sudarmonowati and Henshaw, 1993), the development of cyclic somatic embryogenesis (Raemakers et al., 1993b) improved methods for germination of somatic embryos (Mathews et al., 1993) and suspension culture (Taylor et al., 1995). Especially the last mentioned system has provided possibilities for the development of a transformation protocol in cassava.

Protoplast culture

Regenerated plants from protoplasts have been reported in many important crops. In the 80's rice was an example of plant regeneration from protoplasts (Abdullah et al., 1986). Nowadays procedures have been developed for the regeneration of plants from isolated protoplasts of potato, wheat, tomato, soybean, cabbage, chicory, lettuce, butterbean, winged bean, cucumber, pea, orchids, citrus, kiwifruit, strawberry, cotton, and some tree species (for reviews see Bajaj, 1989a; 1989b; 1993a; 1993b).

The use of protoplasts in plant transformation requires at least three stages: protoplast isolation, the transfer of genes into the protoplasts and regeneration of transformed protoplasts into functional plants (Gallun et al., 1976). Theoretically protoplasts can be isolated almost from any part of the plant. However, the possibility to isolate protoplasts capable of sustained division and plant regeneration is still restricted to a limited number of plant species and explant sources (Blackhall et al., 1994). Leaf mesophyll is frequently employed as source material for protoplast isolation. Other explants used for protoplast isolation are hypocotyl, stem, petiole, cotyledon, florets, callus, suspensions and somatic embryos (Blackhall et al., 1994; Atree et al., 1989). However, sustained division leading to plant regeneration is not routine for most of the cell type sources from protoplasts of many monocotyledonous species. For that reason, embryogenic cell suspension cultures provide the most commonly used sources for protoplasts in cereals.

Protocols for regeneration of transgenic plants from protoplasts of rice are available (Toriyami, et al., 1988; Shimamoto et al., 1989; Davey et al., 1991) and research in this direction for other crops is still on going. These studies mainly are focused on practical applications, involving protoplasts of a wide range of species. Especially for plants which are not amenable to *Agrobacterium*-mediated transformation, protoplast transformation has proven to be a valuable tool to obtain stable transformants (Simpson and Estrella, 1989; De Block, 1993). From these studies it has become clear that many variable factors play a role in the regeneration of stably transformed plants when using protoplasts in combination with direct gene transfer (Schweiger et al., 1993; Lurquin, 1989, De Block, 1993; Fowke and Cutler, 1994).

Generally, three principal factors which govern the regeneration competence of protoplasts are the plant genotype, ontogenetic state of the explant source and the cultural environment. The latter includes medium composition and growth conditions (Blackhall et al., 1994).

Friable embryogenic callus

The most recent regeneration system of cassava is by the use of friable embryogenic callus. By culturing somatic embryogenic tissue on Gresshoff and Doy (1972) basal medium instead of Murashige and Skoog basal medium (1962) friable embryogenic callus could be obtained (Taylor et al., 1995). For enhanced proliferation, the friable embryogenic callus was cultured in liquid Schenk and Hildebrandt (1972) medium as a kind of embryogenic suspension. Morphologically, friable embryogenic callus is composed of globular embryoids varying in size from 50 -100 μm in diameter. The origin of the globular embryoids has been confirmed from one or a few cells at the surface of the older units. Maturation of friable embryogenic callus into normal somatic embryos has been accomplished although the rate of maturation of embryos is very low (Taylor et al., 1995). High levels of transient expression of the GUS gene were established using friable embryogenic callus as the target tissue for particle bombardment (Schöpke et al. 1995).

From the studies described so far, it is obvious that only appropriate regeneration systems will be amenable to transformation. Therefore, the choice of a regeneration system holds an important role in establishing the transformation of cassava. So far the existing regeneration systems have been combined with all the available methods of transformation (see Table 2), but without success. The establishment of a regeneration system which can be used for transformation is a very important task to be able to improve specific traits in cassava.

Outline of the thesis

In this thesis several strategies were studied to find a method for transformation of cassava. In Chapter 2, the effect of several auxins and explant densities on cyclic somatic embryogenesis is described. Improvements of somatic embryogenesis were based on the comparison with existing methods. The relevant aspects of somatic embryogenesis for cassava transformation are

also discussed in Chapter 2. In Chapter 3 the use of cyclic embryos as a source of protoplasts is described. The possibilities and limitations of protoplast culture as an alternative tool for gene transformation are also discussed. The friable embryogenic callus (FEC) system is introduced in Chapter 4 and it is shown that protoplasts of this tissue regenerate into plants. By combining the protoplast regeneration system with transformation the possibility to obtain stably transformed cassava plants is discussed in this Chapter. In Chapter 5 the friable embryogenic callus was successfully used as target tissue for particle bombardment. The transformed nature of the plants was confirmed by Southern blot analysis. In Chapter 6 germination of embryos induced by the new protocol of somatic embryogenesis described in Chapter 2, is investigated using the desiccation method from Mathews et al.(1993). This improved germination protocol is of importance for the germination of transgenic embryos. In Chapter 7 a general discussion is given.

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CHAPTER 2

COMPARISON OF NAA AND 2,4-D INDUCED SOMATIC EMBRYOGENESIS IN CASSAVA

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ABSTRACT

The two auxins NAA and 2,4-D were compared in their ability to induce somatic embryogenesis in cassava. In 6 genotypes primary somatic embryos were induced from leaf explants cultured on 2,4-D supplemented medium. NAA was not effective for this process. Both NAA and 2,4-D were capable of inducing secondary or cyclic somatic embryogenesis. In all 6 genotypes a higher number of somatic embryos was formed in NAA than in 2,4-D containing medium. Furthermore, the time period to become mature was shorter in NAA than in 2,4-D. In NAA the highest number of embryos was formed if the embryos were subcultured every 15 days, in the procedure with 2,4-D this was 20 days. High density culture in NAA did not result into the formation of embryos, whereas in 2,4-D it leads to the formation of globular embryos. In 2,4-D the newly induced embryos were connected vertically to the explant and in most cases they did not possess a closed root pole. In NAA the embryos were connected horizontally to the explant and the embryos possessed a closed root pole. In some genotypes, repeated subculture of embryos in NAA, resulted in a gradual shift from somatic embryogenesis to adventitious root formation. This shift could be reversed by subculture of the material in 2,4-D.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is after rice, sugar cane and maize the fourth most important crop grown in the tropical areas as a dietary source of carbohydrates for human consumption, animal feed and starch industry. Cassava is cheaper in economic terms than many other crops (Cock, 1985). Cassava has further as advantage that it can be grown in marginal areas where there are major constraints for the production of other root crops and grains (Byrne, 1984; Lynam, 1993). Drawbacks for the further utilization of cassava are the low protein content of the roots, the presence of toxic levels of cyanogenic glucosides in the roots and rapid deterioration of roots after harvest. Because cassava is propagated by cuttings derived from stems of plants in the field, diseases and pests are easily transmitted. Large scale multiplication of healthy genotypes will reduce the yield losses caused by diseases and pests. Genetic modification could be used in those cases where there is no genetic variation available for a trait in the gene pool of cassava by introducing genes with the desired trait from other species.

For these applications regeneration methods should be available. In cassava the only routine method of regeneration is somatic embryogenesis. In cassava, somatic embryogenesis starts with the induction of primary embryos from zygotic embryos (Stamp and Henshaw, 1982) or leaves (Stamp and Henshaw, 1987a, Szabados et al., 1987, Mathews at al., 1993, Raemakers et al., 1993a). The primary somatic embryos can be used as source of explants to initiate a new cycle of embryogenesis (Stamp and Henshaw, 1987b; Raemakers et al. 1993b,c, Mathews et al., 1993). This phenomenon has been described as secondary somatic embryogenesis. 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 (Williams and Maheswaran, 1986). Cyclic somatic embryogenesis is attractive for mass production of clonal plantlets since the multiplication rates generally exceeds those attainable with other tissue culture regeneration systems, like shoot micropropagation. In *Junglans regia* (McGranahan et al. 1988) and in *Glycine max* (McCabe et al., 1988) secondary somatic embryogenesis has been used successfully to obtain transformed plants. 2,4-D is the most commonly used auxin for induction of primary and secondary somatic embryogenesis in cassava. The characteristics of somatic embryogenesis initiated by 2,4-D have several disadvantages for use in either mass propagation

or genetic modification. For use in mass propagation it is important that the system of embryogenesis should be as simple as possible and the embryos should mature readily and develop into plants. Optimal for plant transformation would be a single epidermal cell origin. In cassava, the embryos originate from groups of cells. This group consists of either mesophyllic cells only or mesophyllic plus epidermal cells (Stamp, 1987; Raemakers et al., 1996). In *Glycine max* it was shown that the origin of embryos induced by NAA differed from embryos induced by 2,4-D (Hartweck et al., 1988). In *Cucurbita pepo* NAA induced embryos matured more easily than 2,4-D induced embryos (Jelaska et al., 1985). In this report the effect of the auxin NAA on somatic embryogenesis is compared with 2,4-D in several cassava genotypes.

MATERIAL AND METHODS

Plant Material

The cultivars MCol.22 and MCol.1505 (CIAT, Cali, Columbia), TMS90853 (IITA, Nigeria), Gading, Adira 1 and Adira 4 (kindly provided by Dr. A. Dimyati, AARD-Indonesia) were used in this study. Nodal *in vitro* cuttings (2-3 cm long) were cultured on basal medium (BM) supplemented with 8 g/l Daichin agar. BM consisted of Murashige and Skoog salts and vitamins (1962), and 20 g/l sucrose. The pH was adjusted to 5.7 before autoclaving.

The temperature in the growth chamber was 30^0 C, the photoperiod 12 hours and the irradiance 40 $\mu mol^{-2}s^{-1}$.

Influence of auxins on primary somatic embryogenesis

After 20 days of growth young leaf lobes (0.5-1.0 mm) were isolated from the sprouted nodal explants and cultured in light on solid BM supplemented with 1-8 mg/l 2,4-D or 0.1-40 mg/l NAA (step 1). After 20 days the explants were transferred to BM supplemented with 0.1 mg/l BAP (step 2). Forty leaf explants were cultured per treatment. Embryo induction and maturation was evaluated after respectively 15 and 40 days.

Influence of auxins on secondary somatic embryogenesis

A new cycle of somatic embryogenesis was started by culturing 0.2 g. of chopped mature embryos (0.25-0.50 mm^2) in 300 ml flasks with 75 ml of BM supplemented with 1-8 mg/l 2,4-D or 0.1-40 mg/l NAA. After 20 days the 2,4-D cultured explants were transferred to BM supplemented with 0.1 mg/l BAP. A new cycle of embryogenesis was started after 20 (NAA) or 30 (2,4-D) days. The jars were cultured in light on an orbital shaker (LAB-Line Instruments Inc. Model 3519) at 120 rpm. The experiments were set up as a factorial, completely randomized design with four replications. The number of mature embryos was counted and the presence and morphology of roots was recorded. A mature embryo was defined as an embryo with fully developed, green cotyledons with a clear hypocotyl.

Influence of subculture duration on secondary somatic embryogenesis

The optimal concentration of NAA and 2,4-D respectively 10 mg/l and 8 mg/l (see 2.3) was chosen to determine the influence of the length of an embryo cycle on the number of embryos produced. Ten to 30 days after the start of a new cycle of embryogenesis, embryos of Adira 4 were chopped again and 0.2 g was subcultured in the same medium. The experiment was set up as a factorial, completely randomized design with three replications. The number of mature embryos was counted.

Influence of explant density on secondary somatic embryogenesis

Somatic embryos of Adira 4, subcultured every 15 days in liquid BM supplemented with 10 mg/l NAA or every 20 days in BM supplemented with 8 mg/l 2,4-D, were used to study the effect of explant density. A new cycle of embryogenesis was started by culturing 0.1 to 1.5 g of chopped embryos of Adira 4 in 200 ml flasks with 60 ml of BM supplemented with 10 mg/l NAA or 8 mg/l 2,4-D. The experiment was set up as a factorial, completely randomized design with four replications. The number of mature embryos per flask (ME) was counted. The number of ME per flask divided by the density (g/flask) gives the number of ME per gram (ME/g). The total biomass was recorded, divided into mature embryos, immature embryos, and non-embryogenic callus. Immature embryos were defined as embryos from globular to the torpedo-shaped stage.

Histology of secondary somatic embryogenesis

Mature embryos were slightly fragmented and cultured on solid BM supplemented with 10 mg/l NAA. After 14 days of culture explants were fixed in glutaraldehyde (5%) for at least 2 hours, before dehydrating in a series of alcohol solutions (50, 70, 90 and 100 %) for 30 minutes. After dehydration clearing of the explants was done by keeping them in solutions with different concentrations of infiltration solution and absolute alcohol (3:1, 1:1, 1:3, 1:0, and 1:0) for 2 hours. The explants were then embedded in Technovit 7100 on blocks and after hardening sections of 6 μm in width were made using a Reichart-Jung 2050 Supercut microtome. Sections were mounted on glass slides and stained with Toluidine Blue. Photographs were taken with a Carl Zeiss bright field Axiophot photomicroscope (model number 7082).

RESULTS

Influence of auxin type on primary embryogenesis

Leaf explants cultured on NAA or 2,4-D formed callus which appeared after 4 days of culture. On NAA the callus was soft. Somatic embryos or embryo-like structures were not formed. Instead, all explants formed abundantly adventitious roots. On 2,4-D two types of callus were observed: soft and compact. The soft callus never produced embryos or roots. Depending on the genotype the compact callus had a yellowish or white colour. Parts of this compact callus was organized in embryo-like structures or globular embryos. Genotypic differences were not apparent in the formation of compact callus. After transfer to step 2 medium mature embryos were formed in all genotypes (Table 1).

Mature embryos appeared after 2-3 weeks, except in Adira 1 where it took 8 weeks. The highest number of mature embryos was found on a medium supplemented with 8 mg/l 2,4-D. Genotypic differences were present. Adira 1 gave the lowest numbers of mature embryos and MCol.22 the highest number (Table 1). On a medium supplemented with 8 mg/l 2,4-D this was respectively 0.2 mature embryos per initial leaf explant (ME/IL) and 4.8 ME/IL.

Table 1. The effect of 2,4-D on the formation of mature somatic embryos per cultured leaf explant (40 leaf explants were cultured per treatment).

2,4-D (mg/l)	TMS90853	Adira 1	Adira 4	MCol.1505	MCol.22	Gading
1	-	0.0	0.1 a	0.1 a	0.1 a	0.4 a
2	-	0.1	0.2 a	0.2 a	0.2 a	0.2 a
4	-	0.1	0.5 ab	0.7 ab	2.0 b	0.4 a
8	0.6	0.2	1.5 b	1.5 b	4.8 c	1.7 a

Note: mean value followed by the same letter in a column denotes no significant difference by LSD (0.05) test.

Influence of auxins on secondary somatic embryogenesis

Time to become mature and number of mature embryos formed

In all tested genotypes NAA and 2,4-D induces secondary somatic embryogenesis (Table 2). In 10 mg/l NAA torpedo shaped embryos were already visible after 6 days of culture, and after 14 days most of the embryos were mature. The mean weight of a mature embryo varied between 11 and 92 mg. In 2,4-D the first torpedo shaped embryos were visible after 20 days. For further maturation these embryos were cultured for another 10 days in liquid step 2 medium. After that maturation phase the mean fresh weight per embryo varied between 5 mg and 26 mg. Because of this difference in the development NAA and 2,4-D induced embryos were subcultured for a new cycle of embryogenesis, respectively, after 20 and 30 days. In this way embryos of all tested genotypes were maintained for three months on 10 mg/l NAA. In Table 2 the results of a population of embryos subcultured for at least three months in NAA is given and compared with 2,4-D. Both auxins at a concentration of 0.1 mg/l did not result in mature embryos (data not shown). At a concentration of 1 mg/l 2,4-D only a few were formed (data not shown), whereas with 1 mg/l NAA there was a significant increase in the number of embryos produced. The highest number of embryos was formed with 8 mg/l 2,4-D and 10 mg/l NAA. In all genotypes more embryos were formed in NAA than in 2,4-D. Higher concentrations of both auxins reduced significantly the number of embryos produced.

Root Formation

NAA induced embryos formed roots after several subcultures in NAA. 2,4-D induced embryos never formed roots, even not after 2 years of continuous subculture. The formation of roots was dependent on the genotype and the NAA concentration. TMS90853 and Adira 1 had the highest percentage root formation. In MCol.1505 it was observed in maximum 18% of the mature embryos. In Adira 4 and MCol.22 root formation never occurred and in Gading it was observed occasionally. At 4 and 10 mg/l NAA the highest percentages of embryos had formed roots. It declined with increasing concentrations of NAA. In first instance the roots formed were clearly tap roots and it was not correlated with a decrease in the number of mature embryos. After continued subculture of TMS90853 and Adira 1 embryos in NAA the nature of the roots shifted

Table 2. Effect of NAA and 2,4D on the number of mature embryos and the percentage of embryos with roots (between brackets).

Concentration (mg/l)	Genotype					
	TMS90853	Adira1	Adira4	MCol.22	MCol.1505	Gading
NAA:						
1	59b(42d)	53abc(13a)	45a(0)	34a(0)	22a(6ab)	-
4	213c(70e)	123d(47b)	126c(0)	108ab(0)	74b(13b)	-
10	360d(23c)	292e(35b)	323d(0)	268c(0)	376d(18bc)	327(5)
20	64b(13b)	83bc(5a)	89b(0)	143b(0)	91b(2a)	-
30	17a(0a)	26ab(0a)	60a(0)	44a(0)	38ab(0a)	-
40	9a(0a)	8a(0a)	13a(0)	17a(0)	5a(0a)	-
2,4-D:						
4	100b(0a)	74a(0)	38a(0)	99b(0)	108b(0)	-
8	162c(0a)	123b(0)	119b(0)	187c(0)	148c(0)	151(0)
32	14a(0a)	22a(0)	85b(0)	28a(0)	13a(0)	-

Mean value followed by the same letter in a column (for auxins separately) denotes no significant difference by LSD (0.05) test; 4 replications, per replication 0.20 gam/flask.

to adventitious roots. This phenomenon was not observed in the other genotypes. The adventitious roots grew long and slender, while the taproots were thick and short. The adventitious roots developed from callus and were associated with a decrease in the number of embryos formed. Root formation was not dependent on the developmental stage at which embryos were used to start a new cycle of embryogenesis. Rooted NAA cultures could be made embryogenic

again by subculture in 2,4-D. Already after one cycle in 2,4-D only embryos without roots were formed. If that one cycle in 2,4-D was followed by subculture in NAA than in the next 1 to 2 cycles embryos without roots were formed followed by embryos with taproots which after continuous subculture shifted to adventitious roots.

Influence of time of subculture

The amount of embryos produced was dependent on the subculture regime. The main characteristics are in all genotypes the same and in Table 3 these are given with Adira 4 as example.

Table 3: Influence of subculture duration on formation of the number of new embryos.

Genotype	Auxin	duration in days 10	15	20	30
Adira 4	10 mg/l NAA	830 b	801 b	788 b	207 a
	8 mg/l 2,4-D	65 b	30 a	120 c	79 b

Mean value followed by the same letter in one line denotes no significant difference by LSD (0.05) test, 4 replications, per replication 0.20 gam/flask.

A shorter subculture duration resulted in starting material which consisted of less developed embryos. Embryos cultured in 2,4-D for 10 days were globular shaped and those cultured for 30 days were mature. Embryos cultured for 10 days in NAA were almost mature with light green cotyledons and if cultured for 30 days the embryos were mature with darkgreen cotyledons. The previously used subculture regime of 20 days for NAA was not optimal. In NAA the highest number of embryos was produced if embryos were subcultured for 10 days. For 2,4-D the optimal subculture duration was 20 days. In 2,4-D a relatively large fraction of embryos were single embryos whereas in NAA almost all embryos were multiple embryo clusters. The above described characteristics of different subculture regimes in NAA and 2,4-D were also observed in the other genotypes. In Adira 4 all tested subculture durations gave a higher yield of mature embryos in NAA than in 2,4-D. In other genotypes NAA gave a higher yield from 10-20 days whereas with 30 days 2,4-D outyielded NAA (results not shown). In the

following experiments embryos in NAA were subcultured after 15 days and in 2,4-D after 20 days.

The effect of explant density

The amount of embryos used to start a new cycle of somatic embryogenesis had different effects in NAA and 2,4-D. These effects are shown using Adira 4 as example. In Figs. 1A and B the number of mature embryos (ME) formed is given. It is expressed as number of ME per flask (ME/flask). The number of ME/flask divided by the density (g/flask) gives the number of ME per gram (ME/g). With NAA the number of ME/flask increases up to a density of 0.8 g/flask with 2489 ME (Fig. 1A). Than it decreases to 207 ME/flask at a density of 1.5 g/flask. For 2,4-D the optimal density is 0.2 g/flask with 186 ME. At a density higher than 0.8 g/flask no mature embryos were formed. If converted to ME/g than in both NAA (Fig. 1A) and 2,4D (Fig. 1B) containing medium the highest number of ME/g is formed at a density of 0.1 g/flask. In Figs. 1C and D the total biomass produced per flask is given. With NAA containing medium a density of 0.1 g/flask produced 11.1 g and 89% of the biomass were mature embryos, 5% immature embryos and 6% callus (Fig. 1C). In NAA the amount of biomass production was relatively constant over a density of 0.1 to 1.5 g/flask. At a density of 1.5 g/flask only 14% of the embryos were mature embryos. At this density most of the biomass (58%) was callus. In 2,4-D there is a significant increase in biomass with increasing densities (Fig. 1D). Furthermore, there is a shift from mature embryos at the lower densities to immature embryos at the higher densities. The percentage callus is relatively constant. The mass of immature embryos consisted almost completely of globular embryos. Small amounts of these masses were cultured on solid step 2 medium for maturation. It was found that at a density of 1.5 g NAA has the potential to produce 3000 mature embryos.

Histology of NAA induced embryos.

NAA induced embryos differed from 2,4-D induced embryos in the way they were attached to the explant. In 2,4-D, the shoot pole points vertically upwards and the root pole was not visible since it was embedded in explant tissue (Raemakers et al., 1996). In NAA the shoot-root pole axis of the embryo was almost horizontal to the explant. The broader side of the embryo was

the shoot pole and the more narrow the root pole. In Fig. 3A longitudinal and in Figs 3B and 3C transversal sections are shown. The transversal sections are from the same two embryos: Fig. 3B is from near the shoot pole and Fig 3C from near the root pole. The embryos seem to lie on the epidermis. Near the shoot and root pole the embryo was connected to the explant by a thin layer of cells.

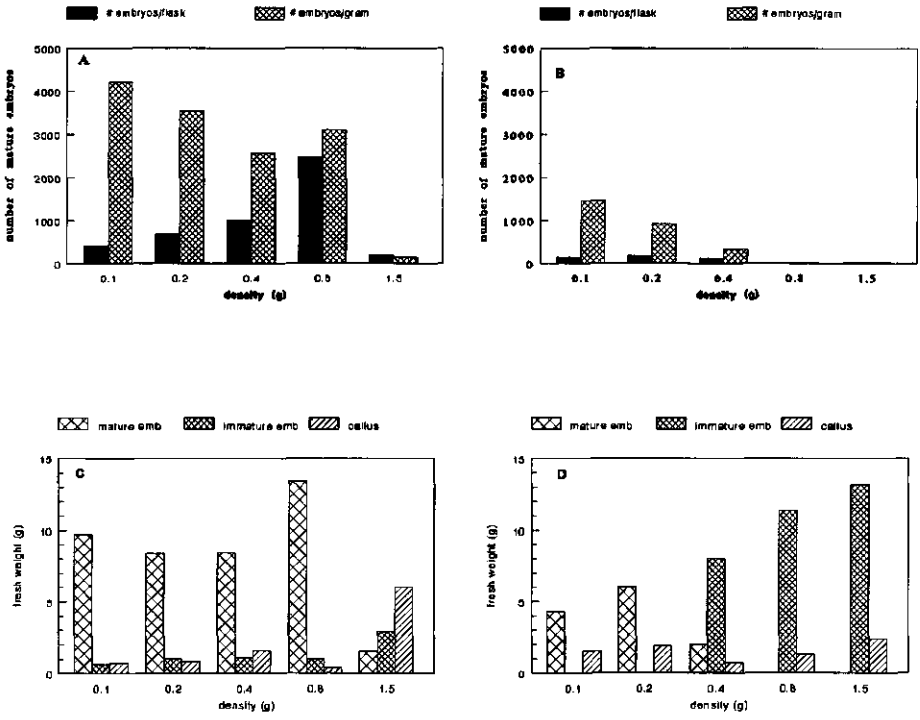

Figure 1: Influence of explant density in 10 mg/l NAA (A) and 8 mg/l 2,4-D (B) on the production of mature embryos and on the fresh weight production in 10 mg/l NAA (C) and 8 mg/l 2,4-D (D).

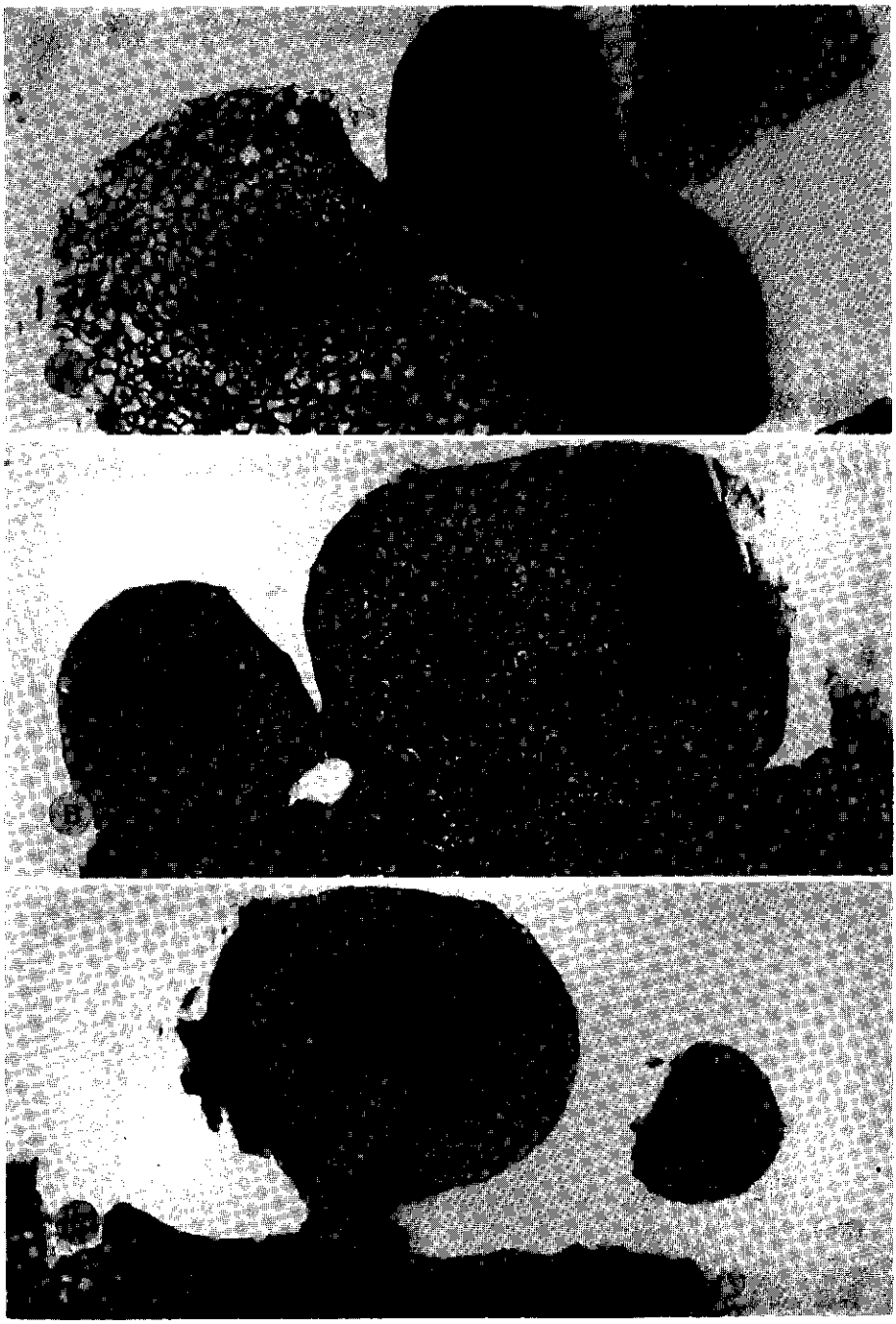

Figure 3. Longitudinal (A) and transverse sections (B, C) of NAA induced embryos (after 15 days of culture).

DISCUSSION

Somatic embryogenesis offers promises for rapid multiplication and plant transformation. For both applications somatic embryogenesis starts with the induction of primary embryos. In cassava NAA does not initiate primary somatic embryogenesis. 2,4-D has that capability and all cassava genotypes tested responded with the formation of mature embryos. Also in *Bambusa oldhamii* 2,4-D has the capacity to induce primary embryogenesis and NAA not. Chang, 1991). However, in *Pisum sativum* (Ozcan et al., 1993), and *Arachis hypogeae* (Ozias-Akins, 1989) NAA is more efficient than 2,4-D in inducing primary somatic embryogenesis. All cassava genotypes formed secondary somatic embryos in a much higher frequency than primary embryos. The usefulness of secondary somatic embryogenesis for plant multiplication is dependent on several variables. One of them is the time period for embryos to become mature and the number of embryos produced. In NAA an embryogenic cycle leading to mature embryos is finished in 14 days compared to 30 days in 2,4-D. Also in *Cucurbita pepo* (Jelaska, 1980) it was found that NAA induced embryos maturated more easily than 2,4-D induced embryos. In all genotypes NAA was initially superior for a high production of mature embryos. However, after several cycles in NAA there is, in some genotypes, a shift from embryogenesis to adventitious rooting. This shift was also observed in some genotypes of *Pisum sativum* (Ozcan et al., 1993) and *Glycine max* (Mante et al., 1989). In other genotypes of *Pisum sativum* (Ozcan et al., 1993) and *Glycine max* (Lazzeri et al., 1987) and in *Cucurbita pepo* (Jelaska, 1980) NAA induced embryos have an higher capacity to germinate into normal plants than 2,4-D induced embryos. Also in cassava germination of 2,4-D induced embryos is poor and a slow process. In Chapter 6 the germination ability of NAA induced embryos will be compared with 2,4-D induced embryos.

For transformation it is beneficial to have regeneration of single epidermal cells. In 2,4-D the embryos are formed from groups of cells which originate from internal tissue (Stamp, 1986; Raemakers et al., 1995). Bombardment of 2,4-D induced embryos led to the recovery of chimeric embryos. The chimeric sector was restricted to the epidermis with as a result that these

transformed cells could not be transferred to a new cycle of embryos (results not shown), because the new embryos initiated from deeper cell layers. The NAA induced embryos differed in the way they were attached to the explant. This suggests a different origin. Unfortunately, the histological data of NAA induced embryos gave no evidence for single epidermal cell origin. Bombardment of NAA induced embryos resulted in transient GUS activity of the same level as 2,4-D induced embryos and also with NAA medium the transformed sector of an embryo could not be increased (data not shown). Two other observations might be of importance with respect to transformation. One is the fact that in 2,4-D also globular embryos could be multiplied and the other is that high density culture in 2,4-D leads to the production of large numbers of globular embryos. Maybe a further reduction of the subculture period, combined with high density culture will result in more friable cultures. In such cultures it is more likely that either the embryos originate from single epidermal cells or that they originate from multiple cells but that the epidermis divides anticlinal to contribute to deeper cell layers. Bombardment of such cultures might lead to chimeric transformed embryos of which the transformed sector also encompasses deeper cell layers.

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CHAPTER 3

THE INVESTIGATION OF SOMATIC EMBRYOS AND LEAVES AS SOURCES FOR PROTOPLAST CULTURE IN CASSAVA

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ABSTRACT

While regeneration of cassava from protoplasts is still very difficult, it remains an important possibility for transformation. Therefore more basic knowledge about protoplast culture in cassava is needed. A mixture of the cell wall degrading enzymes cellulase RS Onozuka, macerozyme and pectolyase gave the highest yield of protoplasts irrespective of the tissue source. Protoplasts isolated from somatic embryos and leaf mesophyll from genotypes Adira 1, Adira 4, Gading, TMS90853, Line 11, MCol.22 and MCol.1505 were cultured in different media. A density of 2 x 10^5 protoplasts/ml plated in agarose drops gave the highest plating efficiency. Cell divisions and green calli were easily obtained from protoplasts isolated of both tissue sources. Root formation occasionally occurred but shoots were not observed.

INTRODUCTION

Cassava, *Manihot esculenta* Crantz, belongs to the family Euphorbiaceae. It is grown in a broad range of soils and climatic conditions in the tropics. Cassava is originally a crop of the tropical lowlands, but in the Andes of South America and the highlands of East Africa it is cultivated at 2000 meter or more above sea level, where the average temperature may be as low as 17°C (Jennings and Hershey, 1985). Because of its ability to survive under many types of stress such as soil acidity, drought, low soil phosphorus and low ambient temperature cassava has become an important crop in the tropical areas. Compared to other tuber crops and cereals, cassava is very efficient in producing carbohydrates (Cock, 1985). Since conventional breeding of cassava is hampered due to its heterozygosity, low fertility, poor seed set and low seed germination (Jennings and Hershey, 1985) biotechnological methods such as somatic hybridization and genetic transformation via protoplasts might play an additional role in its future improvement.

Because protoplasts are single cells without cell walls, they are excellent recipients for macromolecules such as DNA (Shimamoto et al., 1989). Therefore, intensive studies to regenerate plants from protoplasts have been performed in many plant species. This resulted in plant regeneration from protoplasts in different species like *Solanum tuberosum* (Shepard et al., 1977) and *Mentha piperita* (Sato et al., 1993). However, *Manihot esculenta* seems to be still recalcitrant for protoplast regeneration. In literature there is only one successful report on protoplast regeneration (Shahin and Shepard, 1980). Until now, no one has been able to repeat this observation despite a number of different unpublished and unsuccessful experiments. Young leaves of cassava were successfully used as starting material for inducing somatic embryos (Stamp and Henshaw, 1987, Szabados et al., 1987; Mathews at al., 1993, Raemakers, 1993). Because of the stagnation of protoplast regeneration in cassava, alternative sources of protoplasts should be investigated other than young leaf material. Somatic embryogenesis in cassava is a reliable system, so that it is worthwhile to be tested as an alternative source of protoplasts.

Somatic embryos apparently originate from embryogenic groups of cells within adaxial leaf explants (Stamp, 1987). In these somatic embryos, the place of embryogenic cells was suggested

to be present at a distance of two to five cell layers from the epidermis (Raemakers et al., 1995). In sorghum (Wernicke and Brettell, 1980), pea (Mroginski and Kartha, 1981), and leek (Buiteveld et al., 1993) it was indicated that the competence of such cells declined as they differentiated into organized tissues. It is also assumed that in cassava the embryogenic cells in somatic embryos are relatively more frequently competent than in young leaf lobes.

Genotype dependence for the ability to produce protoplasts has been reported in many crops including cassava, showing that some genotypes are more amenable, whereas others are more recalcitrant (Shepard et al., 1980; Radke and Grun, 1986). In other recalcitrant species such as *Coffea canephora* (Schöpke et al., (1987), *Citrus mitis* (Sim et al., 1988), *Picea glauca* (Attree et al.,1989), and *Oryza sativa* (Shimamoto et al., 1989) it was shown that only protoplasts of embryogenic tissue have the capacity to develop into plants. In this study somatic embryos and leaf mesophyll of several genotypes were investigated as a protoplast source with the aim to develop a protoplast based regeneration system.

MATERIALS AND METHODS

Plant material and growth conditions

The cultivars used were Adira 1, Adira 4 and Gading from Indonesia (kindly provided by Dr. A. Dimyati, Indonesia (AARD), MCol.22 and MCol.1505 from Columbia (CIAT); the genotype TMS90853 from Nigeria (IITA) and Line 11 from Zimbabwe. First expanded leaves together with the apical bud from in vitro grown shoots and mature somatic embryos (green cotyledons and a distinct hypocotyl) were used for the isolation of protoplasts. Shoots were maintained by regular subculture of nodal cuttings on a medium supplemented with Murashige and Skoog (1962) salts and vitamins (MS), 7 g/l Daichin agar and 20 g/l sucrose. Somatic embryos were induced from leaf explants and maintained by cyclic somatic embryogenesis as described by Raemakers et al. (1993). Shoots and somatic embryos were incubated in a growth chamber with a day/night temperature of 30/28 ^{0} C, a daylength of 12 hours and a light intensity of 40 μmolm^{-2}s^{-1}. Protoplasts were cultured in the same environment unless stated otherwise.

Isolation of protoplasts

Prior to tissue plasmolysis and cell wall digestion, the plant materials were stored for 6 hours in the dark, at 4^0 C. Then, 2 g of harvested leaves was sliced into small strips (1 - 2 mm) and placed in Petri dishes (ϕ 9 cm) containing 10 ml of cell wall digestion solution. Cell wall digestion solution consisted of a mixture of enzymes (Cellulase RS Onozuka 10 g/l, Macerozyme 200 mg/l, Pectolyase 10 mg/l); growth regulators (NAA 1 mg/l, 2,4-D 1 mg/l, Zeatin 1 mg/l); major salts (368 mg/l $CaCl_2$; 34 mg/l KH_2PO_4; 740 mg/KNO_3; 492 mg/l $MgSo_4.7H_2O$); minor salts (19.2 mg/l Na-EDTA; 14 mg/l $FeSO_4.7H_2O$) and osmoticum (91 g/l D-mannitol) and 0.5 g/l MES. These incubation media are indicated by an "e" followed by a number; for example "e5". After 18 h of incubation, 10 ml of washing medium was added to the solution. Washing medium with an osmolarity 0.530 mOsm/kg consisted of major salts (see cell wall digestion solution), 45.5 g/l mannitol and 7.3 g/l NaCl. The digested tissue was filtered through a 73 μM pore size filter (PA 55/34 Nybolt - Switzerland) into a 250 ml beaker glass. The filtrate was divided equally over two 12 ml conical screw cap tubes, and centrifuged at 600 rpm for 3 min (Mistral 2000). The washing procedure was repeated once after removal of the supernatant. The protoplast solution was resuspended by floating on 9.5 ml solution containing major and minor salts (see cell wall digestion solution) and 105 g/l sucrose. The pH was 5.8 and the osmolarity 0.650 mOsm. The solution with protoplasts was allowed to equilibrate for 5 minutes before 0.5 ml of washing medium was gently added on the top. After centrifugation at 700 rpm for 15 min (Mistral 2000), the protoplasts were concentrated in a band between the sucrose and washing medium. The protoplast layer was harvested with a pasteur pipette and the yield was counted in a standard haemocytometer chamber.

Protoplast culture

Four protoplast densities ($1x10^5$, $2x10^5$, $4x10^5$, $6x10^5$ pp/ml) were tested. Protoplast viability was tested by using a Fluorescein diacetate (FDA) staining technique according to Widholm (1972). Plating efficiency was defined as the number of protoplasts that had undergone the second mitotic division according to Anthony et al. (1994). Observations on protoplast growth were done daily until day 10. On the basis of the plating efficiency 2×10^5 pp/ml was chosen for further experiments. Protoplasts were plated in Sea Plaque agarose 0.2 % w/v, disc type

culture (Dons and Bouwer, 1986) in petri dishes (ϕ 9 cm) containing 10 ml of medium A. The Petri dishes were sealed with parafilm. Medium A (medium for cell division and micro callus induction) consisted of MS salts and vitamins, 4.5 g/l myo-inositol, 4.55 g/l mannitol, 3.8 g/l xylitol, 4.55 g/l sorbitol, 0.098 g/l MES, 40 mg/l adeninsulphate and 150 mg/l casein-hydrolysate, 0.5 mg/l d-calcium-panthotenate, 0.1 mg/l choline-chloride, 0.5 mg/ ascorbic acid, 2.5 mg/l nicotinic acid, 1 mg/l pyridoxine-HCl, 10 mg/l thiamine-HCl, 0.5 mg/l folic acid, 0.05 mg/l biotine, 0.5 mg/l glycine, 0.1 mg/l L-cysteine and 0.25 mg/l riboflavine. Glucose (59.40 g/l) or sucrose (36.03 g/l) were used as carbohydrate sources. The cultures were incubated in the dark for 6 days after which they were adapted gradually to the standard light conditions.

Micro-callus culture

After 10 days the agarose droplets with small micro-calli were fragmented and cultured in Petri dishes (ϕ 9 cm) containing solid medium for callus induction (medium B). Medium B consisted of MS (salts and vitamins) solidified with 7 g/l agar (Daichin), 54 g/l D-mannitol, 2.5 g/l glucose, 0.098 g/l MES supplemented with 0.1 mg/l 2,4-D or 0.1 mg/l NAA and 0.5 BAP mg/l. Micro-calli were kept under diffuse light (25 $\mu molm^{-2}s^{-1}$) for 5-7 days before they were grown in standard light intensity (40 $\mu molm^{-2}s^{-1}$). Every 3 - 4 weeks pale-white or creamy calli were transferred to fresh medium B. After two weeks of culture micro-calli were transferred to medium C for callus development and regeneration. Medium C contained MS, salts and vitamins, 7 g/l agar (Daichin) supplemented with (0.01-1 mg/l) IAA, or (0.01-1 mg/l) NAA, or 2 mg/l 2,4-D and 10 mg/l Zeatin, or 0.1 mg/l TDZ, or 0.1-10 mg/l BAP, and 0.3 mg/l GA and vitamins at B5. After 4 and 8 weeks of culture in medium C, calli were evaluated for their colour : white (translucent/pale white/creamy), green (light green, green) and brown; and for their morphological appearance: friable or compact. Green calli were transferred individually to media with different combinations of plant growth regulators to induce regeneration of shoots (medium D) or to fresh medium C for long term storage. Twenty-five individual calli were grown in a Petri dish (ϕ 9 cm) containing medium D. After 4 and 8 weeks calli were evaluated for their development.

RESULTS

Protoplast yield

In pilot experiments different enzyme mixtures were tested on leaves (LM) and somatic embryos (SE) of the genotype MCol. 1505 (Table 1). Protoplasts could be isolated easily from both sources. In both type of explants the enzyme mixtures e4 and e6 (cellulase RS Onozuka, macerozyme and pectolyase) yielded the highest amount of protoplasts. The other enzyme mixtures gave a significantly lower yield.

Table 1. The influence of different enzyme mixtures on the protoplast yield (10 ^{5}/gram) of leaves and somatic embryos of genotype MCol. 1505.

No.	Enzyme mixture (%)	leaf	somatic embryos
e1	Cel.RS (1)/Mac (0.02)	1.89 b	0.60 a
e2	Cel.R 10 (1)/Mac (0.02)/Pec. (0.001)	1.89 b	0.29 a
e3	Cel.RS (1)/Mac (0.02)/ Dri(0.02)	2.35 b	0.87 b
e4	Cel.RS (1)/Mac (0.02)/Pec (0.001)	6.54 d	1.79 c
e5	Cel.R10 (10/Mac (0.02)/Dri (0.02)	1.08 a	0.52 a
e6	Cel.RS On.(1) /Mac (0.02)/Pec (0.01)	5.21 c	1.89 c
e7	Cel.RS On.(1)/Mac (0.01)/Pec (0.01)	2.30 b	0.78 b
e8	Cel.RS (1)/Mac (0.01)/Dri (0.02)	2.08 b	0.50 a

Note: The mean value followed by the same letter denotes no significant differences according to Duncan Multiple Range (0.05) test. Cel.RS=Cellulase RS; Cel.R10=Cellulase R 10; Cel.RS On=Cellulase RS Onozuka; Mac=Macerozyme; Dri=Driselase; Pec=Pectolyase.

Protoplasts from LM were greener, larger and more vacuolated than those isolated from SE. Protoplasts from LM treated with enzyme mixtures e1 and e5 had a brownish-green colour. In the subsequent experiments the enzyme mixtures e4 and e6 were used. Results from the FDA test showed that viability differed from one experiment to another. The highest viability of protoplasts isolated from SE was 60 % and from LM 40 % (data not shown). Table 2 summarizes the influence of enzyme mixtures e4 and e6 on the yield and the plating efficiency of protoplasts isolated from LM and SE of several genotypes. Protoplasts could easily be isolated

from all genotypes. In contrast to the pilot experiments shown in Table 1 the protoplast yield from LM was on average higher than from SE (Table 2). This yield varied from 2.34 to 31.4 x 10^5 protoplasts/g for LM and 0.22 to 2.35 x 10^5 protoplasts/g for SE. However for the plating efficiency, protoplasts from LM were less effective than those originating from SE.

Table 2 . Effect of genotype, tissue source and enzyme solution on protoplast yield and plating efficiency.

Enzyme mixture	Genotype	Protoplast yield[1] (10^5)		Plating efficiency (%)	
		LM	SE	LM	SE
e4= Cel.RS(1)/Mac(0.02) /Pec(0001)	Adira 4	2.95	1.41	0.48	0.42
	Adira 1	-	0.22	-	0.18
	Gading	-	1.9	-	0.93
	TMS90853	2.34	1.44	0.06	0.18
	MCol. 22	6.01	-	0.35	-
	MCol. 1505	6.13	2.35	0.19	0.91
e6=Cel.RS On.(1)/Mac(0.02) /Pec(0.01)	Adira4	3.30	1.39	0.31	0.62
	L 11	31.4	-	0.39	-
	Adira 1	-	0.53	-	0.33

Note: Cel.RS=Cellulase RS, Cel.RS On=Cellulase RS Onozuka, lm=leaf mesophyll, Mac=Macerozyme, Pec=Pectolyase, se=somatic embryo. [1]Protoplast yield was calculated starting from 2 g of fresh leaves or somatic embryos.

The plating efficiency ranged between 0.06 to 0.48 % for protoplasts originating from LM and between 0.18 to 0.93 % from SE (Table 2). Leaves of genotype Line 11 gave the highest protoplast yield, followed by MCol. 1505, MCol. 22 and Adira 4. There was no association between the yield of protoplasts and the plating efficiency. The effect of protoplast density on plating efficiency was tested with LM protoplasts of genotype MCol. 1505. In a density of 2 X 10^5 pp/ml and 4 X 10^5 pp/ml cell division occurred earlier and at a higher frequency than with densities of 1 X 10^5 and 6 X 10^5 pp/ml (data not shown). A plating density of 2 X 10^5 pp/ml was most optimal for a relatively high frequency of cell divisions. A density of 1 X 10^5 pp/ml produced less cell divisions, while at a density of 6 X 10^5 pp/ml the development of micro-calli was inhibited.

Table 3. Effect of the carbohydrate source in medium A on the occurrence of the first cell division of cultured protoplasts isolated from leaf mesophyll.

Geno-type	Carbohydrate source			
	Glucose		Sucrose	
	#Days [1])	#Tetrads[2]) at day 11 (PE)	#Days [1])	#Tetrads[2]) at day 11 (PE)
Gading	5 - 8	587 (0.29)a	4 - 6	662(0.33)b
TMS90853	6 - 9	365 (0.18)a	5 - 7	500 (0.25)a
MCol. 22	5 - 7	486 (0.24)a	3 - 5	696 (0.35)b
MCol.1505	4 - 9	530 (0.26)a	4 - 6	852 (0.43)c

Note: PE=plating efficiency, 1) = Number of days until the first cell division, 2) The mean value followed by the same letter of a row denotes no significant difference according to Duncan Multiple Range (0.05) test.

Protoplast culture

Generally, protoplasts of all genotypes underwent the first cell division after 3 - 9 days of culture in medium A. When sucrose was used as energy source, the first two-cell divisions of protoplasts of MCol.22 occurred 3 days after culture, whereas with glucose it occurred mostly one day later. In cultured protoplasts of MCol. 1505, Gading and TMS90853 the first cell divisions were observed after 5 days. The plating efficiency was in a medium with sucrose also significantly higher than in a medium with glucose (Table 3). Almost 25% of protoplasts of TMS90853 had changed their colour from green to brownish, their chloroplast and other organelles aggregated and concentrated at one pole. These were clear indications for a poor protoplast quality and low cell division competence. This phenomenon was also observed, albeit in a lower frequency, in cultured protoplasts of the other three genotypes. Culturing of protoplasts in medium supplemented with 2-4 mg/l 2,4-D for 2 weeks (medium A) resulted in cell elongation (Fig. 1) and low cell division rate, whereas during a treatment of more than 24 hours cell division was stopped completely (data not shown)

Micro-callus induction

After 3 weeks of culture in medium A, micro-calli were transferred to medium B for callus development. The presence of a low concentration of 2,4-D (0.1 mg/l) combined with BAP (0.5 mg/l) resulted in the development of a white translucent callus (Table 4). A low concentration of NAA (1-5 mg/l) combined with 0.5 mg/l BAP produced a compact creamy-yellowish callus. The diameter of the callus in NAA medium was larger than of callus grown in medium containing 2,4-D. Increase of the 2,4-D or NAA concentration inhibited callus proliferation. Without growth regulators the micro-calli did not develop well; after 10 days their growth ceased and their colour turned from creamy white to pink.

Table 4. Influence of growth regulators on appearance and growth of microcalli from protoplasts of somatic embryos from genotype MCol. 1505 cultured on medium B. Data were taken 26 days after protoplast culture.

hormone supplement (mg/l)	Induction of callus	Callus growth (%)	Callus colour
0	slow	5	c
2,4D(0.01) + BAP(0.5)	fast	72	w*
2,4D (1) + BAP(0.5)	medium	45	w
2,4D (4) + BAP(0.5)	slow	10	w
NAA (0.1) + BAP(0.5)	fast	64	c
NAA (1) + BAP(0.5)	medium	40	c
NAA (5) + BAP(0.5)	slow	24	c
NAA (10) + BAP(0.5)	slow	10	g

Note:c=creamy colour, g=green, w=white colour, *=translucent.

After 14 days of culture in medium B, calli of ca ϕ 3 mm were transferred to medium C supplemented with different growth regulators for plant regeneration. At 4 and 8 weeks after culture in medium C growth of green callus was observed in all treatments (Table 5). A combination of 2 mg/l 2,4-D with 1 mg/l IAA and 0.5 mg/l BAP gave the highest growth rate and the highest percentage of green callus. This medium was able to maintain the callus in a green state for more than 3 months whereas callus on the other medium combinations turned brown. For that reason this medium was used for long term maintenance of green callus. The

hormone combinations of 0.2 mg/l 2,4-D and 0.5 mg/l BAP followed by a medium with 1 mg/l NAA and 10 mg/l Zeatin ranked second best on green callus formation. The percentage varied from 50 to 60%. The other growth regulator combinations gave lower percentages of green callus. Development of callus pieces in the same medium varied considerably, particularly with respect to morphology. For example, some of the compact calli were arranged in a spiral form with 3 to 4 turns and of more than 0.7 cm height. This type of callus was usually pale yellow or creamy in the border area and green at the top. Disk-shaped callus was developed laterally reaching a size of 3 to 4 cm in diameter. Morphologically it was a green-compact callus, sometimes irregular in form. The disk-shaped calli never produced roots. Another type of callus had a hemispherical shape, was ca 0.5 cm high and, bright green in colour. This type consisted of globular callus, of circa 2 mm in diameter.

In order to stimulate regeneration, green callus was cultured on medium D. Renewal of the medium, addition of vitamins (pyridoxine, nicotinic acid and/or ascorbic acid) and reduction of the sugar concentration did not stimulate these green calli to form shoots or embryos. After culturing of green calli for more than 2 months nodular structures were occasionally formed, they never developed into somatic embryos, shoots or leaf like structures but roots were initiated occasionally instead. The highest frequency of root formation was 2%. The roots were always initiated from the green callus part and about 20 % of the rooted calli had only one long slender root and the rest produced 2-5 roots per callus (Fig. 2).

As elaborated earlier, protoplasts were cultured on media containing different growth regulators. Table 6 summarizes the protocols of medium sequences, in which the protoplasts of different genotypes were cultured. It shows that in all selected protocols green calli could be obtained; in 8 out of 17 protocols also roots were produced. In 6 out of 11 protocols, roots were formed from protoplasts isolated from somatic embryos of all tested genotypes. It was difficult to indicate a causal factor of root formation. The effect of growth regulators in regeneration media (medium D), on rooting were inconsistent. For example 0.1 mg/l NAA in combination with 10 mg/l BAP produced roots in protocols 2 and 6 but not in 5 and 9. The same was found with BAP alone in medium D producing roots in protocols 4 and 13 but not in protocols 14 and 16.

Culturing of green callus in auxin-free media (protocol 10) resulted in root formation. However, root formation was frequently related to the presence of NAA in the first culture medium, except in protocol 11.

DISCUSSION

Many research groups have failed to regenerate cassava plants from protoplasts. In most cases only callus proliferation and sometimes roots were obtained (Anonymous, 1985; Nzoghe, 1989). Generally protoplasts are excellent for DNA uptake experiments. They have been used successfully for plant transformation in several crops like maize (Lyznik et al. 1989), orchadgrass (Horn et al. 1988), rice (Kyozuka et al., 1988) and soybean (Dhir et al. 1992).

Plant regeneration from protoplasts would enable the generation of transgenic cassava plants via protoplast transformation. In this report mature somatic embryos and leaf mesophyll cells were used as a source of protoplasts. It was shown (Table 1) that plating efficiency of protoplasts of somatic embryos tended to be higher, although the yield of protoplasts was lower than those obtained from LM. A disadvantage of using LM is the difficulty in obtaining leaves which are physiologically identical. A leaf of the first node differs from a leaf of the second node and from nodes of the further order. This might be one of the reasons for the considerable variation in protoplast production which was observed. Furthermore, leaf mesophyll is not a uniform source of protoplasts. It consists of different tissues (Sinnott and Wilson, 1963). Although there were differences in plating efficiency, protoplasts of both LM and SE developed easily into callus. Studies with tobacco callus showed that a high ratio of auxins to cytokinins in the medium favoured root formation and the reverse shoot formation. Although this approach is not demonstrated to be successful universally (Brown and Thorpe, 1980), this concept played an important role in the basic understanding of the regulation of growth and development. The role of auxins and cytokinins in plant regeneration from protoplasts has been believed to be as a determinative factor for many crops. The embryogenic callus derived from protoplasts of pea were induced in the presence of 0.9 mg/l 2,4-D, but for induction of somatic embryos hormone-free medium

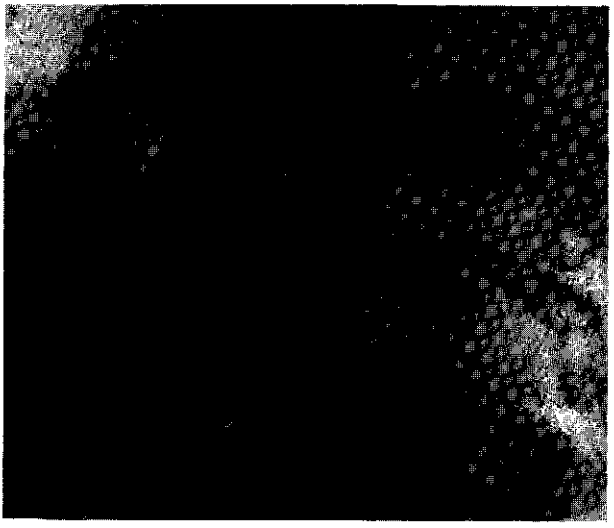

Figure 1: Elongation of protoplasts cultured for few days in 2,4-D containing B medium.

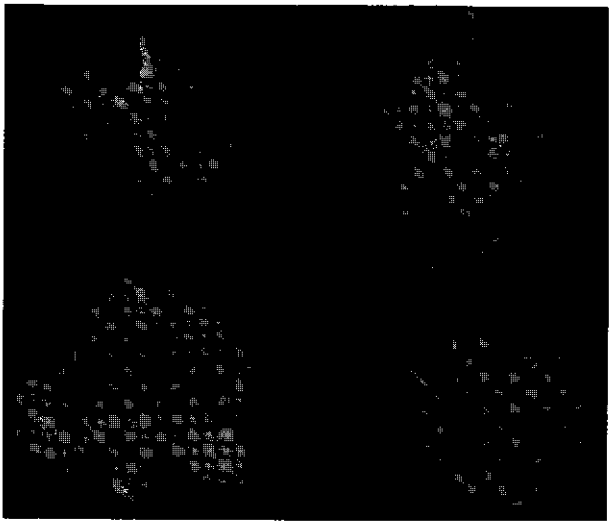

Figure 2: Adventitious root formation on callus derived from protoplasts.

Table 5. Response of micro-calli regenerated of protoplasts isolated from somatic embryos of cassava genotype M 1505 after 4 and 8 weeks culture in media with different combinations of growth regulators (medium C).

Hormone	Callus colour (%)						Callus
(mg/l)	morphology 4 weeks			8 weeks			
	Wh	Gr	Br	Wh	Gr	Br	
IAA (0.01) + Zea (10)	20	55	25	5	5	90	fr
IAA (0.1) + Zea (10)	0	60	40	0	15	85	co
IAA (1) + Zea (10)	10	50	40	0	5	95	co
IAA (0.1) + TDZ (0.1)	10	75	15	5	5	90	fr
IAA (1) + TDZ (0.1)	10	80	10	0	30	70	fr
NAA (0.01) + Zea (10)	0	60	40	0	0	100	co
NAA (0.1) + Zea (10)	15	60	25	0	10	90	co
NAA (1) + Zea (10)	15	75	10	10	50	40	co
NAA (0.2) + BAP (10)	0	70	30	0	25	75	fr
NAA (0.2) + GA (0.3)	0	40	60	0	5	95	co
NAA (0.2) + BAP (0.01) + GA (0.3)	0	20	80	0	10	100	co
NAA (0.2) + BAP (0.1) + vit B5	10	50	40	0	5	95	co
2,4D (2) + TDZ (0.25) + vit B5	10	50	40	10	40	40	co
2,4D (2) + IAA (1) + BAP (0.5)	0	100	0	0	100	0	co
2,4D (2) + BAP (0.5)	30	50	20	0	60	40	fr/co

Note : Br=brown, co=compact callus, fr=friable callus, Gr=green, Wh=white.

Table 6. Medium sequences of most promising protocols for the induction of cell divisions, callus and regeneration in different cassava genotypes.

Protocol	Genotype	Explant	(A) cell division induction	(B) Callus induction	(C) Callus development	(D) Regeneration induction	Regeneration ability
1.	Gading	lm	NAA (1) 2,4D (1) Zea (1)	2,4D (0.1) BAP (0.5)	2,4D (2) IAA (1) BAP (0.5)	IAA (0.1) TDZ (0.1)	gc gc
2.	MCol.1505	se	NAA (1) 2,4D (1) Zea (1)	2,4D (0.1) BAP (0.5)	ditto	NAA (0.1) BAP (10)	root/gc
3.	MCol.22	lm	NAA (1) 2,4D (1) Zea (1)	2,4D (0.1) BAP (0.5)	ditto	BAP (0.1) TDZ (0.1)	root
4.	TMS90853	se	NAA (1) 2,4D (1)	2,4D (0.1) BAP (0.5)	ditto	BAP (0.1)	root
5.	Gading	se	2,4D (1) Zea (1)	2,4D (0.1) BAP (0.5)	ditto	NAA (0.1) BAP (10)	gc
6.	MCol.22	lm	NAA (1) 2,4D (1) Zea (1)	2,4D (0.1) BAP (0.5)	ditto	NAA (0.1) BAP (10)	root
7.	Gading	lm	2,4D (1) Zea (10)	NAA (0.1) BAP (0.5)	ditto	IAA (0.1) Zea (5)	gc
8.	MCol.1505	se	NAA (1) 2,4D (1) Zea (1)	NAA (0.1) BAP (0.5)	ditto	IAA (0.1) TDZ (0.125)	root
9.	MCol.22	se	NAA (1) BAP (1)	NAA (0.1) BAP (0.5)	ditto	NAA (0.1) BAP (10)	gc

Table 6. Medium sequences of most promising protocols for the induction of cell divisions, callus and regeneration in different cassava genotypes (Continuation).

Protocol	Genotype	Explant	(A) cell division induction	(B) Callus induction	(C) Callus development	(D) Regeneration induction	Regeneration ability
10.	MCol.22	se	NAA (1) 2,4D (1) Zea (1)	NAA (0.1) BAP (0.5)	2,4D (2) IAA (1) BAP (0.5)	vitamin B5	roots
11.	TMS90853	se	NAA (1) 2,4D (1) Zea (1)	2,4D (0.1) BAP (0.5)	ditto	IAA (0.1) Zea (10)	gc
12.	TMS90853	se	NAA (1) 2,4D (1)	NAA (5) BAP (0.5)	ditto	TDZ (0.1)	roots
13	TMS90853	se	NAA (1) 2,4D (1) Zea (1)	NAA (5) BAP (0.5)	ditto	BAP (0.1)	roots
14.	TMS90853	se	2,4D (1) Zea (1) TDZ (0.25)	NAA (2) IAA (1)	ditto	BAP (0.1)	gc
15.	MCol.1505	lm	2,4D (1) Zea (1)	2,4D (2) Zea (0.5)	ditto	ZEA (10)	gc
16.	Adira 4	lm	2,4D (1) BAP (1)	2,4D (2) BAP (0.5)	ditto	BAP 10)	gc
17.	Adira 4	se	2,4D (1) Kinetin (1)	2,4D (2) Kinetin (0.5)	ditto	Kinetin (10)	gc

Note: gc=green callus, lm=leaf mesophyll, se=somatic embryo. Data were taken from independent experiments.

was required and finally for maturation cytokinin was needed (Lehminger-Mertens and Jacobsen, 1989).

In peppermint, plant regeneration from protoplasts was induced by the presence of 1 mg/l NAA and 0.4 mg/l BAP followed by 0.1-0.5 mg/l NAA and 1-5 mg/l BAP for shoot maturation (Sato, 1993). In cassava, Shahin and Shepard (1980) used 1 mg/l NAA combined with 0.5 mg/l BAP as the first (cell division induction) and second media (micro-calli induction). In the subsequent medium NAA was reduced or replaced by IAA, while BAP was replaced by Zeatin. In this report a wide range of auxin-cytokinin ratios has been tested. The presence of the auxin NAA or 2,4-D in combination with cytokinin promoted callus growth (Tables 4 and 5). Callus never developed into somatic embryos nor shoots although callus was transferred into media with different concentrations and combinations of cytokinin. The failure of protoplasts to undergo embryogenesis or to form adventitious shoots was either, the loss of genetic competence of protoplasts to regenerate for example because of polyploidisation or that genes involved in regeneration were not induced. This was possibly due to the improper supply of plant regulators, to the physical environment stress or to the fact that the protoplasts were not competent for regeneration (Obsorne, 1993). For the expression of regeneration the physiological state of the plant source material, the protoplast isolation conditions, the culture medium composition and the environment are critical factors (Constable et al., 1975 and Fowke et al., 1973). To study every single factor determining the ability to regenerate is not easy. Many workers have found that the plant material as a source of protoplasts is one of the most important basic factors. It has been reported that in many plant species belonging to the Gramineae, the use of embryogenic cell suspension cultures as the protoplast donor resulted in plant regeneration (Roest and Gilissen, 1993). In recalcitrant woody species as for example *Citrus* (Base et al., 1991) regeneration was accomplished by using protoplasts of embryogenic tissues. It was assumed that cells of embryogenic tissue are more frequently competent for plant regeneration than those from mature somatic embryos or leaf mesophyll.

The embryogenic competent cells in somatic embryos of cassava are regularly located two to five cell layers deep (Raemakers et al., 1995). Histologically it was shown that the number of

embryogenic cells increased after 14 days of culture. It could imply that the use of young stages of somatic embryos (immature embryos) or globular stage embryos possibly could increase the frequency of embryogenic competent cells enabling plant regeneration. In leek (*Allium ampeloprasum* L.), Buiteveld and Creemers-Molenaar (1994) have demonstrated that regeneration of protoplasts can be obtained if suspension cultures were used as source of protoplasts. This suspension culture was initiated with friable embryogenic callus derived from immature embryos (Buiteveld et al., 1994).

Recently, Taylor et al. (1995) established a new system for producing friable embryogenic callus of cassava. These calli possibly have more competent cells than somatic embryos, which means that relatively a high frequency of protoplasts isolated from these cells have the competence to regenerate. The successful application of this material for protoplast regeneration will be described in Chapter 4.

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CHAPTER 4

PLANT REGENERATION FROM PROTOPLASTS ISOLATED FROM FRIABLE EMBRYOGENIC CALLUS OF CASSAVA

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ABSTRACT

Suspensions derived from friable embryogenic callus (FEC) of cassava genotype TMS60444 were incubated in a solution consisting of enzymes (Cellulase RS Onozuka, Macerozym and Pectolyase), growth regulators (1 mg/l 2,4-D, 1 mg/l NAA and 1 mg/l Zeatin) and major and minor salts. Two-weeks old suspensions yielded the highest number of protoplasts (1.4 x 10^6 protoplasts/g fresh weight). Protoplasts plated at a density of 1-10 x 10^5 in a TM2G medium supplemented with 0.5 mg/l NAA and 1 mg/l Zeatin resulted in a plating efficiency of as high as 2.5 %. After 2 months of culture 60% of the developed calli were highly friable and in appearance identical to the original FEC. The protoplasts derived FEC were maintained in a proliferative state by culture on Gresshoff and Doy medium plus 10 mg/l Picloram. Transfer of FEC to liquid medium resulted in suspension cultures which had a proliferation rate slightly higher than that of the original material. Culture of FEC for maturation resulted in a maximum of 124 torpedo shaped and mature embryos per 10^4 of initially cultured protoplasts. Mature embryos were multiplied by secondary somatic embryogenesis at high efficiency on a Murashige and Skoog medium supplemented with 8 mg/l 2,4-D. Culture of mature embryos on MS2 plus 1 mg/l BAP resulted in shoots which were rooted easily on the same medium without BAP.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial shrub cultivated in the lowland tropics for its starch containing roots. Improvement of the crop by classical breeding is difficult due to low fertility and poor seed set. Somatic hybridization of protoplasts could be an alternative to create intra- or interspecific hybrids of sexual incompatible genotypes. For this, an efficient regeneration system of these protoplasts is a prerequisite. Such a system could also be used to transfer cloned genes to agronomicaly established genotypes by protoplast electroporation. Cassava is very recalcitrant for plant regeneration of protoplasts. There is only one report of shoot regeneration from protoplasts of cassava (Shahin and Shephard, 1980). They used well expanded leaves for the isolation of protoplasts. Despite considerable efforts, plant regeneration from protoplasts has never been repeated since then (Anonymous, 1985; Nzoghe, 1991; Anthony et al., 1995, Chapter 3). A logical approach was to use tissues which contain embryogenesis competent cells. Such cells are found in the apical meristems, young leaves and somatic embryos cultured on auxin supplemented media (Stamp and Henshaw, 1987; Raemakers et al., 1993). However, protoplasts isolated from these tissues gave in the best case green callus and adventitious roots (Chapter 3).

Recently, a new type of somatic embryogenesis was developed. In this in vitro system the embryos do not develop beyond the pre-globular stage and the embryogenic callus is highly friable (Taylor et al., 1995). Transfer of this friable embryogenic callus to liquid medium resulted in a suspension-like culture. In leek (Buitenveld and Creemers, 1994), petunia (Power et al., 1979), rice (Kyozuka et al., 1988), sugarcane (Chen, et al., 1988), and wheat (Chang et al., 1991) such cultures were an excellent source for protoplast regeneration. Furthermore, electroporation of protoplasts derived from suspension cultures led to the transformation of maize (Rhodes et al., 1988), rice (Toriyama et al., 1988) and orchardgrass (Horn et al., 1988).

In this report the use of friable embryogenic callus as source for protoplasts has been described. Starting with these protoplasts two routes of plant regeneration have been described. One leading to friable embryogenic callus and the other directly to mature embryos. The mature embryos

could be converted into plants.

MATERIALS AND METHODS

Plant material

Friable embryogenic callus (FEC) of cassava genotype TMS60444 was kindly provided by Dr. Nigel Taylor of University of Bath, United Kingdom. Every three weeks FEC was subcultured on Gresshoff and Doy (1972) medium supplemented with vitamins and minerals, 20 g/l sucrose and 10 mg/l Picloram (GD2). Suspension cultures were initiated by transferring 0.5 g of FEC into a 300 ml flask with 50 ml of liquid medium supplemented with Schenk and Hildebrandt (1972) salts and vitamins, 60 g/l sucrose and 10 mg/l Picloram (SH6).

The medium was refreshed every 2 days and after 14 days the content of a each flask was subdivided over 5 new flasks. Cultures were agitated on a L.E.D. Orbit Shaker (Lab-Line Instruments, Inc.) at 130 - 150 rpm. All cultures were kept in a growth chamber with a photoperiod of 12 hours, a temperature of 30 °C and an irradiance of 40 $\mu mol^{-2}s^{-1}$.

Isolation of protoplasts

In a preliminary experiment the yield of FEC cultured on GD2 and of suspensions cultured for 1 to 4 weeks in liquid SH6 medium was determined. For protoplast isolation this tissue was sieved (mesh 1 mm^2) and 2 grams of the sieved tissue was placed in a Petri-dish (ϕ 9cm) containing 10 ml of a mixture of enzymes (10 g/l Cellulase RS Onozuka, 200 mg/l Macerozym, 10 mg/l Pectolyase), growth regulators (1 mg/l NAA, 1 mg/l 2,4-D, 1 mg/l Zeatin), major and minor salts (368 mg/l $CaCl_2$, 34 mg/l KH_2PO_4, 740 mg/KNO_3, 492 mg/l $MgSO_4.7H_2O$, 19.2 mg/l Na-EDTA, 14 mg/l $FeSO_4.7H_2O$).

Cells were incubated for about 16 hours on a L.E.D. Orbit Shaker (Lab-Line Instruments, Inc.) at 30 rpm in darkness. Further steps of the protoplast isolation procedure were as described in Chapter 3.

Assessment of protoplast viability and the presence of cell walls

The viability of freshly isolated protoplasts was observed using FDA (Widholm, 1972). FDA (0.5 mg/ml in acetone) was mixed with washing solution (Chapter 3) in a ratio of 1:50. An equal volume of the FDA solution was added to protoplasts. After five minutes the protoplasts were examined with a fluorescence Axiophot Photomicroscope using an aniline blue filter. Calcofluor White was used to check the remnant of cell walls. An equal volume of 0.4 M mannitol solution containing 0.1 % w/v calcofluor white (Hughes and Gunning, 1975) were mixed with protoplasts. After 10 minutes the protoplast suspensions were examined with a fluorescence Axiophot Photomicroscope using a blue filter.

Protoplast culture

Protoplasts were cultured and plated in media solidified with Sea Plaque agarose 0.2 % w/v as described in Chapter 3. In three experiments the influence of different parameters was studied. In the first experiment, protoplasts were cultured at a density of 10^5 protoplasts/ml (pp/ml) on TM2G medium (Wolters et al., 1991) supplemented with different growth regulators. In experiment two, protoplasts were cultured at densities of $2x10^5$, $5x10^5$ or $1x10^6$ pp/ml on TM2G supplemented with 0.5 mg/l NAA and 1 mg/l Zeatin. In experiment three, protoplasts were cultured at densities of $1x10^5$ or $1x10^6$ pp/ml on TM2G medium or medium A (Murashige and Skoog (1962) salts and vitamins, 4.5 g/l myo-inositol, 4.55 g/l mannitol, 3.8 g/l xylitol, 4.55 g/l sorbitol, 0.098 g/l MES, 40 mg/l adeninsulphate and 150 mg/l caseinhydrolysate, 0.5 mg/l d-calcium-panthotenate, 0.1 mg/l choline-chloride, 0.5 mg/ ascorbic acid, 2.5 mg/l nicotinic acid, 1 mg/l pyridoxine-HCl, 10 mg/l thiamine-HCl, 0.5 mg/l folic acid, 0.05 mg/l biotine, 0.5 mg/l glycine, 0.1 mg/l L-cysteine and 0.25 mg/l riboflavine and 59.40 g/l glucose, 0.5 mg/l NAA and 1 mg/l Zeatin). The media were refreshed every 10 days, by replacing 9 ml with fresh medium. The plating efficiency was calculated after 20 days.

After two months of culture of protoplasts and (micro)calli in the first medium, high quality FEC was selected and either cultured for further proliferation or for maturation. For proliferation FEC was transferred to Gresshoff and Doy (1974) medium supplemented with 40 g/l sucrose, 7 g/l Daichin agar and 2 mg/l Picloram (GD4). After 3 weeks the FEC was

transferred to a Gresshoff and Doy medium supplemented with 20 g/l sucrose, 7 g/l agar and 10 mg/l Picloram (GD2). Suspension cultures were initiated by transferring 1.0 g of FEC to liquid SH6% medium supplemented with 10 mg/l Picloram. Two weeks later the suspension was divided over new flasks with an initial packed cell volume of 1.0 ml. The increase in packed cell volume was observed for two weeks and compared with the original material.

For maturation FEC was cultured on a medium consisting of Murashige and Skoog (1962) salts and vitamins, 0.1 g/l myo-inositol, 20 g/l sucrose, 18.2 g/l mannitol, 0.48 g/l MES, 0.1 g/l caseinhydrolysate, 0.08 g/l adenine sulphate, 0.5 mg/l d-calcium-panthotenate, 0.1 mg/l choline chloride, 0.5 mg/l ascorbic acid, 2. mg/l nicotinic acid, 1 mg/l pyridoxine-HCl, 10 mg/l thiamine HCl, 0.5 mg/l folic acid, 0.05 mg/l biotin, 0.5 mg/l glycine, 0.1 mg/l L-cysteine, 0.25 mg/l riboflavine and 1 mg/l Picloram. This maturation medium was refreshed every 3 weeks. The torpedo shaped embryos were isolated and cultured on medium supplemented with Murashige and Skoog (1962) salts and vitamins, 20 g/l sucrose, 7 g/l agar (MS2) and 0.1 mg/l BAP.

Subculture of mature embryos

Mature embryos were either cultured directly on MS2 plus 1 mg/l BAP for shoot development, or on solid or in liquid MS2 supplemented with 10 mg/l NAA or 8 mg/l 2,4-D for secondary embryogenesis.

RESULTS

Protoplast isolation and culture

The suspension cultures consisted predominantly of small clusters of dense cytoplasmic cells which have a light-brown colour under a light microscope. The protoplasts isolated from suspension cultures were heterogeneous both in their size and in the amount of cytoplasm. The age of the suspension culture was a critical factor.

The protoplast yield of friable embryogenic callus harvested directly from solid GD2 medium

was much lower than of the suspension cultures. Two weeks old suspensions gave the highest and 4 weeks old suspension cultures the lowest yield of protoplasts (Table 1).

Table 1. The effect of suspension culture duration on protoplast yield, viability and with the calcofluor white test on cell wall remnants (mean from 3 replicates).

Duration (weeks)	Protoplast yield (10^5/gr)	Viability determined by FDA (% positive cells)	Presence of cell walls determined by calcofluor white (% positive cells)
0	0.5	32±2	nd
1	7	30±8	4.75
2	14.5	33±5	3.77
3	8.16	28±2	3.5
4	1.18	26±1	nd

nd=not determined

Fig. 1A shows a sample of protoplasts isolated from suspensions. With FDA viable protoplasts showed a yellow-green fluorescence. These protoplasts were spherical and displayed cytoplasmic streaming. Independent of the suspension culture duration around 30% of the total amount of isolated protoplasts was viable (Table 1). Results from the calcofluor white staining showed that about 4 % of the protoplasts fluoresced brightly, indicating the presence of cell walls. However, most of these protoplasts did not have a spherical shape and showed no reaction with FDA.

Plating efficiency and micro-callus development

In the first experiment protoplasts were cultured in TM2G medium supplemented with different growth regulators (Table 2). Protoplasts, cultured in 0.5 mg/l NAA and 1 mg/l Zeatin, or in 0.5-4 mg/l Picloram and 1 mg/l Zeatin divided after three to four days. In a medium without growth regulators or in a medium with only Zeatin this was observed after 8-12 days, and in medium with 10 mg/l Picloram plus 0.5 mg/l Zeatin there was almost no division of protoplasts.

Table 2. The effect of growth regulators on plating efficiency of protoplasts after culture for 30 days in medium TM2G and on the formation of torpedo shaped and mature embryos after 60 days on maturation medium.

Growth regulators (mg/l)	PE (%)	Days to first cell division	# torpedo shaped and mature mature embryos per 10^4 protoplasts
No hormone	0.02	12	nd
Zea (1)	0.06	8-9	4.75a
NAA(0.5) + Zea(1)	0.67	3-4	184.2c
Pic(0.5) + Zea(1)	0.45	3	6.75a
Pic(2) + Zea(1)	0.49	3	47.40b
Pic(4) + Zea(1)	0.08	3	3.4a
Pic(10) + Zea(1)	< 0.01	nd	nd

Data were taken as an average from 3 to 4 replications (one drop of 100 μl protoplasts solution), mean followed by the same letter denote no significant difference according to LSD (0.05) test; nd=not determined; PE=plating efficiency.

The plating efficiency varied from 0.02 % in a medium without growth regulators to 0.67% in TM2G medium supplemented with 0.5 mg/l NAA and 1 mg/l Zeatin. The media supplemented with 0.5-4 mg/l Picloram gave intermediate plating efficiencies. Plating efficiency was also influenced by the density of the cultured protoplasts. In Table 3 it can be seen that a density of 5 x 10^5 gave the highest (0.23 %) and a density of 10^6 the lowest plating efficiency (0.02 %).

Table 3. The effect of protoplast density on plating efficiency (after 21 days of culture) in TM2G supplemented with 0.5 mg/l NAA and 1 mg/l Zeatin and on the formation of torpedo shaped and mature embryos (after 2 months in the first medium followed by 2 months in maturation medium).

Densities (10^5)	Plating efficiency (%) at 21 days	# torpedo shaped and mature embryos per 10^4 protoplasts
2	0.10±0.04	124
3	0.15±0.03	85
5	0.23±0.04	30
10	0.02±0.01	5

Data were taken as an average from 3-4 replications (one drop of 100 μl of protoplast suspension).

Plating efficiency varied considerably between experiments. In general, however, the same trend was observed. In Table 4 it can be seen that 10^6 gave a lower plating efficiency than 10^5. Furthermore, it can be seen that TM2G medium is superior over medium A. After 20 days of culture in TM2G medium supplemented with 0.5 mg/l NAA and 1 mg/l Zeatin the first micro-calli, consisting of at least 2 types (Fig. 1B), became visible. In the first type, cell division occurred inside the boundaries of the initial cell wall, so the daughter cells became smaller than the original protoplasts whereas the shape of the micro-calli was irregular. In the second type, most probably cell division occurred in a different way because the cells could not be observed as easily as in the first type. Some of the cells had a banana-like shape and were transparent.

Table 4. The effect of basal medium and protoplast density on plating efficiency (after 21 days of culture) and production of micro-calli (after 2 months of culture).

	10^5 pp/ml		10^6 pp/ml	
Medium (growth regulators)	PE (%)	Micro-calli per 10^4 pp	PE (%)	Micro-calli per 10^4 pp
TM2G (0.5 mg/l NAA + 1 mg/l Zeatin)	2.5	1058±158	0.91	64±17.25
A (0.5 mg/l NAA + 1 mg/l Zeatin)	0.24	240±85	0.18	25±9

Data were taken as an average from 5 to 10 agarose drops at 55 days of culture (one drop is equal to a 100 μl protoplasts suspension), PE = plating efficiency.

After 2 months of culture, 10^4 protoplasts cultured in TM2G supplemented with 0.5 mg/l NAA and 1 mg/l Zeatin at a density of 10^5/ml produced 1058 micro-calli, whereas 10^4 protoplasts cultured at a density of 10^6/ml only produced 64 micro-calli. Replacing TM2G medium with medium A reduced at both densities the number of micro-calli significantly (Table 4). At this stage at least three types of calli could be distinguished. One type consisted of globular shaped embryos (Fig. 1C) which were mostly observed in protoplasts cultured at a density of 10^6. Some of them developed cotyledon like structures, light green in colour. However, these embryos could not be germinated properly. Another type was fast growing and consisted of a large compact callus, they were observed in protoplast cultures of both densities. This callus never

developed embryos. The third type was highly friable callus and was observed at both densities (Fig. 1C). At a density of 2-5 x 10^5 (medium TM2G) about 60 % of the calli were friable and embryogenic. The FEC was either subcultured for further proliferation or for maturation.

Proliferation of FEC

Following selection of FEC, 0.1 g of it cultured for three weeks on GD 4 plus 2 mg/l Picloram increased into 0.7 g of tissue. More than 95 % of the tissue consisted of high quality FEC. Subsequently, this tissue was maintained by subcultures of three weeks on GD2 medium supplemented with 10 mg/l Picloram. To initiate suspension cultures FEC was transferred to liquid medium. A suspensions cultured for 6 weeks in liquid SH6 medium is shown in Fig. 1D. The increase in packed cell volume (PCV) of this material was slightly higher than that of the original material (data not shown).

Maturation of FEC

In an attempt to induce maturation of embryos, FEC isolated after two months of culture in TM2G was cultured on maturation medium. On this medium there is a gradual shift from proliferation to maturation. As a result the packed cell volume had increased with a factor 4 after two weeks of culture in liquid maturation medium. Also after transfer to solid maturation medium there is proliferation. After two weeks on solid medium most of the embryos had reached a globular shape (Fig. 1E) and only a few of these globular embryos developed further. The first torpedo shaped embryos became visible after one month of culture on solid maturation medium (Fig. 1F). The number of mature and torpedo shaped embryos was not correlated with the plating efficiency but with the density of the initially cultured protoplasts (Table 3). No such embryos were obtained if protoplasts were cultured on TM2G without growth regulators (Table 2). The highest number of mature and torpedo shaped embryos was formed from protoplasts cultured on TM2G supplemented with 0.5 mg/l NAA and 1 mg/l Zeatin. If NAA was replaced by Picloram than the number of torpedo shaped and mature embryos was significantly lower (Table 2). From the tested Picloram concentrations 2 mg/l gave the best results. After 3 months of culture between 60 and 200 torpedo shaped and mature embryos were isolated per agarose drop. Torpedo shaped embryos became mature at high frequency if they were cultured on fresh

maturation medium or on MS2 plus 0.1 mg/l BAP.

Secondary somatic embryogenesis and shoot development

Table 5. Response of mature embryos on the induction of secondary embryogenesis on several media.

Treatment	# explants	# embryos which form secondary embryos
solid MS2 + 8 mg/l 2,4-D	30	28 (93)
liquid MS2 + 8 mg/l 2,4-D	30	25 (83)
solid MS2 + 10 mg/l NAA	30	10 (33)
liquid MS2 + 10 mg/l NAA	30	14 (45)
7 days solid 2,4-D/ 20 days liquid NAA	30	24 (80)

Between brackets percentage

Only a few torpedo shaped embryos formed secondary embryos if cultured on liquid or solid MS2 medium supplemented with 10 mg/l NAA or 8 mg/l 2,4-D (data not shown). Mature embryos were better explants for secondary embryogenesis. In both liquid and solid medium 2,4-D was superior for induction of secondary embryogenesis as compared to NAA. If mature embryos were first cultured in 2,4-D and than in liquid NAA the response was comparable with culture in 2,4-D alone. Also embryos which first had undergone a cycle of secondary somatic embryogenesis in medium with 2,4-D, produced highly efficient secondary embryos in NAA supplemented medium. About 30% of the mature embryos cultured on MS2 plus 1.0 mg/l BAP developed into shoots. These shoots were easily rooted on MS2 (Fig. 1G).

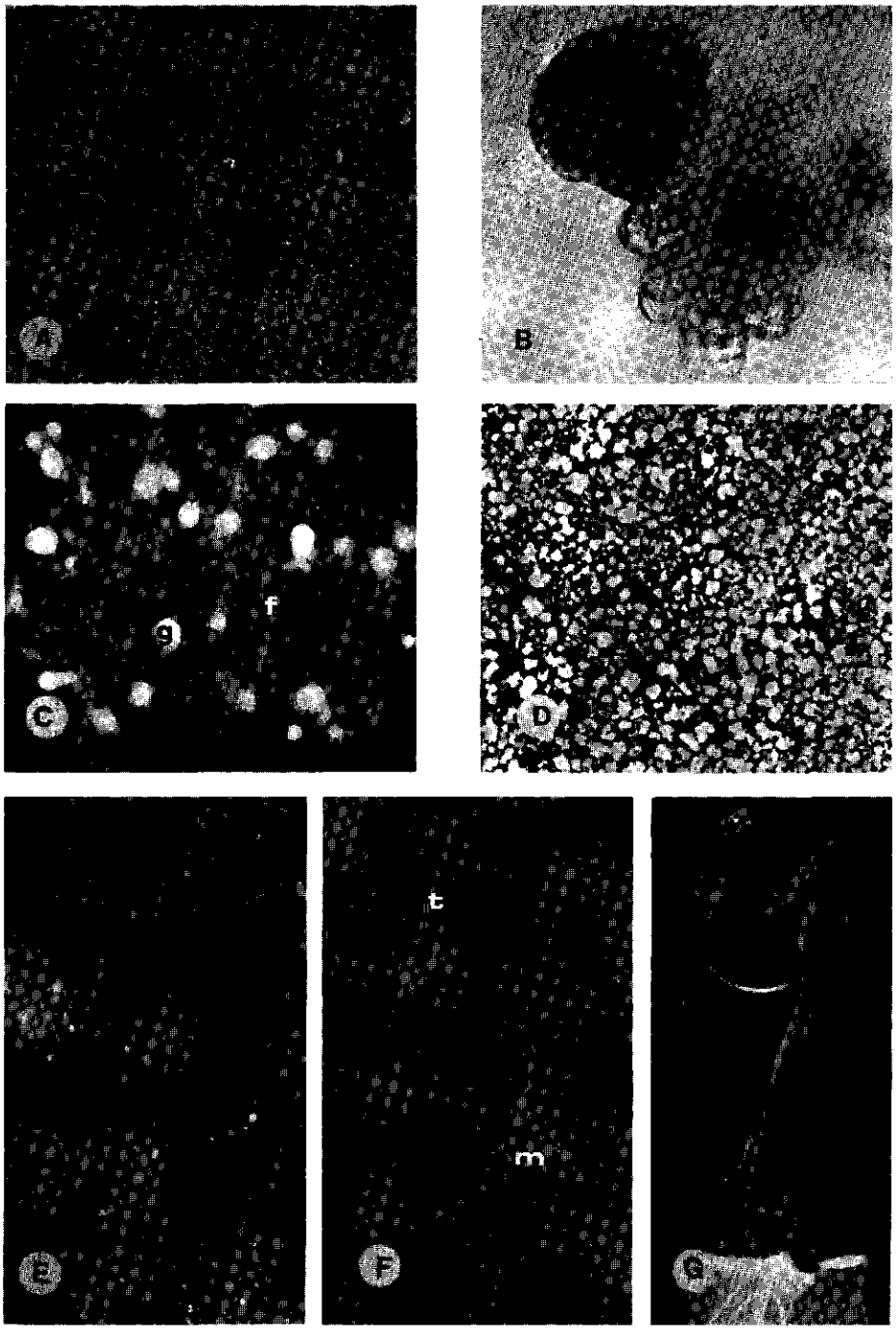

Fig. 1: Regeneration of plants from protoplasts: isolated protoplasts A), development of two types of micro-callus after 20 days of culture B), development of friable embryogenic callus (f) and globular shaped embryos (g) after 2 months of culture C), suspensions made of friable embryogenic callus D), formation of globular embryos from friable embryogenic callus E), formation of torpedo shaped (t) and mature embryos (m) and rooted plantlet G).

DISCUSSION

Nowadays in rice, leek (Buiteveld and Creemers-Molenaar, 1993) and maize regeneration from protoplasts is no longer a barrier for the genetic improvement of these crops (Abdullah et al., 1986, Kyozuka et al., 1987 and Rhodes et al., 1988). With one exception (Shahin and Shepard, 1980) in cassava regeneration of plants from protoplasts has been tried, without success, using many types of explants as source for protoplasts (Anonymous, 1985; Nzoghe, 1989; Chapter 3). The plant regeneration from protoplasts described here brings new possibilities for the improvement of this crop. The most important aspect of this procedure is the use of friable embryogenic callus or suspensions derived from it. Important for high frequency regeneration of protoplasts is the use of suspensions instead of FEC as source of protoplasts (Table 2), and is the culture of protoplasts on TM2G medium supplemented with NAA and Zeatin (Table 3, 5) in a density of $1x10^5$ protoplasts/ml (Table 3 and 4).

De Jong et al., (1993) showed that the percentage of suspension cells of carrot suspension cultures that regenerated into plants was not more than 2%. In the best case 2.5 % of the cassava protoplasts underwent cell division and formed micro-calli (Table 4). Although not all microcalli produced embryos. Anthony et al., (1995) improved plating efficiency by culturing protoplasts of cassava with short glass rods in ammonium-free MS medium. Buiteveld and Creemers-Molenaar (1994) improved plating efficiency by plating protoplasts of leek isolated from suspension cultures in Ca-alginate medium. These procedures might also increase plating efficiency of cassava protoplasts derived from FEC.

Two months after isolation of protoplasts two different routes of regeneration could be chosen: maturation of embryos or further proliferation of the FEC. This is exactly the same as the response obtained with the original material. In some treatments the number of torpedo shaped and mature embryos exceeded the number expected from the plating efficiency. However, maturation occurred at a low frequency; less than 10% of the individual friable embryos developed into mature embryos. The fact that in some treatments the number of obtained organized embryos exceeded the plating efficiency can be explained by the fact that during the

first two months of culture micro-calli are in reality proliferating FEC and by the fact that there is a gradual shift from proliferation to maturation. Another bottle neck was the germination of mature embryos into plants. This problem could be solved by first multiplying single mature embryos through secondary embryogenesis. In this way the chance that plants were derived from individual mature embryos was maximized. However, the whole procedure always will be a time consuming process and it is expected to be genotype dependent as was shown by Raemakers (1993) for secondary somatic embryogenesis.

With the availability of a system by which protoplasts can regenerate into plants a new road is open to genetic transformation of cassava. Electroporation or PEG mediated DNA transfer or cocultivation with *Agrobacterium* might be ways to obtain transformed friable callus. Initial transformation experiments by electroporation of protoplasts derived from secondary embryos has shown that this way is certainly an attractive possibility (data not shown). Furthermore, the protoplast regeneration system described here is repeatable and is an important first step, towards the application of interspecific protoplast fusion in cassava.

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CHAPTER 5

PRODUCTION OF TRANSGENIC CASSAVA PLANTS BY PARTICLE BOMBARDMENT USING LUCIFERASE ACTIVITY AS SELECTION MARKER.

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ABSTRACT

Cassava embryos derived from friable embryogenic callus of the genotype TMS60444 were bombarded with DNA of the constructs pJIT100 or PJIT64. Both constructs contain the firefly luciferase (LUC) gene driven by the 35S promoter. The influence of several particle gun machine parameters and pretreatment of plant material on transient LUC activity were studied. Two weeks after bombardment pieces of friable calli with one spot showing LUC activity were selected. In total 67 independent LUC spots, derived from 5 experiments, were further cultured either in liquid or on solid medium. One spot was cultured per plate or flask. In the subsequent selection rounds all spots of one individual plate or flask were cultured as one individual group. In this way different transformation events were separated and multiplied. Eight weeks after bombardment 34 cultures still contained LUC activity and the mean number of LUC spots per culture had increased from 1 to 4.6 in liquid medium and to 2.5 in solid medium. After two more months of subsequent culture and LUC selection the transformed nature of these cultures was confirmed on the molecular level using the polymerase chain reaction assay. Friable embryos derived from one transformation event were cultured for maturation. In total 24 of the 467 tested mature embryos were LUC positive. After multiplication of the LUC positive mature embryos by cyclic somatic embryogenesis they were allowed to grow into shoots and to root on basic medium. The analyzed plantlets contained multiple copies of the inserted DNA. The presented method enables us to transform this particular cassava genotype, thus allowing the genetic improvement of the important tropical crop cassava by transgenesis.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a short lived shrub of 1-5 m in height which is cultivated for its starch containing tuberous roots (Byrne, 1984; Cock, 1985). Since 1990 a number of laboratories have attempted to establish a transformation procedure for this crop (Schöpke et al. 1993; Arias-Garzon and Sayre, 1993; Raemakers, 1993; Luong et al., 1995; Sarria et al., 1995). All these groups have utilized somatic embryogenesis as method of regeneration. It is initiated from zygotic embryos (Stamp and Henshaw, 1982) or from leaves (Stamp and Henshaw, 1987a; Szabados et al., 1987; Taylor et al., 1993; Mathews et al., 1993; Raemakers et al., 1993a). The primary embryos produced in this fashion can then be induced to enter continuous cycles of secondary somatic embryogenesis by repeated subculture of embryos on auxin supplemented medium. The embryos generated by this culture system are highly organized and develop readily into mature embryos (Raemakers et al., 1993b,c; Mathews et al., 1993). There is only one report of successful use of this secondary system of embryogenesis for cassava transformation. This was done using a wild type *Agrobacterium tumefaciens* strain (Sarria et al., 1995). However, because the plants contain the wild type Ti-plasmid of the *Agrobacterium* strain, including the oncogenes, they are probably of no agronomical value. Furthermore, the duration of the experiments and the efficiency in which only a few -presumed- transgenics were obtained makes this procedure not feasible. Attempts to use strains without oncogenes were all unsuccessful. Secondary somatic embryogenesis in conjunction with particle bombardment (Schöpke et al. 1993, Arias-Garzon and Sayre, 1993) or electroporation (Luong et al., 1995) has led to chimeric transformed embryos. However, subculture of these embryos for repeated cycles of secondary somatic embryogenesis did never result into stably, fully, transformed plants.

In cassava a low percentage of the embryogenic tissue has a friable nature. This friable tissue can be isolated, purified and multiplied, resulting in almost complete friable embryogenic cultures. In this system with friable embryogenic callus the embryos are less organized. The embryos do not develop beyond the pre-globular stage, but instead proliferate new embryos from the surface of the units (Taylor et al., 1995). In cassava, this is the first report which describes the use of this friable embryogenic system in combination with particle bombardment.

Furthermore, it is the second report where in a crop recalcitrant for transformation, the luciferase reporter gene (isolated from *Photinus pyralis*), was used for selection of transformants. Previously this selection system was used successfully to obtain transformed plants in *Dendrobium* (Chia et al., 1994). The luciferase enzyme requires luciferin, ATP, Mg^{2+}, and O_2 to produce light (560 nm). Plant cells expressing luciferase and supplied with luciferin emit photons (Ow et al., 1986) which can be detected with a luminometer. It is shown that this specific, friable, form of somatic embryogenesis, combined with the detection of transformed tissue using luciferase activity, leads to cultures with transgenic friable embryos which after culture for maturation and germination develop into luciferase positive plants. It was shown by Southern hybridization that the selected plants contain the introduced DNA stably integrated in their genome.

MATERIAL AND METHODS

Plant material

The initiation of friable embryogenic callus cultures was as described before (Taylor et al., 1995). Highly friable embryoids of genotype TMS60444 were maintained on a medium consisting of Gresshoff and Doy (1972) vitamins and salts, 7 g/l Daichin agar, 20 g/l sucrose and 10 mg/l Picloram (solid GD2). Every three weeks the friable embryos were subcultured on the above mentioned medium. In order to initiate liquid suspension cultures 0.5 g of friable embryos was transferred in a flask of 200 ml with 50 ml of liquid medium supplemented with Schenk and Hildebrandt (1972) salts and vitamins, 60 g/l sucrose and 10 mg/l Picloram (liquid SH6). The medium was refreshed every 2 days and after 14 days the content of each flask was divided over 5 new flasks. The pH was adjusted to 5.7 before autoclaving. The temperature in the growth chamber was 30^0C, the photoperiod 12 hours and the irradiance 40 $\mu molm^{-2}s^{-1}$.

Constructs and coating of DNA on the particles.

In one experiment, DNA from the plasmid PJIT64 and in all other experiments DNA from pJIT100 (kindly provided by J.F. Guerineau, John Innes Institute, UK) were used. The plasmid

PJIT64 contains the firefly luciferase under control of two 35S promoters and the CaMV polyadenylation region and the plasmid pJIT100 contains the firefly luciferase and the phosphinothricin acetyl transferase gene both under control of the 35S promoter and the CaMV polyadenylation region (Guerineau and Mullineaux, 1993). Plasmid DNA was isolated using Wizard™ Maxipreps DNA purification system of Promega.

A method adjusted from McCabe et al. (1988) was used to coat DNA on the particles. Eighty μg of DNA was mixed with 10 mg of gold particles (1.6 μm, BioRad), 30 μl 5 M NaCl, 5 μl 2 M tris HCl pH 8.0, 115 μL H_2O, 100 μl 25% PEG 1550, 100 μL 0.1 M spermidine and 50 μl 2.5 M $CaCl_2$. After centrifugation the pellet was resuspended in 10 ml of absolute alcohol and briefly sonificated. One hundred sixty μl of the gold suspension was pipetted through the appature of an inverted macrocarrier holder onto a standard macrocarrier (Bio Rad). After 5 minutes the macrocarrier holder was removed. The macrocarrier covered with a thin layer of gold beads was dried in an oven (10 minutes, 40 °C) and used for bombardment.

Bombardment of suspensions culture

Suspension culture derived friable embryos, cultured in liquid SH6 medium for a period of as short as 5 min to as long as 7 weeks, were sieved (mesh 1 mm^2) and collected on filters (81 μm). Per bombardment 0.05 g of friable embryos (approximately 2500 units) was spread in the centre of a plate with solid (7 g/l Daichin agar) SH6 medium. Plates were bombarded using the PDS-1000He biolistic device (BioRad). The distance between the rupture disc and the macro-carrier was 0.5 cm and between the macrocarrier and the stopper plate 0.5 cm. The effect of different helium pressures (450-1800 p.s.i.) combined with different vacuum levels (15 or 26 inches Hg vacuum) and distances of the plate to the stopper plate (5 or 8 cm) were tested on the transient luciferase (LUC) activity one day after bombardment. After bombardment the friable embryos were cultured for one day on solid SH6 after which they were either transferred to liquid SH6 or solid (first 3 days on Gresshoff and Doy (1972) medium supplemented with 7 g/l Daichin agar, 40 g/l sucrose and 10 mg/l Picloram (GD4) than on a Greshoff and Doy medium with 20 g/l sucrose, instead of 40 g/l).

In another experiment the effect of different pretreatments on transient LUC activity was tested. For this solid GD2 cultured friable embryogenic callus was sieved and cultured in liquid SH2, SH4 or SH6 medium for three days. The cultures were bombarded at 450 p.s.i and the friable embryos were divided over solid SH2, SH4 and SH6.

Selection and multiplication of luciferase (LUC) positive friable embryos

The first selection of LUC positive explants was done two weeks after bombardment. In the experiments used for optimization of transient activity this was the second and in the other experiments the first LUC assay. The friable embryos in a 1 cm radius around a LUC positive spot were cut out of the agar and subcultured in liquid (SH6) or solid (first 3 days GD4 than GD2) medium. One spot was cultured per flask or plate. In the next selection rounds (every 2 weeks) the positive spots of individual cultures were isolated and cultured together.

Maturation and germination of friable embryos

Cultures containing at least 1% LUC positive friable embryos were grown for maturation on a medium consisting of Murashige and Skoog (1962) salts and vitamins, 0.1 g/l myo-inositol, 20 g/l sucrose, 40 g/l mannitol, 0.48 g/l MES, 0.1 g/l caseinhydrolysate, 0.08 g/l adenine sulphate, 0.5 mg/l d-calcium-pantothenate, 0.1 mg/l choline chloride, 0.5 mg/l ascorbic acid, 2. mg/l nicotinic acid, 1 mg/l pyridoxine-HCl, 10 mg/l thiamine HCl, 0.5 mg/l folic acid, 0.05 mg/l biotin, 0.5 mg/l glycine, 0.1 mg/l L-cysteine, 0.25 mg/l riboflavine. The pH was adjusted to 5.7 before autoclaving. Every two weeks the LUC positive explants were transferred to fresh medium until they became mature. LUC positive mature embryos were multiplied by cyclic somatic embryogenesis in liquid medium supplemented with Murashige and Skoog (1962) salts and vitamins, 20 g/l sucrose (MS2) and 10 mg/l NAA as described previously (Raemakers et al., 1995a). Multiplied mature embryos were cultured for shoot development on medium supplemented with Murashige and Skoog (1962) salts and vitamins, 20 g/l sucrose, 8 g/l Daichin agar (MS2) and 1 mg/l BAP. Shoots were rooted on MS2.

Luciferase (LUC) assays

Tissues cultured in liquid medium were collected on filters (82 μm) and spread on solid GD4

medium. The explants were sprayed with 0.25 mg/ml luciferin (Promega, E160) and assayed for luciferase (LUC) activity with a VIM intensified CD camera and Argus-50 photon counting image processor from Hamamatsu Phototonic Systems. Cultures of 1 day, 2 and 4 weeks after bombardment were measured for 1 minute. In the later assays this was less than 30 seconds.

Molecular analysis.

Genomic DNA was isolated for PCR from approximately 50 mg friable embryos and for Southern hybridization from 300 mg green leaf tissue of in vitro plantlets using a method described by Salehuzzeman et al. (1993). Primers LUC1 (ACG GTT TTG GAA TGT TTA CTA C) and LUC2 (CGG TTG TTA CTT GAC TGG CGA C) were synthesized (Eurogentec) and yielded a 792 bp fragment corresponding to an internal portion of the luciferase gene. A 50 μl PCR reaction mix contained the primers (50 pM), Taq polymerase (0.2 U), dNTP (80 μM of each), 1XPCR reaction buffer and 50 ng of the isolated DNA. The reaction mix was overlaid with 50 μL mineral oil. PCR conditions were 92°C initial melting, for 10 min, 30 cycles of 92°C/2 min and 47 °C/2 min and a 72°C/2 min final extension. Southern hybridization was performed as described (Salehuzzeman et al., 1993) using 8 μg of EcoRV or BglII restricted DNA of cassava and the luciferase gene as a probe.

RESULTS

Optimization of transient activity.

Nine different experiments were conducted in order to optimize bombardment treatments for transformation. The tested variables are presented in Table 1. In the first 5 experiments the influence of some variables concerning the particle gun and pretreatment on the transient LUC activity were studied. These transient studies were not performed in the last four experiments.

Machine variables

In the first experiment (Table 1) friable embryos were placed 5 cm below the stopperplate and

bombarded with different helium pressures at 26 inch Hg vacuum. A helium pressure of 450 p.s.i. was found to result in the highest transient luciferase (LUC) activity with more than 30.000 photons being detected in one minute. A Petri dish bombarded with pJIT100 and sprayed with water, instead of luciferin, produced 56 photons/min and a Petri dish bombarded with a control construct without the LUC gene and sprayed with luciferine produced 87 photons/min. An example of transient LUC activity of a culture bombarded with 450 p.s.i. is shown in Fig. 1A. The scale in this figure represents different numbers of emitted photons. At this magnification it is difficult to count individual spots, but at a higher magnification at least 300 distinct spots/cm^2 could be identified. The luciferase activity decreased rapidly. Fifteen minutes after application of luciferin the LUC activity had decreased by 29% and after 2 hours only 8% of the original activity could be detected. As the BioRad gun cannot operate at helium pressures lower than 450 p.s.i. the distance to the stopper plate, combined with the partial vacuum pressure was varied to reduce the momentum of the particles (Table 1: experiment 2). The transient activity of cultures bombarded under the same conditions as in experiment 1 was 10.623 photons/min which is a factor three lower than in this first experiment. The same level of transient activity was observed after the vacuum level was decreased to 15 inc. Hg or after the distance of the material to the stopperplate was increased to 8 cm. However, if both the lower vacuum level and the greater distance were combined, than the luciferase activity decreased to 300 photons/min. In the above described two sets of experiments the cultures were bombarded once. Two shots per plate resulted in a LUC activity which was almost twice as high as compared to one shot per plate (Table 1: experiment 3).

Table 1: Overview of the particle bombardment experiments of friable embryos of cassava genotype TMS60444. (experiment 1 to 8: plasmid pJIT100, experiment 9 plasmid pJIT 64).

Exp.	Pre-culture		# shots/	Machine parameters			Post culture[A]	Number of bombarded plates	Photons per minute (day 1)	LUC spots per bombarded plate (day 14)
no.	Medium[A]	Duration	Plate	p.s.i.	vac.	dist.				
1	L	5 days	1	450	26	5	L	6	32569	1.00
"	"	"	"	900	"	"	"	5	17339	1.20
"	"	"	"	1100	"	"	"	6	24151	0.33
"	"	"	"	1800	"	"	"	4	17300	1.50
2	L	6 weeks	1	450	26	5	S	4	10623	0.50
"	"	"	"	"	15	"	"	4	12623	0
"	"	"	"	"	26	8	"	4	10417	0
"	"	"	"	"	15	"	"	4	300	0
3	L	6 weeks	1	450	26	5	L	4	23721	0.25
"	"	"	2	"	"	"	"	4	50490	0.50
4	2%[B]	3 days	1	450	26	5	2%[C]	5	7944	nd
"	"	"	"	"	"	"	4%[C]	5	10227	nd
"	"	"	"	"	"	"	6%[C]	5	4587	nd
"	4%[B]	"	"	"	"	"	2%[C]	5	7704	nd
"	"	"	"	"	"	"	4%[C]	5	11127	nd
"	"	"	"	"	"	"	6%[C]	5	13158	nd
"	6%[B]	"	"	"	"	"	2%[C]	5	8880	nd
"	"	"	"	"	"	"	4%[C]	5	17907	nd
"	"	"	"	"	"	"	6%[C]	5	22014	nd
5a	L	5 min	1	900	26	5	S	5	9107	0
"	"	2 hours	"	"	"	"	"	5	36515	0.2
"	"	7 weeks	"	"	"	"	"	5	44955	1.6
5b	"	"	"	"	"	"	"	5	nd	5.0
6	L	2 days	2	1100	26	5	L	11	nd	3.27
7	L	4 days	2	450	26	5	L	3	nd	2.33
8	L	7 weeks	1	450	26	5	S	4	nd	2.25
"	"	"	"	900	"	"	"	4	"	2.25
"	"	"	"	1100	"	"	"	4	"	2.50
"	"	"	"	1550	"	"	"	4	"	3.75
9	L	1 week	2	1100	26	5	L	1	nd	3.00

[A]:L= liquid medium SH6%, S= solid GD2%, [B]:liquid SH with 2, 4 or 6% sucrose, [C]: solid SH with 2, 4 or 6% sucrose (see material and methods); nd=not determined.

Plant material

In experiment 4 (Table 1) the effect of different pre- and post-transformation culture media on transient LUC activity was studied. Pre-culture of friable embryos in SH2 medium gave the lowest and in liquid SH6 medium the highest transient LUC activity, independently of the post-treatment. There was an interaction between the post-culture media and the pre-culture media on transient LUC activity. Post-culture in SH6 medium gave the highest transient LUC activity in friable embryos pre-cultured in SH6 and SH4. However, post-culture in SH4 gave the highest transient activity in friable embryos pre-cultured in SH2. In experiments 1 to 4 the friable embryos were cultured for a period varying from 2 days to 6 weeks in liquid SH6 before they were bombarded. Between the experiments there was not a clear relation between the pre-culture duration in SH6 and the transient LUC activity (Table 1), probably this might be caused by experimental variations. In order to assess the effect of the time in liquid culture, friable embryos were transferred to liquid medium for 5 min, 2 days or 7 weeks before they were bombarded (Table 1: experiment 5a). Friable embryos cultured for 5 minutes in liquid SH6 produced only 9.107 photons and this increased to 36.515 photons/min after they were cultured for 2 hours in liquid SH6. The highest activity was found with friable embryos which had been subcultured for 7 weeks.

Detection of LUC positive friable embryos 14 days after bombardment

Fourteen days after bombardment the LUC activity was seen to be decreased to less than 2% of the level observed after 1 day (data not shown) and it was possible to distinguish distinct LUC spots (Fig. 1B). In the different experiments, the number of LUC spots per bombarded plate (LUC/bp) is given in the last column of Table 1.

The highest number of LUC spots per bombarded plate was observed in the experiments 5b, 6, 7, 8 and 9 where the first LUC assay was performed 14 days after bombardment. In the experiments 1 to 5a where also transient activity was measured, a maximum of 1.6 LUC spots/bp was recorded (Table 1: experiment 5a). In the two experiments where different helium pressures were compared there was either no effect of the pressure on the number of LUC spots per bombarded plate (Table 1: experiment 1) or a slight increase (Table 1: experiment 8) with using higher pressures. In experiments 1 and 8, respectively, 1.0 and 0.5 LUC spots/bp were detected. No spots were detected if the acceleration of particles was

further reduced by either a lower level of vacuum or a longer distance of the plant material to the stopper plate (Table 1: experiment 2). In experiment 5a (Table 1), where different preculture durations were tested, a preculture of 5 minutes did not result in LUC spots whereas 7 weeks gave clearly more spots than 2 hours.

Subculture and multiplication of LUC positive friable embryos

The friable embryos around a LUC spot were cut out of the agar and subcultured in either liquid SH6 or on solid GD2 medium. To separate the different transformation events one LUC spot was cultured per flask or plate.

Table 2: The efficiency of luciferase selection for the recovery of cultures with transformed friable embryos.

		# of cultures with LUC spots			
		Weeks after bombardment			
Exp	Treatment	2	4	6	8
1	liquid	6	4	3	3
5	solid	33	20	16	15
7	solid	4	4	4	4
	liquid	3	3	3	2
8	solid	18	12	10	9
9	liquid	3	1	1	1
	Total	**67**	**44**	**37**	**34**

In the next selection rounds each flask or plate was treated as one individual culture and all the spots of one culture were subcultured as belonging to one group. A total of 67 LUC spots was subcultured: 12 in liquid and 55 on solid medium. Two weeks later 44 cultures still contained LUC spots: 8 in liquid and 36 on solid medium. This was not correlated with the strength of the spot isolated two weeks after bombardment. In some cases very bright spots had disappeared whereas low intensity spots became very bright after 4 weeks. In the next selection round the decrease in LUC positive cultures was lower. Four more disappeared during subculture from week 6 to 8 because of infection. Eight weeks after bombardment 34 cultures were still LUC positive. The mean number of LUC spots was in liquid cultures 4.6 and in solid cultures 2.5. From 8 weeks on in all 34 cultures the number of LUC positive

explants per culture doubled every 2-4 days. The highest efficiency of the transformation procedure, calculated as the number of cultures which still contain LUC spots 8 weeks after bombardment per bombarded dish was 2.0 in experiment 7. In this experiment 7 spots were detected 2 weeks after bombardment (Table 1) of which one spot was lost during culture (Table 2).

Maturation of LUC positive friable embryos and development into shoots

Because there was no experience with maturation of friable embryos, one culture, containing transformed friable embryos (Table 1: experiment 9; friable embryos were transformed with pJIT64) was, taken to optimize this process. For this the fraction of transformed friable embryos was increased by continuous LUC selection.

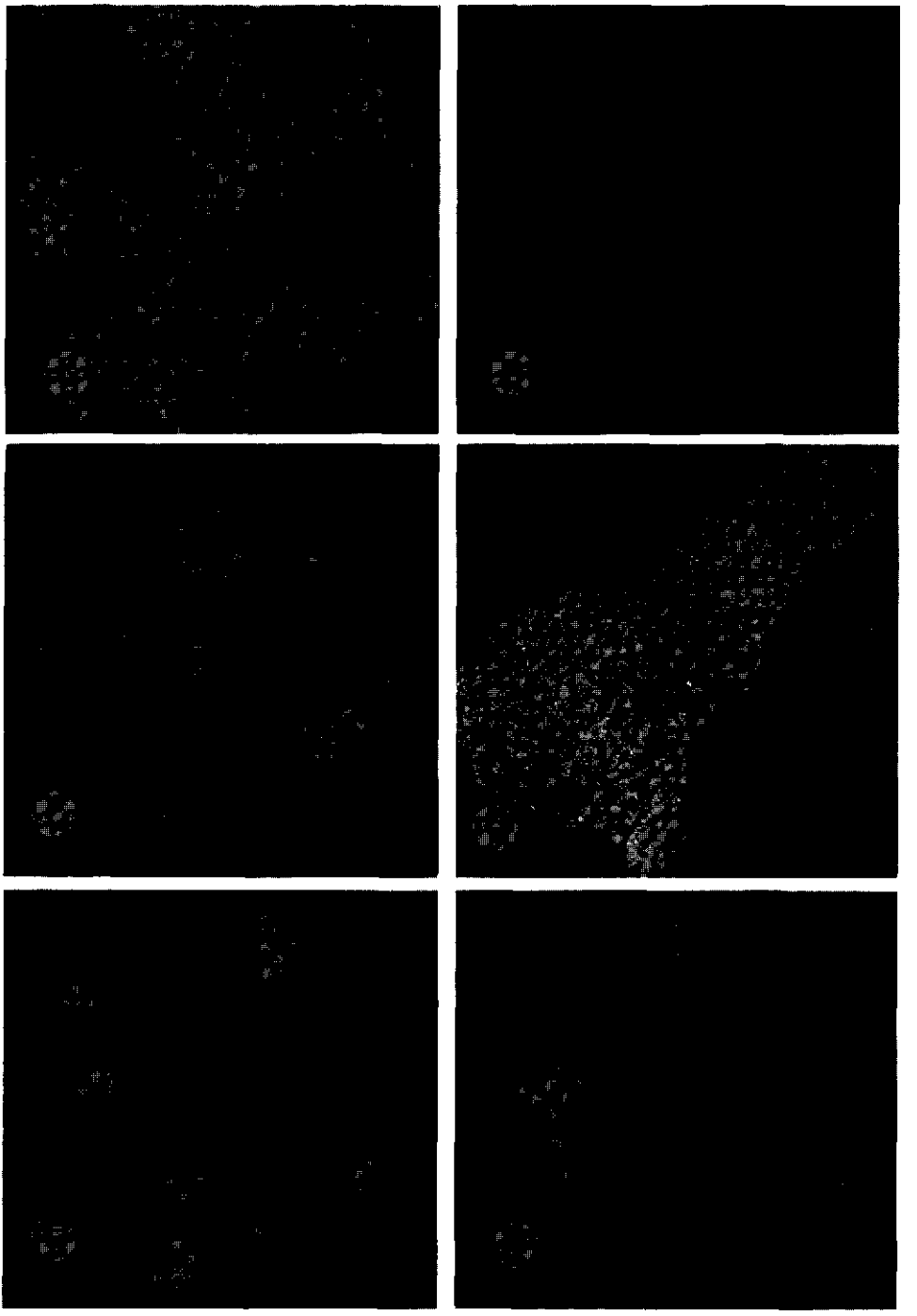

Figure 1: Luciferase activity of suspension derived callus bombarded with pJIT64: A) 1 day after bombardment (x 4), B) 2 weeks after bombardment (x 1.2), C) 4 months after bombardment (x 1.2), D) mature embryo derived from friable embryogenic callus (x 15), E) mature embryo multiplied by secondary somatic embryogenesis (x 1.2), F) plants developed from secondary embryo cultures (x 1.2).

Friable embryos as shown in Fig. 1C were used for maturation. After 4 weeks of culture on maturation medium the first mature embryos were isolated and assayed for LUC activity. In total 467 mature embryos derived from one transformation event were assayed of which 24 were LUC positive (Fig. 1D). LUC positive mature embryos were multiplied by culturing in liquid MS2 medium supplemented with 10 mg/l NAA or in solid MS2 medium supplemented with 8 mg/l 2,4-D. In the first medium 5 out of 12 and in the second medium 10 out of 12 of the mature embryos formed secondary embryos (Fig. 1E). Of the initial 22 LUC positive mature embryos 12 initiated LUC positive secondary cultures and two were LUC negative. In total about 300 mature embryos were cultured for shoot development on MS2 medium supplemented with 1 mg/l BA. The first shoots appeared one month after culture and in total 21 shoots were obtained. Al these shoots were LUC positive (Fig. 1F).

Molecular analysis

From 8 cultures friable embryos with LUC activity were analyzed by PCR. All cultures gave a band of the expected size for both the luciferase gene and the phosphinothricin acetyl transferase gene (data not shown), indicating the stable integration of the construct in these cultures. DNA from two secondary cultures of mature embryos and of two regenerated plants derived from experiment 9 (Table 1) were restricted with EcoRV or BglII. The banding patterns of the four different samples are identical (Fig. 2). Because EcoRV, and BglII cuts the luciferase gene once it can be concluded that a minimum of four copies are stably integrated in the plant genome.

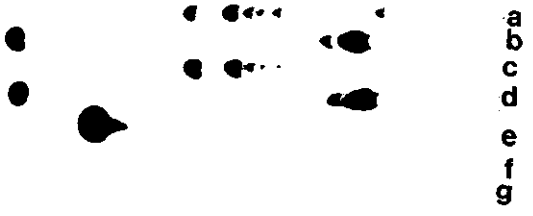

Figure 2: Southern blot hybridization of DNA isolated from secondary embryogenic culture (a and b), regenerated trnsformed plant (c and d) and control non-transformed plant (f and g).DNA was restricted with BgII (a,c,f), and EcoRV (b,d,g). Lane e is a marker lane.

DISCUSSION

Previously, cassava embryos induced by secondary or cyclic somatic embryogenesis were used as target tissue for transformation (Schöpke et al. 1993, Arias-Garzon and Sayre, 1993, Luong et al., 1995, Sarria et al., 1995, Chapter 2). In only one case, by using *Agrobacterium tumefaciens*, this resulted in transformed plants (Sarria et al., 1995). However, the plants contained the wild-type Ti plasmid and the procedure was not repeatable. This paper is the first report were cassava has been transformed reproducibly with only genes of interest. This was accomplished by combining a new system of somatic embryogenesis (Taylor et al., 1995) with a selection system based on the activity of the firefly luciferase gene (Ow et al., 1986). This friable embryogenic callus or suspension culture system differs in several aspects from cyclic somatic embryogenesis. In the friable system embryos do not develop beyond the pre-globular stage whereas in cyclic embryogenesis the embryos develop readily into mature embryos. In both types of somatic embryogenesis there is a phase were new embryos are initiated, followed by a phase of growth and development of the initiated embryo. The time period between two moments of initiation and growth or development is much shorter in friable than in cyclic somatic embryogenesis. As a consequence the cells in friable embryos are more often in a state of initiation whereas in cyclic somatic embryogenesis they are for a longer period in a state of growth and development. An initiation state is advantageous for plant transformation because a cell has than the chance to act independently from neighbouring cells and form a new embryogenic unit. Another difference is the fact that in friable embryogenesis the embryos originate from epidermal cells, possibly single cells (Taylor et al., 1995). In cyclic somatic embryogenesis the embryos originated from a group of cells and this group consisted of epidermal and mesophyllic cells (Stamp, 1987; Raemakers et al., 1995b).

LUC based selection has many advantages over selection with ß-glucuronidase (GUS). The most important one is the non-destructive nature of the LUC assay; positive explants can be selected and subcultured. The GUS assay is destructive. The GUS protein is very stable; it has in mesophyll cells a half life time of more than 50 hours (Jefferson et al.,1987). The LUC protein has a half life time of about 3 hours in mammalian cells (Thompson et al., 1991). Although data on the half life time in plant cells are not available, the fact that two hours after supply of luciferin the LUC activity is reduced to 8% suggests that the enzyme is not long lived. A consequence of a longer half life time is that the transiently produced enzyme will be active for a longer time period thus overestimating the transformation efficiency. A short enzymatic half life time has as advantage that there is no build up of enzyme and that gene/enzyme activity can be tested repetitively (Luehrsen et al., 1992).

Furthermore, plant tissue does not contain endogenous LUC activity which might interfere with the assay as is observed for the GUS enzyme.

LUC based selection has also advantages above chemical selection (reviewed by Wilmink and Dons, 1993) by antibiotics as kanamycin or herbicides as phosphinothricin. On one extreme the chemical selection might be to rigorous leading to the loss of the regenerative capacity of the transformed tissue. This was observed in transformation of friable embryogenic callus of barley (Stiff et al., 1995), Norway spruce (Robertson et al., 1992), white spruce (Bommineni et al., 1993) and banana (Sági et al., 1995) where no plants could be regenerated from chemically selected transformed callus. In such cases LUC based selection would ensure regeneration. On the other extreme chemical selection might not be strong enough resulting in a high percentage of recovered plants which are not transgenic. If this were the case LUC selection combined with chemical selection would reduce labour because only positive explants are subcultured and the ones without LUC activity are discarded. The LUC gene is also an option in plants where chemical selection does not work as was reported for *Dendrobium* (Chia et al., 1994).

In spite of the fact that pJIT100 contains the phosphinothricin acetyl transferase gene, ppt was not used for the selection of transgenic events. Since ppt, even at a concentration as high as 10 mg/l, was not able to completely inhibit the growth of non-transformed tissue, it was not used in the experiments described here. It is however, obvious that the presence of this marker gene in the transformed tissue (FEC, maturing FEC, mature embryos and plants) will be used to investigate the use of ppt resistance as a selection system in transformation of cassava.

From the here presented data it seems that for the optimal result it is important to use friable embryos which have been subcultured for at least one week in liquid SH6 medium. The friable embryos should be bombarded with particles which have a momentum equal or higher than that is obtained with 450 p.s.i., vacuum of 26 inc Hg and a distance of the plant material to the stopperplate of 5 cm. The efficiency of this procedure was as high as 2 (Table 1,2 experiment 7) successful transformation events per bombarded dish. At this moment all cultures which were used in maturation experiments yielded LUC positive mature embryos (results not shown). The obtained efficiencies are in agreement with other crops where embryogenic suspension cultures were used as target tissue for bombardment. For example in maize the number of transformation events yielding transformed plants varied between 0.6 and 1 per bombarded dish (Fromm et al., 1990, Gordon-Kamm et al., 1990). In peanut 2 transformation events leading to transformed plants were observed per bombardment (Ozias-Akins

et al., 1993).

After bombardment this transformation system counts 4 phases. First friable embryos originating from one transformation event are allowed to proliferate. If enough transformed friable embryos are available than a part can be stored in liquid nitrogen and the other part is used for maturation (second phase). In the third phase mature embryos are multiplied by cyclic somatic embryogenesis before they are allowed to grow into plants (fourth phase). This 4 phases system will guarantee that the percentage of transformation events leading to transformed plants is maximized. If there is a future demand than friable embryos of a specific transformation event can be taken out of the liquid nitrogen and can be used for further molecular analysis or for the introduction of additional genes of interest. It is obvious that we have a method for cassava transformation available which needs to be extended to other genotypes and genes. However, the fact that a repeatable transformation system is within reach opens up the possibilities to improve this important crop cassava by genetic modification.

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CHAPTER 6

COMPARISON OF GERMINATION OF NAA AND 2,4-D INDUCED SOMATIC EMBRYOS OF CASSAVA (*MANIHOT ESCULENTA* CRANTZ).

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ABSTRACT

In cassava the germination of cyclic somatic embryos, induced in liquid medium by the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthalene acetic acid (NAA), was compared for four different genotypes. In all genotypes desiccation stimulated normal germination of NAA induced embryos. However, the desiccated embryos, required a medium supplemented with cytokinins such as benzylaminopurine (BAP) for high frequency germination. The morphology of the resulting seedling was dependent on the concentration of BAP. With 1 mg/l BAP plants with thick and short taproots and branched shoots with short internodes were formed. With 0.1 mg/l BAP the taproots were thin and slender and the shoot had only one or two apical meristems. If the embryos were desiccated sub-optimally, higher concentrations of BAP were needed than if the embryos were optimally desiccated to stimulate germination. Also desiccated embryos which were cultured in the dark required a lower concentration of BAP and, furthermore, these embryos germinated faster than embryos cultured in the light. Complete plants were obtained four weeks after the start of somatic embryo induction. 2,4-D induced embryos showed a different response. In only one genotype desiccation enhanced germination of 2,4-D induced embryos and in three other genotypes it did not. In all genotypes desiccation stimulated root formation. Embryos cultured in the dark formed predominantly adventitious roots, whereas embryos cultured in the light formed predominantly taproots.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial crop of the family Euphorbiaceae, grown mainly for its enlarged tuberous roots. The crop has many advantages: it is drought tolerant, can be grown in diverse edapho-climatic conditions, in depleted soils and is able to recover from damage caused by severe incidence of pests, diseases and bushfires (Cock, 1985). These advantages and the adaptability to indigenous farming systems explain the rapid spread of the crop on the African and Asian continents (Byrne, 1984). Cassava has a potential yield of 90 tonnes per hectare per year (Cock, 1985). However, the average yield in Indonesia is 10.3 tonnes per hectare (Soenarjo et al., 1987), and in Africa it is even lower (Anonymous, 1993). This low yield has been attributed to the poor fertility of the soils where cassava is grown, deterioration of roots and severe incidence of pests and diseases. The African Cassava Mosaic Virus, for example, can cause yield losses of up to 95% (Bocks and Woods, 1983). Beside the lack of resistant genotypes, the use of stakes infected with diseases and pests enhanced their persistence. There is lack of good healthy planting materials. Such planting material can be produced via meristem culture. In 1975 this technique was used to free cassava from Cassava Mosaic Virus (Kartha and Gamborg 1975). Healthy plants can be produced in large numbers by multiple shoot culture (Smith et al., 1986). However, this technique requires several tissue culture steps (mechanical isolation of cuttings, rooting of cuttings and hardening of plants) which makes the procedure labour intensive. In principle, labour could be reduced if planting materials are produced via somatic embryogenesis. Somatic embryos have the developmental program to grow into complete plants without mechanical isolation and separate shooting and rooting steps (Parrot et al., 1991). In cassava somatic embryogenesis was first reported by Stamp and Henshaw (1982) and since then by several groups as an efficient regeneration system (Szabados et al., 1987; Mathews et al., 1993; Raemakers et al., 1993abc). Embryos are induced on leaves or zygotic embryos cultured on auxin supplemented medium. The primary embryos recultured on auxin supplemented medium produce secondary embryos and continuous subculture of embryos allows the development of cyclic embryogenic cultures (Stamp and Henshaw 1987; Mathews et al., 1993; Raemakers et al., 1993bc). The auxin 2,4-D initiates both primary and cyclic embryogenesis, NAA in contrast, only cyclic embryogenesis (Chapter 2). To use somatic embryogenesis for plant multiplication the embryos should germinate efficiently into seedling-

like cassava plants. To achieve this goal, Raemakers et al., (1993b,c) cultured mature 2,4-D induced embryos on a medium supplemented with 1 mg/l BAP. Depending on the genotype between 10% (MCol.1505) and 70% (MCol.22 and TMS90853) of the embryos developed into plants (Raemakers, 1993). The process of germination was slow, the plants were initially malformed and lacked taproots. Adventitious roots were formed after culture of plants on growth regulator-free medium. A germination process whereby genotype-independent complete normal plants with taproots are produced within a short period of time is desirable. Mathews et al., (1993) improved germination by desiccating embryos. With this method 85% of MCol.1505 embryos, induced on 2,4-D supplemented solid medium, germinated into complete plants compared to 5% for undesiccated embryos.

High frequency germination of mature embryos is also of importance for the application of the protoplast and genetic modification technology for cassava improvement. It was shown that only a low percentage of the mature embryos derived from protoplasts (Chapter 4) and from transformed mature embryos (Chapter 5) developed into plants.
In this report germination of embryos induced in liquid medium is described. It is shown that NAA induced embryos differ from those induced by 2,4-D. Furthermore, it is shown that besides desiccation the use of cytokinins and darkness during culture for germination are important factors in obtaining complete plants in a number of different cassava genotypes.

MATERIALS AND METHODS

Plant material and growth conditions

The cassava (*Manihot esculenta* Crantz) genotypes TMS 90853, Line 11, Adira 4 and Gading were used in this study. Primary embryos were produced following the method described by Raemakers et al., (1993a). In brief: young leaf lobes (1-6 mm) were cultured on solid (8 g/l Daichin agar) basal medium (BM) supplemented with 20 g/l sucrose (BM) plus 8 mg/l 2,4-D (step one). BM consisted of Murashige and Skoog (1962) salts and vitamins. After 20 days explants were transferred to BM supplemented with 0.1 mg/l BAP for maturation (step two). Mature embryos are defined as structures with a distinct hypocotyl and green cotyledons. Mature embryos were isolated from the leaf explants, chopped into fine pieces and cultured in 300 ml flasks with 75 ml liquid BM supplemented with 8 mg/l 2,4-D or 10 mg/l NAA for

the production of cyclic embryos. Cultures were placed on a gyrator (LAB-Line Instruments Inc., model 3519) at 120 rpm. After 15 (NAA) and 30 (2,4-D) days of culture mature embryos were chopped again and cultured in the same media for a new cycle of embryogenesis. In this way the embryos used in this study were maintained for 2 years (TMS 90853), 1 year (Adira 4) and ½ year (Gading and Line 11). All cultures were kept in a growth chamber with a photoperiod of 12 hours, a temperature of 30 °C and an irradiance of 40 $\mu mol^{-2}s^{-1}$.

Methods to achieve desiccation and subsequent germination of embryos.

Mature NAA induced embryos of Adira 4 were subjected to various desiccation treatments for a period of 7 days. After the desiccation the embryos were cultured for germination on solid BM supplemented with 1.0 mg/l BAP.

Petri-dish desiccation

Mature embryos (12 g of fresh weight) induced in liquid medium were transferred to sterile Whatmann 3 mm filter paper to absorb hanging water. After 1 hour the weight of the embryos had decreased to 35 percent. Four grammes of the dried embryos were transferred to empty Petri plates, weighed, sealed with parafilm and stored in the growth chamber. The Petri-dishes were changed every other day to remove condensed water. The percentage of desiccation was calculated from the ratio between the weight of the dried embryos after and before Petri-dish desiccation, multiplied by 100.

Desiccation using glycerol

After removal of hanging water, 4 gr of dried mature embryos were transferred to an empty Petri-dish and placed in a desiccator (2 l) containing 200 ml of a 87% glycerol solution to control the internal humidity.

Desiccation by using sugar solutions

Four grammes of mature embryos were transferred to solid BM supplemented with 0-160 g/l sucrose, or liquid BM supplemented with 0-60 g/l sucrose, or H_2O supplemented with 0-60 g/l sucrose, or liquid BM supplemented with mannitol (0-10 g/l).

The effect of different growth regulators on embryo germination.

To study the effect of growth regulators on embryo germination, NAA induced Petri-dish desiccated embryos of Adira 4 were cultured on BM, BM supplemented with TDZ (0.1, 1, 4 mg/l), BAP (0.5, 1, 4 mg/l), Kinetin (0.5, 1, 4 mg/l), IBA (1, 5, 10 mg/l) or NAA (1, 5, 10 mg/l).

Effect of degree of moisture loss on embryo germination.

To study the relation between moisture loss and germination, Petri-dishes with mature NAA induced embryos were either placed in 500 ml jars filled with 50 ml of 0, 0.05, 0.2 mg/l NaCl (Gading) or were placed in 2 l jars filled with 50 ml of a 0, 60, 87% glycerol solution (Adira 4). After 7 days the embryos were cultured for germination on BM supplemented with 0-4 mg/l BAP.

Influence of light/dark regime on germination of desiccated embryos.

Mature NAA or 2,4-D induced embryos of TMS 90853, Line 11, Adira 4 and Gading were desiccated using the Petri-dish method and cultured on BM supplemented with 0-4 mg/l BAP, either in the dark or in the light.

Histology

Taproots of embryos were fixed in aqueous FAA solution (4 % formalin, 5 % acetic acid and 50 % ethanol), dehydrated 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.

Evaluation

Embryos cultured in the dark/light experiment were evaluated after 14 and 28 days of culture and in all other experiments after 28 days. The embryos were evaluated individually for presence or absence of roots, the morphology of the roots (thin or thick, taproots or adventitious) and presence or absence of shoots. A shoot was present when an internode had appeared with at least one primary leaf. An embryo was considered to be germinated if it had both a root and a shoot.

RESULTS

The effect of different desiccation methods on embryo germination.

Different desiccation methods were used to stimulate germination from mature somatic embryos of the cassava genotype Adira 4 (data not shown). Non-desiccated embryos did not germinate into complete plants with roots and shoots. Only a small number of the cultured embryos formed either roots or shoots (17 and 3% respectively). In the Petri-dish and glycerol desiccation methods the moisture loss could be determined. In the Petri-dish method the dried embryos lost a further 40% of their fresh weight and in the glycerol method 55 percent. In both methods 88% of the embryos germinated into complete plants with taproots and shoots. The remaining embryos formed only shoots or roots. To obtain an effective desiccation it was essential that the liquid cultured embryos were first dried on filter paper to remove hanging water. If this was not done only a few embryos germinated. Because water condensed on the lids of the Petri-dishes it was further essential that every other day the embryos were transferred to a new Petri-dish to remove the water.

In the desiccation methods using sugar solutions the moisture loss could not be determined exactly. At lower concentrations of sugars the embryos continued to grow, indicated by an increase of fresh weight. When higher concentrations of sugars were used the growth ceased. In all treatments less than 10 % of the embryos germinated. Although the glycerol desiccation was comparably as good as the Petri-dish desiccation the latter was chosen because of its simplicity for further experimentation.

The influence of growth regulators on embryo germination.

The embryos used in this experiment lost 20% of their fresh weight. The dried embryos were cultured on different media (Table 1). None of the embryos cultured on growth regulator-free medium germinated or formed shoots and only 1 embryo formed roots (Table 1). The auxins IBA and NAA stimulated only root formation. For this NAA was more effective than IBA. With 4.0 mg/l NAA 66% of the embryos formed roots (Table 1). The roots formed were abundant, thin and, in contrast to IBA where tap roots were formed, adventitious of origin.

All the tested cytokinins stimulated germination and/or shoot formation. TDZ gave the lowest

percentage of germination; at all the concentrations tested not more than 12 percent germinated. Germination increased with higher concentrations of kinetin. On a medium supplemented with 4 mg/l kinetin 36% of the embryos germinated and 18% formed only shoots (Table 1). The roots of these embryos were normal thin tap roots (Fig. 1A) and the shoots had more than one apical meristem. The highest percentage of shoot development (with and without roots) was observed when embryos were cultured on BAP supplemented medium. The optimal BAP concentration was found to be 1.0 mg/l. At this concentration about 67% of the embryos developed shoots and 14% germinated. The morphology of the roots and shoots of embryos was dependent on the BAP concentration. Embryos cultured on 1 or 4 mg/l BAP formed thick tap roots (Fig. 1B). The formation of thick tap roots was correlated with shoot formation. The shoots had short internodes and were branched. Sometimes more than 10 apical meristems were formed from one embryo (Fig. 1B). Histological studies showed that the thickness of the roots was caused by a loosely arrangement of cells around the stele (Fig. 1C). Embryos cultured on 0.1 mg/l BAP formed thin and slender taproots (Fig. 1D). Thin root formation was not always associated with shoot formation, however, the shoots formed had normal internodes and only one or two apical meristems (Fig. 1D).

The relation between moisture loss and embryo germination.

In the earlier experiment on the effect of different desiccation methods 88% of the Petri-dish desiccated embryos cultured on 1 mg/l BAP germinated. In the experiment presented in Table 1 the same treatment gave a germination of 14 percent. A reason for this difference in frequency of germination might be the amount of moisture loss, respectively 40 and 20 percent. To test this hypothesis embryos of Adira 4 and Gading were subjected to treatments which gave different levels of moisture loss. The results are presented in Table 2.

Embryos of both genotypes with a moisture loss of 20% did not germinate into complete plants after culture on a growth regulator free medium. Germination occurred if embryos were cultured on a medium with BAP. For Gading this frequency was still very low, but 45% of the Adira 4 embryos cultured on 1 mg/l BAP germinated. In both genotypes embryos with a moisture loss of 40 % gave a higher percentage of germination. For Gading 0.1 mg/l BAP gave the best germination (72%) and for Adira 4 this was 1 mg/l BAP (87%). With higher BAP concentrations there was a shift from germination to shoot formation without roots.

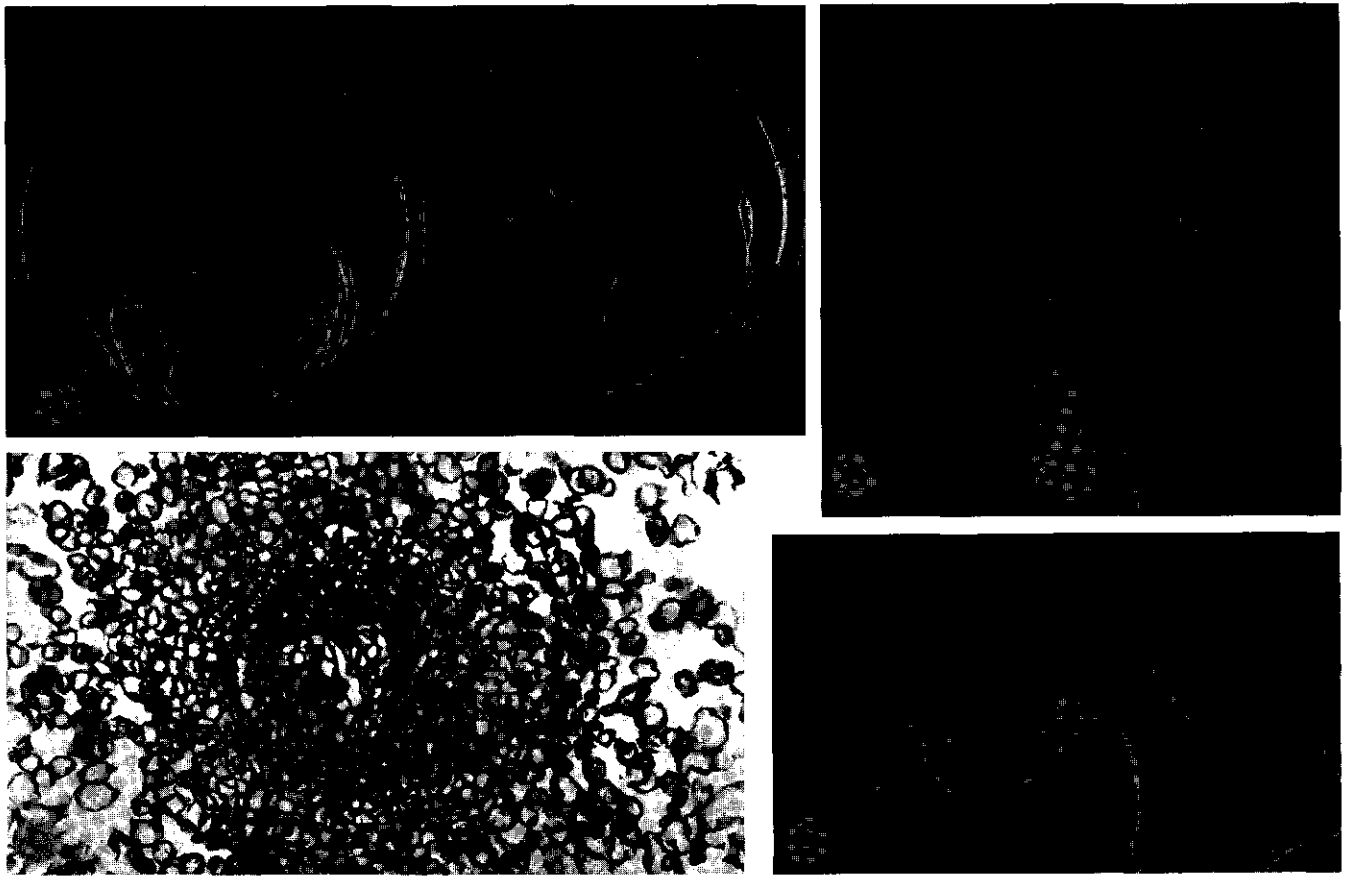

Figure 1: Germination of embryos cultured on medium with 5 mg/l kinetin (A), germination of embryos cultured on medium with 1 mg/l BAP: with thick tap roots and multiple shoots (B), histological section through thick roots (C), germination of embryos cultured on medium with 0.1 mg/l BAP: with thin tap roots and one apical meristem (D).

Table 1: Influence of different growth regulators on shoot and root formation and germination of Petri-dish desiccated somatic embryos (moisture loss 20%) of genotype Adira 4.

Hormone	Concentration (mg/l)	# of embryos	roots [A)]	shoots [A)]	germination[A)]
	-	20	1 (5)	0 (0)	0 (0)
TDZ	0.1	25	2 (8)	2 (8)	3 (12)
	1.0	24	1 (4)	1 (4)	2 (8)
	4.0	23	4 (17)	3 (13)	1 (4)
Kin	0.5	24	1 (4)	0 (0)	2 (8)
	1.0	20	0 (0)	1 (5)	1 (5)
	4.0	22	0 (0)	4 (18)	8 (36)
BAP	0.5	15	0 (0)	6 (40)	2 (13)
	1.0	21	1 (5)	14 (67)	3 (14)
	4.0	22	0 (0)	7 (32)	2 (10)
NAA	0.1	15	9 (60)	0 (0)[A)]	2 (13)
	1.0	20	11 (55)	0 (0)	0 (0)
	4.0	11	7 (64)	0 (0)	2 (18)
IBA	1.0	20	3 (15)	0 (0)	0 (0)
	5.0	10	3 (30)	0 (0)	0 (0)
	10.0	15	0 (0)	0 (0)	0 (0)

[A)] between brackets percentage.

Table 2: Investigation of moisture loss and BAP concentration on shoot and root growth and germination of somatic embryos.

Moisture loss	BAP (mg/l)	# of embryos	roots [A)]	shoots [A)]	germination [A)]
Gading					
20%	0.0	36	1 (3)	4 (11)	0 (0)
	0.1	28	1 (0)	1 (4)	3 (8)
	1.0	36	2 (6)	11 (31)	1 (3)
	4.0	35	1 (3)	7 (19)	0 (0)
40%	0.0	23	11 (48)	2 (9)	6 (26)
	0.1	32	7 (21)	0 (0)	23 (72)
	1.0	40	14 (35)	5 (13)	19 (48)
	4.0	40	7 (18)	10 (25)	16 (40)
75%	0.0	24	11 (45)	0 (0)	3 (13)
	0.1	23	3 (13)	1 (4)	10 (43)
	1.0	22	0 (0)	7 (31)	9 (41)
	4.0	28	0 (0)	11 (39)	8 (29)
Adira 4					
20%	0.0	42	7 (17)	0 (0)	0 (0)
	1.0	78	1 (1)	24 (31)	35 (45)
	4.0	56	0 (0)	21 (38)	28 (50)
40%	0.0	38	16 (42)	1 (3)	0 (0)
	1.0	61	0 (0)	5 (8)	53 (87)
	4.0	23	0 (0)	12 (52)	10 (43)
75%	0.0	46	11 (24)	2 (4)	0 (0)
	1.0	56	5 (6)	8 (15)	32 (58)
	4.0	42	0 (0)	6 (10)	16 (38)

[A)] between brackets percentage.

In both genotypes complete plant germination decreased when embryos had lost 75% moisture (Table 2). Also in this experiment thick roots and stems with multiple shoots were produced in the higher BAP concentration. In the next section a method is described which decreases the need for using high BAP concentrations.

Influence of light/dark conditions on development of normal plants from somatic embryos. NAA and 2,4-D induced embryos of four genotypes were compared for their ability to germinate. Undesiccated embryos, both 2,4-D and NAA induced, cultured for 14 days on a medium with 0-4 mg/l BAP, did not germinate or formed only shoots or roots (results not shown). Also, after 28 days germination or root formation alone did not occur and only shoots were formed at a very low frequency (<10%). The embryos lost between 38 and 53% of their fresh weight.

Desiccated, NAA induced, embryos of TMS90853 (Fig. 2A), Gading (Fig. 2B) and Line 11 (Fig. 2C) germinated at much higher frequencies than 2,4-D induced embryos. This was observed independent of the light/dark conditions and the used BAP concentration in the germination medium. For Adira 4 (Fig. 2D) only the NAA embryos cultured on 0 and 0.1 mg/l BAP germinated at a higher frequency than 2,4-D embryos and on a medium with 1 and 4 mg/l BAP there were no differences.

In all four genotypes NAA embryos cultured in the dark showed the highest percentage of germination in media supplemented with 0 or 0.1 mg/l BAP and this was higher than in the comparable treatments in the light. Under dark conditions root development occurred within 4 days followed by the elongation and curvature of the hypocotyl and finally the opening of the hypocotylar hook after 10 days. The stems of the shoots were slender with long internodes. Embryos cultured in the dark on 0 or 0.1 mg/l BAP formed mostly single shoots (Fig. 3A) whereas those on 1 mg/l BAP formed mostly two apical meristems (Fig. 3B). The shoots were etiolated which was corrected after one week transfer to the light. In all four genotypes NAA induced embryos cultured in the light showed the highest percentage of germination on a medium supplemented with 1 or 4 mg/l BAP and this was higher than in the comparable treatments in the dark. Root formation occurred within 4 to 8 days after culture and shoots followed a few days later. Shoots of NAA embryos cultured in the light

on 0.1 mg/l BAP had mostly one or two apical meristems (Fig. 1D) whereas those cultured on 1 mg/l BAP had more than 4 apical meristems and sometimes a higher number up to 10 (Fig. 1B.). Germination of 2,4-D embryos was very low in TMS 90853 (Fig. 2A), Gading (Fig. 2B) and Line 11 (Fig. 2C) both in the light and the dark. In Adira 4 (Fig. 2D) 40% to 50% of the embryos cultured in the light on medium with 1 or 4 mg/l BAP germinated. This was higher than in the comparable treatments in the dark. Desiccation of 2,4-D embryos had a clear effect on root development in all genotypes.

The roots appeared after 4 to 7 days of culture and the form was dependent on the light/dark conditions; predominantly adventitious in the light (Fig. 3C) and predominantly taproots in the dark (Fig. 3D). The results presented in Fig. 2 were taken 14 days after culture for germination. Two more weeks of culture gave only a significant increase of germination for NAA embryos cultured in light. There was no or only a small improvement of germination for NAA embryos cultured in the dark and for 2,4-D embryos cultured in the light or the dark.

DISCUSSION.

Morphological and biochemical evidence has shown that somatic embryogenesis mimics zygotic embryogenesis *in planta* (Amirato, 1983). *In planta* zygotic embryos undergo desiccation during maturation. Desiccation terminates the developmental process of embryo formation and activates the switch from maturation to germination (Kermode, 1990). Similar, in somatic embryogenesis desiccation improves germination of somatic embryos. This was reported for the first time for cassava by Mathews et al., (1993). In their method 85% of the desiccated MCol.1505 embryos germinated into complete plants, compared to less than 5% in non-desiccated embryos. In their study, embryos were induced on solid medium supplemented with 2,4-D. In our study embryos induced in liquid medium were used and a comparison was made between embryos induced by NAA or 2,4-D. NAA induced embryos indeed required desiccation for normal germination, however, to be effective cytokinins were needed in the medium to germinate desiccated embryos. In auxin induced embryos cytokinins are known to organise the shoot apex during embryo maturation (Amirato, 1983) and to break dormancy in poorly germinating somatic embryos (Gray and Puhit, 1991).

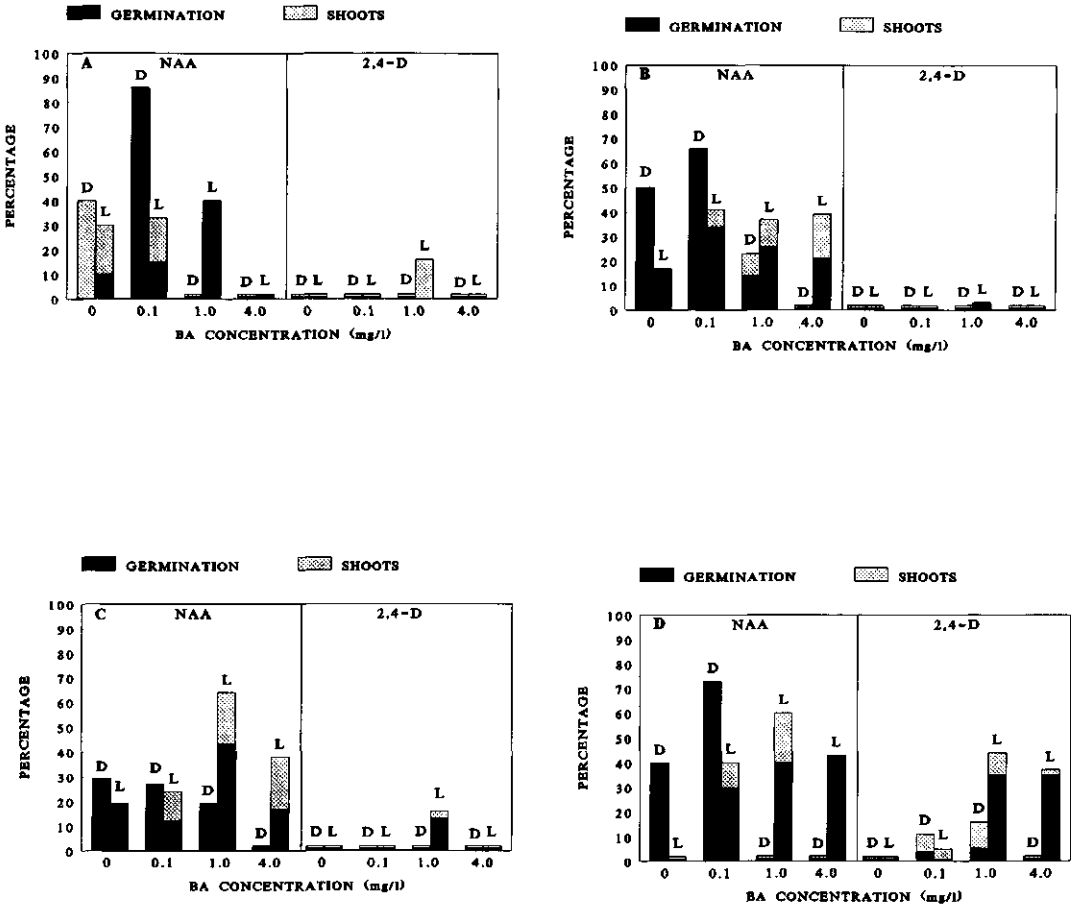

Figure 2: Germination and shoot development of desiccated NAA and 2,4-D induced embryos of TMS90853 (A), Gading (B), Line 11 (C) and Adira 4 (D) cultured in the dark (D) and the light (L) on a medium supplemented with 0-4 mg/l BAP (24-30 mature embryos per treatment).

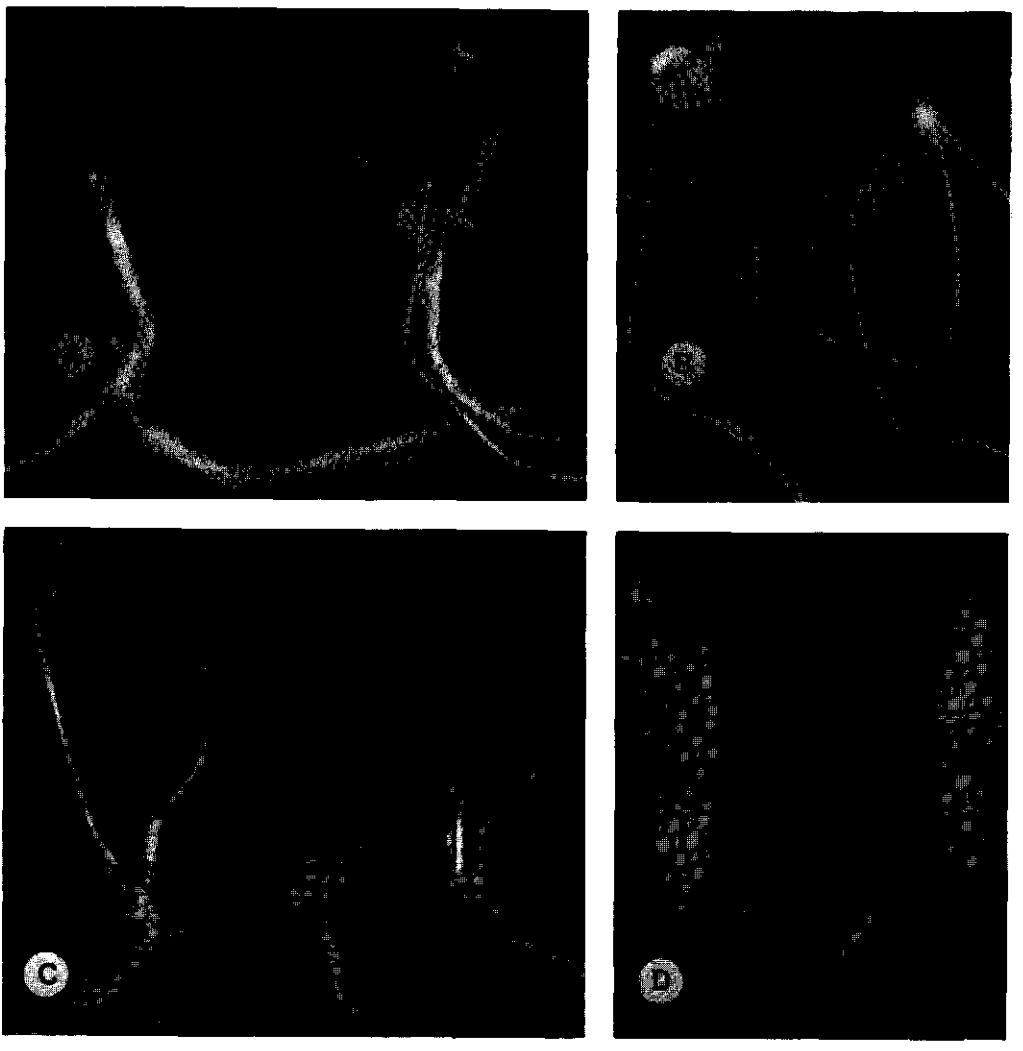

Figure 3: Influence of light/dark regimes on germination of desiccated Gading embryos: NAA/dark cultured on 0.1 mg/l BAP: one apical meristem per embryo (A); 2,4-D/light: adventitious roots (B); NAA/dark cultured on 1.0 mg/l BAP: one or two apical meristems per embryo (C); 2,4-D/dark: tap roots (D).

Of the cytokinins tested in cassava, BAP improved germination or shoot development of embryos more than TDZ and kinetin. BAP stimulated both taproot formation and shoot formation. This is in contrast with multiplication of stem cuttings where 0.1 mg/l BAP already inhibits adventitious root formation (Raemakers, 1993).

The BAP concentration needed for high frequency germination seemed to be dependent on the level of moisture loss in the somatic embryo. If this is optimal, lower levels of BAP are needed than if it is suboptimal. High concentrations of BAP had distinct effects on the morphology of the resulting seedling. The BAP concentration required for embryo germination in the dark is lower than in the light and as a consequence the shoots resembled more the morphology of seedlings of zygotic embryos. A disadvantage was the etiolated nature of the plants. More research is needed to determine the minimum period of darkness required to stimulate germination to stimulate germination.

In this study desiccation of 2,4-D induced embryos did not stimulate germination in three of the four genotypes, but had only a positive effect on root formation. This response was not affected by the light/dark conditions during culture for germination. However, in the light predominantly adventitious roots and in the dark taproots were formed. The results described here with 2,4-D induced embryos are in contrast with those described by Mathews et al., (1993) and by Raemakers et al., (1993b,c) previously. It was shown that embryos formed in liquid medium supplemented with 8 mg/l 2,4-D do not possess a root meristem (Raemakers, 1993). Culture of these embryos on a medium with BAP, without desiccation stimulated shoot formation without roots (Raemakers et al., 1993b,c). It seemed that desiccation of 2,4-D induced embryos triggered the development and the subsequent outgrowth of a root meristem. This process might have been on the expense of shoot development. The way 2,4-D embryos were induced by Mathews et al., (1993) differed from this study. In their study the 2,4-D exposure time was shorter, the concentration was lower and the embryos were transferred to charcoal supplemented medium for maturation. This culture system might have resulted in mature embryos with an active root meristem which easily can be activated and as a consequence shoot formation was not inhibited.

In the procedure described by Mathews et al., (1993) 4 steps were needed (induction,

maturation, desiccation of embryos and culture of embryos for germination). This has been reduced to three steps in the procedure described in this report. It is further an advantage that the embryos are induced in liquid medium. Compared to solid media, liquid media reduce labour and improves production. Seedlings are produced 4 weeks after start of a new cycle of embryogenesis compared to 10 weeks in the method of Mathews et al., (1993). The described procedure of germination makes somatic embryogenesis further competitive with the classical method of multiplication by stem cuttings.

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CHAPTER 7

GENERAL DISCUSSION

Somatic embryogenesis and regeneration in cassava

In figure 1 an overview of somatic embryogenesis in cassava is given. Until 1993 systems with only primary and secondary somatic embryogenesis were well developed. Somatic embryogenesis starts with the induction of embryos from primary explants. Recently it was shown that besides zygotic embryos (Stamp and Henshaw, 1982) and young leaf explants (Stamp and Henshaw, 1987; Szabados et al., 1987; Raemakers et al., 1993) also floral tissue has the capacity to undergo primary embryogenesis (Mukherjee, 1995). Primary embryos derived from both leaf explants and zygotic embryos form secondary somatic embryos and continuous cultures are obtained by cyclic culture of embryos on auxin supplemented medium. In all these reports mostly Murashige and Skoog (1962) medium supplemented with 2,4-D was used to initiate primary and secondary somatic embryogenesis. It was shown by Sudarmonowati and Henshaw (1992) that also Picloram and Dicamba have this capability.

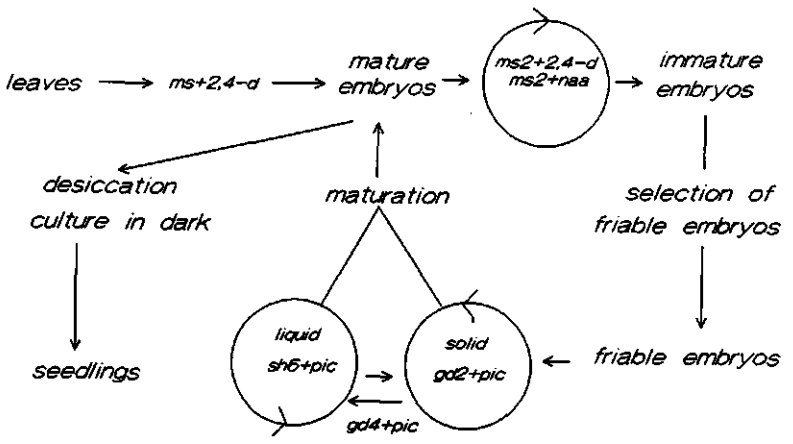

Figure 1: Schematic representation of somatic embryogenesis in cassava, including primary, secondary somatic embryogenesis, selection of friable embryogenic callus, maturation and desiccation followed by germination.

gd2= medium supplemented with Gresshoff and Doy salts (1974) and vitamins plus 20 g/l sucrose.
gd4= medium supplemented with Gresshoff and Doy salts (1974) and vitamins plus 40 g/l sucrose.
ms2= medium supplemented with Murashige and Skoog salts and vitamins plus 20 g/l sucrose.
pic= 10 mg/l Picloram, NAA=10 mg/l naphthalene acetic acid, 2,4-D=8 mg/l, 2,4-dichlorophenoxy acetic acid. sh6= medium supplemented with Schenk and Hildebrandt (1972) salts and vitamins plus 60 g/l sucrose.

As shown in this thesis, NAA can also induce secondary somatic embryogenesis, however in contrast to Picloram and Dicamba it failed to induce primary embryogenesis (Chapter 2). NAA induced embryos differed in many respects from 2,4-D induced ones. For example, histologically, the by 2,4-D newly induced secondary embryos were attached vertically to the explants whereas those by NAA were horizontally. Another prominent difference of NAA compared to 2,4-D induced embryos was the faster maturation. In NAA containing medium mature embryos are obtained in 14 days, whereas in 2,4-D it required a minimum of 21 days. Shortening the culture duration has a beneficial effect, particularly, when operating on a large scale.

For many genotypes and in several laboratories there is still a problem in obtaining embryogenic cultures of cassava (Mroginski and Scocchi, 1992; Taylor et al., 1992; Narayanaswamy et al., 1995; Sudarmonowati and Bachtiar, 1995). The main problem is not that embryogenic tissue from primary explants can be obtained, but the large scale multiplication of this tissue by secondary embryogenesis. For this purpose, either tissue consisting of immature embryos or mature embryos can be used. Multiplication of immature embryos is highly genotype dependent (Nigel Taylor, personal communication), while multiplication of mature embryos is largely genotype independent (Raemakers, 1993; Chapter 3). Both primary and secondary somatic embryogenesis are characterized by the formation of propagules with a bipolar structure. Therefore, Taylor et al., (1995) proposed the term organized embryogenesis. Organized cells are defined as a group of actively dividing cells, having the tissues and organs formed into a characteristic unified whole (Walker, 1989).

A less organized type of somatic embryogenesis was developed by Taylor et al. (1995). With continuous selection, organized embryogenic tissue cultured on a Gresshoff and Doy (1972) medium salts and vitamins supplemented with Picloram converted gradually into a less organized tissue. This tissue consisted of a callus-like mass of pro-embryos which was very friable. Therefore, this tissue was called friable embryogenic callus (FEC). The cells in FEC are continuously in a state where they break away from group control and because of that they are not organized into a unified structure. Suspension cultures were initiated by culturing FEC in Schenk and Hildebrandt (1972) medium supplemented with 6 % (w/v) sucrose and 10 mg/l Picloram. Every 2-3 days this medium was refreshed. Maturation of friable

embryos was accomplished by transferring FEC to maturation medium (Chapter 4, 5). Mature embryos could be induced into secondary somatic embryogenesis by culturing on Murashige and Skoog (MS) medium supplemented with auxins.

Primary and secondary somatic embryogenesis are relatively easy to establish in a wide range of genotypes (Chapter 2), while FEC is for the time being restricted to a few genotypes. The prospect of FEC for a new system of somatic embryogenesis and genetic transformation is promising, although further research is needed to make this system applicable to more genotypes. Essential for this process is the availability of high quality organized tissue and the ability of this tissue to convert into FEC. Taylor et al. (1995) "used organized tissues" which were multiplied at the immature state to initiate FEC. In this case two steps (initiation of organized tissue and conversion into unorganized tissue) are determinative for the successful initiation of FEC. Both steps are genotype dependent. If organized tissue is multiplied in the mature state as described by Raemakers et al., (1993) then only the ability of this tissue to convert into FEC is a determinative step to initiate FEC. It remains to be investigated whether or not organized tissue can be used as starting material. If organized tissue cannot be used, then this tissue should be first multiplied in the immature state before it can be used to initiate FEC. In chapter 2 it is shown that this readily can be accomplished. Either by culturing explants at a high density or by reducing the cyclic duration.

Regeneration of protoplasts

Protoplasts isolated from leaf mesophyll cells and somatic embryos of different genotypes developed readily into green callus. The callus occasionally formed roots, but not shoots or embryos (Chapter 3). In rice (Toriyama et al., 1985), maize (Rhodes et al., 1988; Lyznik et al., 1989), white spruce (Attree et al., 1989) and coffee (Acuna and Pena, 1991) regeneration of plants from protoplasts was achieved after using suspension cultures as starting material. Also in cassava protoplasts isolated from suspension cultures obtained from friable embryogenic callus were capable of regenerating into plants (Chapter 4). The yield of protoplasts isolated from FEC was four-fold higher as compared to that from somatic embryos and leaf mesophyll cells. It was shown that the callus derived from these protoplasts behaved as the original material: culture on maturation medium leaded to maturation of friable embryos, culturing of friable callus on GD2 or SH6 leaded to proliferation of FEC

and culturing of mature embryos on auxin supplemented medium to secondary somatic embryogenesis (Fig. 1).

Transformation of cassava

Transformation in higher plants can be defined as the process of introduction and expression of a foreign gene or genes in a genome other than by sexual crossing (Visser, 1989). Two types of gene transfer techniques are mainly employed in plant genetic engineering. One is mediated by *Agrobacterium spp.* (Potrykus et al.,1985). In this method a vector, *A.tumefaciens* often is used. The success of transformation relies in large part on the ability of the bacterium to transfer DNA to the cells. The other method, known as direct gene transfer, is free of a vector. There are different direct gene transfer techniques available. The two most commonly used are particle bombardment or biolistic delivery (Sanford, 1988) and electroporation (Fromm et al., 1986).

(1). Agrobacterium-mediated transfer of DNA

In the past, five strains of *A.tumefaciens* have been tested on secondary somatic embryos of cassava. The strain AM 8706 formed readily blue spots resulting from the transient expression of GUS. Occasionally this resulted in chimeric transformed embryos. In one case an embryo was obtained with a transformed primary root (Raemakers-unpublished). Cabral et al., (1995) have identified a strain of *Agrobacterium spp.* that is more virulent to cassava. Transformation of secondary somatic embryos with this wild type strain had resulted in some putative transformed plants. It remains to be investigated whether this wild type strain can be converted into a useful plant vector.

(2). Particle bombardment

Secondary somatic embryos induced by NAA or 2,4-D have been treated by particle bombardment. In both cases this resulted in chimeric transformed embryos (Chapter 2). However, it was not possible to enlarge the transformed sectors by a new cycle of embryogenesis. Essentially the same results were reported by Fauquet et al., (1992) and Cabral et al., (1995). The nature of somatic embryogenesis in FEC is different from secondary somatic embryogenesis as described by Raemakers (1993). The main difference concerned, the basic origin of new embryos. In FEC it was claimed to be at the surface of

the embryogenic units (Taylor et al., 1995) so that they are more accessible for gene-transformation. Particle bombardment of FEC resulted in stable transformation (Chapter 5). The transformed nature of FEC and of transformed plants was confirmed by PCR and Southern Blot analysis, respectively. Machine parameters which influence the momentum of the particles were not determinative. The physiological state of the material was more crucial for successful transformation. For this purpose, attention must be paid in culturing a good quality of suspensions. Two to three week-old suspension cultures were found to be the best for transformation. Under the conditions that these suspension cultures are competent for regeneration, transformation will be enhanced. Finally more attention must be paid to the post-transformation environment.

(3). Electroporation

Electroporation of chopped secondary embryos resulted in transient GUS activity. However, also this technique did not result with the used explants in stably transformed somatic embryos (Luong et al., 1995). Electroporation of FEC might be an alternative option for successful plant transformation.

Electroporation of protoplasts is one of the direct gene transfer techniques that produced transgenic plants in many important cultivated plant species (see Bajaj, 1989a; 1989b; 1993). This technique was applied by us in cassava using protoplasts isolated from young leaves. Although in a low frequency, it resulted in transformed callus (Chapter 3-results not shown; LUC positive). This transgenic callus was not capable of regenerating into plants. The presence of competent cells for regeneration was the key step in establishing a transformation system. Protoplasts isolated from FEC have the capability to regenerate into plantlets (Chapter 4). Combination of these two results can lead to a much better transformation procedure.

Barrier to cassava transformation

The described transformation procedure in Chapter 5 needs to be improved especially with respect to maturation and germination of mature embryos into plants. The same problems were observed with FEC derived from protoplasts. Maturation of friable embryos was not studied thoroughly. However, an improved procedure for germination of mature embryos is described in Chapter 6. It was shown that desiccation followed by culturing in darkness with

a low BAP concentration increased germination of NAA induced embryos in all tested genotypes. The same procedure was not successful for 2,4-D induced embryos. This positive effect of NAA on germination can not be used directly for mature embryos derived from FEC or protoplasts, because 2,4-D was more efficient than NAA in inducing secondary embryogenesis. However, after one cycle of secondary embryogenesis NAA was also highly efficient in inducing secondary embryogenesis (data not shown) and than these embryos should be used for germination.

Genotype dependency has been reported for both the gene transfer technique and the ability to regenerate. Transformation competence of *Lycopersicon peruvianum* is higher than in cultivated tomato *L. esculentum* (Koornneef et al., 1986). This was also observed for different genotypes of potato (El Kharbotly, 1995). At this moment FEC is established for a few cassava genotypes, and it remains to be determined whether FEC also can be established in other genotypes. It might be the case that a genotype which is bearing useful traits is recalcitrant to transformation, while a genotype with less importance is more amenable. The possible strategy to overcome these problems is transformation followed by sexual crossing and conventional breeding. Embryo rescue can help to increase the low frequency of germination of cassava seeds. Multiplication of the transformed seedling by micropropagation will not be a serious problem because this work is a routine procedure for almost every laboratory. A more serious problem for developing countries is to establish alternative methods for selection of transformed tissues. In chapter 5 selection of transformed tissues was based on the activity of the luciferase gene. Unfortunately the equipment to detect LUC activity is relatively expensive. Selection based on antibiotics like kanamycin (reviewed by Nap et al., 1992), or herbicides like L-phosphinotricin (De Block et al., 1987) seem to be suitable alternatives.

Prospect of gene transformation in cassava

Application of plant gene technology encompasses a multitude of different techniques ranging from isolation of useful genes, their characterization and manipulation, to the reintroduction of modified constructs into the plants (Lonsdale, 1987). Plant gene technology will catalyze progress in plant breeding, as will be exemplified by a few examples of transgenic crops like

rice (Chen et al., 1987; Shimamoto et al., 1989), maize (Gordon-Kamm et al., 1990; Vain et al., 1993), wheat (Marks et al., 1989), and potato (De Block, 1988; Visser et al., 1989). Rapid progress in gene technology has allowed insight into the complex molecular mechanism of plant pathogen recognition and the natural defence strategies of host plants. This technology can also be used for controlled and efficient identification of desirable genotypes, far beyond the possibilities of classical breeding.

Successful attempts have been made to improve resistance against pathogenic viruses like tobamovirus in tobacco (Powel Abel et al., 1986), potexvirus in potato (Hoekema et al., 1989) and in papaya (Fitch et al., 1992). In the above examples the introduced trait was based on the expression of single genes that are coding for the coat protein. In cassava, African cassava mosaic virus (ACMV) and cassava common mosaic virus (CCMV) might be controlled by the coat protein-mediated resistance technique (Fauquet et al., 1992). The genes encoding key enzymes of cyanogenesis have been cloned (Hughes et al., 1994) which makes manipulation of cassava cyanogenesis possible by genetic transformation using the antisense approach.

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SUMMARY

Cassava (*Manihot esculenta* Crantz) is a tropical tuber crop with unique properties. It is predominantly grown because of its starchy roots. Also in Indonesia, where it is known as 'ubi kayu' or 'singkong', it is an important crop. Until the middle of the 1960's, cassava was the major food security crop in rural areas of some provinces in Indonesia. Nowadays, cassava is an alternative crop for co-staple food and a substitutive crop when other crops fail. Even on marginal soils or cultivated with minimum tillage, cassava is one of the best carbohydrate producers. Large portions of cassava are used for animal feed and in starch based industries and are exported to the European Community and Japan. Local demand for fresh cassava for human consumption and for the agro-industry is increasing. Due to a large number of problems further use and expansion of cassava is restricted. One of these problems concerns the rather low yield of the crop. Although yield of over 90 tons/hectare have been reported the yield is rarely exceeding 12 tons/hectare. To some extent this can be attributed to the absence of fertilizer during growth. Another important factor is the susceptibility to all kind of pests and diseases, which is enhanced by the fact that farmers produce, by vegetative propagation, their own planting material. In wet regions like Western-Java or Sumatra Cassava Bacterial Blight is a serious disease, while in dry regions infections by red mites are a threat to the harvest. For human consumption it is necessary to have genotypes with a low cyanide content. Furthermore, the industry needs cassava cultivars especially suited for high starch production and adapted to mechanical harvest methods. At an international level, cassava breeding programmes have responded to ever changing situations. However, breeding programmes are time consuming and difficult because cassava is considered a 'poor man's crop' and thus little research efforts have been directed towards improvement of this important crop. The fact that cassava is a vegetatively propagated, cross breeding allotetraploid crop did not help either. As of 1980 a number of breeding programmes have been initiated amongst others by the Central Institute for Food Crops of the Agency for Agricultural Research and Development (Indonesia) to synthesize better performing genotypes. Because it takes 10 to 15 years to introduce a new variety on the market it is imperative that breeders make use of all available means to obtain these better genotypes. By implementing in vitro multiplication mass clonal propagation of improved varieties can be applied, while genetic modification (by which genes of interest can be transferred into cassava) opens possibilities to add or alter traits which cannot easily be achieved by traditional breeding alone.

This thesis deals with an investigation of several aspects of regeneration and transformation of cassava. The aim was to develop a routine procedure for genetic modification of cassava. In Chapter 1 a general overview is given of those aspects which are determinative in

obtaining successful transformation. Furthermore the available methods of regeneration and their unsuitability to be combined with transformation is discussed. Cassava transformation requires an efficient and reliable regeneration system which can be combined with a gene transformation system. Previously, a regeneration system was developed in which somatic embryos produced new embryos in a cyclic fashion. Considerable efforts to combine this system with a transformation technique had failed. With this system only chimeric transformed embryos were formed. One of the reasons for this was the way in which the embryos developed. When using the auxin 2,4-D new embryos developed from deep within the used tissue. When using another auxin (NAA) it was not possible to obtain primary somatic embryos, but when secondary or cyclic somatic embryos were used this auxin gave rise to the formation of new embryos from the (sub)epidermis. However, this change did not lead to completely transformed embryos either (Chapter 2). The use of NAA had a distinct positive effect on the speed and frequency with which new embryos could be obtained.

As mentioned in Chapter 1 somatic embryogenesis proved to be the only repeatable and efficient way of regeneration available in cassava. In Chapter 3 experiments are described in order to develop a protoplast regeneration system. Previously only leaves or stems were used as source for protoplasts. In Chapter 3 the results of using leaves, but more importantly using somatic embryos as protoplast source are described and compared. The rational behind it is the assumption that protoplasts isolated from somatic embryos might have a higher regeneration capacity as compared to protoplasts isolated from leaves. Different compositions of growth regulators were tested at subsequent stages of culture. None of the tested protocols resulted in regeneration. However, protoplasts isolated of both somatic embryos and leaf mesophyll explants of several genotypes formed readily green callus. Root formation was observed in a few cases, but shoots were never formed. The failure to obtain plants from protoplasts was either due to incompetence of protoplasts to regenerate or to inappropriate media compositions or a combination of both factors.

In Chapter 4 a, for cassava, new somatic embryo culture system based on so called friable embryogenic callus or suspension culture (FEC) is introduced. This was developed by Dr. N. Taylor of the University of Bath in the United Kingdom. This system differs in many respects from the previous system of somatic embryogenesis. Friable embryogenic callus (FEC) occurs almost always in the form of pre-globular embryogenic units with sizes of less than 1 mm. FEC has a high proliferation rate and originates from surface cells of these pro-embryos and (in most cases) from one single cell. Efforts were made to set up a protoplast regeneration system by using FEC as the source for protoplasts. The procedures of protoplast isolation from somatic embryos (Chapter 3) were applied to suspension cultures derived from FEC. Changes were made with respect to the medium composition for the first step in

culturing protoplasts. The first culture period required at least three to four weeks for the induction of cell divisions and development of microcalli. After two months of culture up to 60% of these calli were 'friable'. For maturation of friable embryos, FEC was transferred to maturation medium for two months. Finally, development of shoots was performed by culturing mature embryos on MS2 supplemented with 1 mg/l BAP. Shoots developed after at least four weeks of incubation on this germination medium, but only 30% of the mature embryos germinated.

Because in FEC the new pro-embryos are derived from single cells at the surface of the existing embryogenic units, they were used as starting material for direct gene transfer by particle bombardment (Chapter 5). The FEC of genotype TMS60444 was bombarded with DNA constructs harboring the firefly luciferase gene. This gene, normally not present in plant material, is capable of emitting light which can be detected by a luminometer (a camera highly sensitive for light emission). Machine parameters such as the distance between the target cells and DNA-macro-carrier and the strength of velocity were not determinative. The quality of starting material was crucial for successful transformation. FEC cultured for at least a week in liquid SH6 medium was found to be the best target tissue for transformation. Selection of transient and stably transformed FEC was based on the activity of the firefly luciferase gene. Friable embryos containing only one spot with luciferase activity were subcultured in either liquid or on solid medium. After 8 weeks transformed FEC cultured in liquid medium increased almost fivefold, and in solid medium almost threefold. Friable embryos of cultures containing at least 1% transformed material were cultured for maturation. Transformed mature somatic embryos were multiplied by cyclic somatic embryogenesis before they were allowed to develop into shoots. The transgenic nature of FEC cultures as well as that of regenerated plants was confirmed by PCR and Southern Blot Analysis, using the luciferase gene as a probe.

The developed transformation procedure is reproducible for the investigated genotype. Major efforts are directed to make the protocol applicable to other genotypes as well, to decrease the duration of time needed to obtain transformed shoots and to increase the efficiency. Important bottlenecks in achieving these goals are: maturation of embryos from the FEC cultures and shoot development of the mature embryos by optimising germination.
Because transgenic somatic embryos are in principle clonal of nature, the propagules can be used for mass clonal multiplication. For this application an efficient germination system is a prerequisite. Therefore, the ability of NAA and 2,4-D to obtain shoot conversion is described in Chapter 6. After desiccation, NAA induced embryos germinated in a higher frequency than 2,4-D induced embryos. Cytokinin was required for a high frequency germination. Incubating desiccated embryos in the dark with a low cytokinin concentration

(0.1 mg/l BAP) or in light with a higher concentration (1 mg/l BAP) were comparable in promoting germination. However, they differed in the morphology of the produced shoots and in the germination period of the embryos. If the concentration of BAP was low (< 0.1 mg/l) an embryo developed into a single shoot, in contrast to a high (1.5 mg/l or more) concentration where multiple shoots were formed from one embryo.

This thesis is a major step in the establishment of a routine transformation protocol for cassava using particle bombardment. Furthermore it opens an alternative way for transformation by using the described protoplast regeneration system combined with electroporation or PEG mediated DNA transformation. With this method breeding of cassava by using transgenic plants has become possible.

SAMENVATTING

Cassave (*Manihot esculenta* Crantz), ook wel manioc of tapioca genoemd, is een zeer belangrijk voedselgewas in de tropen. Het wordt met name geteeld voor de zetmeel bevattende wortelstokken. Ook in Indonesie waar het bekend is onder de namen 'ubi kayu' of 'singkong' wordt het gewas op grote schaal geteeld. Met name op het platteland wordt cassava als een hoofdgewas geteeld wanneer de boeren over onvoldoende geld beschikken om gewassen zoals mais te kunnen telen. Ook wanneer de kwaliteit van de grond slecht is zal de boer in veel gevallen voor cassave kiezen. Cassave is een goedkoop gewas zowel in aanschaf als onderhoud, terwijl het ook nog eens een redelijke oogstzekerheid geeft. Alhoewel cassave nog steeds als 'armeluis' gewas wordt beschouwd neemt de economische betekenis van het gewas, niet alleen in Indonesie, maar ook in andere landen in Zuid-Oost Azie toe. Een groot deel van de cassave productie wordt geëxporteerd als chips of pellets voor de veevoeder industrie naar onder andere Europa. Cassave productie voor lokaal gebruik, voor humane consumptie en als zetmeel bron, wordt steeds belangrijker. Met toenemende industrialisatie en het bereiken van een hogere levensstandaard wordt de betekenis van cassave als zetmeel bron steeds belangrijker. Er is echter een groot aantal problemen die, naast een gebrekkige basale kennis van het gewas, verdere toename van gebruik en toepassingen van cassave in de weg staan.

Een algemeen voorkomend probleem is de relatief lage opbrengst van het gewas. Ofschoon opbrengsten van 90 ton per hectare mogelijk zijn, wordt zelden meer dan 12 ton per hectare gerealiseerd. Voor een deel wordt dit verklaard door de relatief slechte gronden waarop cassave geteeld wordt en het niet gebruiken van kunstmest bij de teelt. Voor een ander deel wordt het verklaard door de vatbaarheid van cassave voor plagen en ziekten dat versterkt wordt doordat de boeren hun plantmateriaal zelf vermeerderen. In natte regio's zoals West-Java en Sumatra is de door een bacterie veroorzaakte ziekte "Cassava Bacterial Blight" een groot probleem, terwijl in droge gebieden aantasting door rode mijten een bedreiging voor de oogst vormt. Voor menselijke consumptie is het noodzakelijk dat het cyanide gehalte laag is. Verder zou men om goed en snel te kunnen oogsten willen beschikken over machinaal oogstbare rassen.

Afhankelijk van het doel dat men nastreeft wil men dus beschikken over superieure cassave genotypen. Traditionele veredeling van cassave is langdurig en moeilijk. Omdat cassave lange tijd beschouwd werd als een onbelangrijk gewas is er relatief weinig veredeld. Dit werd nog eens versterkt door het feit dat cassave, een allotetraploid (twee verschillende genomen die elk twee maal voorkomen) en een, slecht bloeiende, kruisbevruchter is die bij voorkeur vegetatief vermeerderd wordt via stekken. Sinds 1980 zijn er veredelingsprogramma's

opgestart onder andere bij het Central Institute for Food Crops van het Agency for Agricultural Research and Development (AARD) om goede genotypen te synthetiseren. Vanwege de tijd die het kost om een nieuw traditioneel veredeld ras op de markt te kunnen brengen (10 tot 15 jaar) is het noodzakelijk dat de veredelaars ook gebruik maken van nieuwe technieken om deze rassen te verkrijgen. Hierbij kan gedacht worden aan massale klonale vermeerdering via *in vitro* technieken en aan genetische modificatie. Genetische modificatie is een techniek waarbij men gericht een eigenschap in een plant kan wijzigen of toevoegen. Dit houdt in dat men, in theorie, het beste ras van dit moment resistent kan maken tegen die ziekte die op dat moment de meeste schade veroorzaakt.

De centrale leidraad van dit proefschrift is het ontwikkelen van een genetisch modificatie systeem voor cassave. Genetische modificatie vereist zeer efficiënte en, bij voorkeur, ras onafhankelijke regeneratie-protocollen die gecombineerd kunnen worden met transformatie. Regeneratie bij planten kan via somatische embryogenese en adventief scheut organogenese verlopen. In Hoofdstuk 1 wordt een kort overzicht gegeven van de aspecten welke bepalend zijn voor het welslagen van een succesvolle transformatie in het algemeen en dus ook bij cassave. Verder wordt een overzicht gegeven van de beschikbare regeneratie methoden bij cassave en hun (on)geschiktheid om ze te combineren met een transformatie protocol. De enige beschikbare regeneratiemethode -somatische embryogenese- had in eerder onderzoek bij de vakgroep Plantenveredeling (Proefschrift Raemakers, 1993) en ook bij andere onderzoeksinstellingen niet tot volledig getransformeerde planten geleid. Slechts met grote moeite konden er embryos en een enkele scheut verkregen worden die, voor het transgen, chimeer bleken te zijn. Eén van de oorzaken daarvan zou de wijze kunnen zijn waarop de embryos uit dieper gelegen delen van de explantaten ontstaan wanneer ze op embryo inducerend, d.w.z 2,4-D (een auxine) bevattend, medium werden gelegd. Bij het gebruik van een ander auxine (NAA), dat niet in staat was om primaire somatische embryogenese te induceren maar wel secundaire of cyclische embryogenese, bleek de ontstaans wijze van de embryos anders te zijn. De nieuwe embryos werden meer aan de oppervlakte van de explantaten gevormd. Desondanks leidde dit niet tot de vorming van volledig getransformeerde embryos wanneer ze in transformatie experimenten gebruikt werden (Hoofdstuk 2). Wel bleek het gebruik van NAA positieve effecten te hebben op de snelheid en frequentie waarmee nieuwe embryos werden verkregen (Hoofdstuk 2).

Zoals gemeld bleek somatische embryogenese de enige wijze te zijn waarop regeneratie kon plaats vinden. Hoofdstuk 3 beschrijft de resultaten van experimenten die erop gericht waren om uitgaande van protoplasten, die uit blad en somatische embryos verkregen werden, tot plant regeneratie te komen. De verwachting was dat protoplasten van somatische embryos wellicht een beter regeneratief vermogen zouden hebben dan protoplasten uit blad en derhalve

in staat zouden moeten zijn om tot planten te regenereren. Dit bleek niet het geval te zijn. Ontwikkeling van protoplasten uit beide uitgangsweefsels tot microcalli en zgn. groen callus bleek zeer eenvoudig. De verdere uitgroei tot planten kon niet gerealiseerd worden, heel incidenteel werden wortels verkregen (Hoofdstuk 3).

In Hoofdstuk 4 wordt een, voor cassave, nieuwe vorm van embryogene callus- en suspensiecultuur geintroduceerd. Deze 'friable' callus- en suspensies-cultures waren afkomstig van Dr. N. Taylor van de Universiteit van Bath in Engeland. Van dit systeem was bekend dat het nieuwe embryos vanuit de buitenste cellaag van de pro-embryos en vanuit één enkele cel vormde. Deze celsuspensie was in de praktijk een zeer goede bron voor protoplasten. Het bleek mogelijk de protoplasten afkomstig van deze suspensies tot micro-callus en callus te laten opgroeien, gebruikmakend van de ontwikkelde media en methoden beschreven in Hoofdstuk 3. Het callus was ten dele 'friable' en kon betrekkelijk eenvoudig tot de productie van volwassen embryos geinduceerd worden of kon prolifereren tot een nieuwe embryogene suspensie. Uitgaande van de volwassen embryos was het tenslotte mogelijk om scheuten te verkrijgen. Dertig procent van de volwassen embryos groeide uit tot planten.

Het gebruik van deze embryogene suspensies in transformatie experimenten door middel van 'particle bombardment' (het beschieten van suspensies met kogeltjes die bedekt zijn met DNA dat - eenmaal in de plant aanwezig- aanleiding geeft tot de productie van licht) wordt in Hoofdstuk 5 beschreven. Het markergen dat zorgt voor de emissie van licht, het zgn. Luciferase gen afkomstig uit het vuurvliegje, werd hier als selectiemarker gebruikt om transgene embryogene suspensies te verkrijgen. Herhaalde selectie van transgeen callus, gebaseerd op de emissie van licht, resulteerde in cultures waarvan een gedeelte op grond van de expressie van het luciferase gen transgeen was. Bij één experiment werd geprobeerd om de suspensies uit te laten groeien tot volwassen embryos. Dit lukte met een zeer lage efficientie. De getransformeerde embryos werden daarna eerst d.m.v. secundaire embryogenese vermeerderd. Een klein aantal van de volwassen embryos groeide tenslotte uit tot scheuten. Deze scheuten bleken ook, op grond van hun Luciferase activiteit, transgeen te zijn. Dit werd door moleculaire analyses m.b.v. PCR en Southern hybridisatie van zowel embryogene cultures als scheuten bevestigd. De scheuten bleken ten minste vier inserten van het ingebrachte construct te bevatten. De methode lijkt bij het cassave genotype TMS 60444 reproduceerbaar, maar met een lage frequentie, te werken. Verder onderzoek zal erop gericht zijn de methode toepasbaar te maken voor meerdere genotypen, terwijl ook getracht zal worden de efficiëntie te verhogen. Belangrijke 'bottle-necks' zijn maturatie van embryos uit de embryogene suspensies en scheutvorming van de volwassen embryos.

In Hoofdstuk 6 zijn resultaten beschreven van experimenten die erop gericht waren de scheut-

conversie te verhogen. Het bleek dat het uitdrogen van NAA geinduceerde embryos leidde tot een snellere en hogere frequentie van scheutvorming. Uitdrogen van 2,4-D geinduceerde embryos gaf dit effect niet. Het was noodzakelijk om cytokinine (BAP) aan de kiemingsmedia toe te voegen. De morfologie van de verkregen scheuten was sterk afhankelijk van de gebruikte BAP concentratie. Wanneer de concentratie van BAP laag was (ca 0.1 mg/l) gaf dit aanleiding tot enkelvoudige scheuten per embryo. Was de concentratie hoog (ca 1.5 mg/l of meer) dan bleken er zich per embryo meerdere scheuten te ontwikkelen. Meer onderzoek is nodig om de scheutconversie bij de embryogene suspensie cultures te verbeteren.

Dit proefschrift heeft een belangrijke bijdrage geleverd aan de ontwikkeling van een transformatieprotocol voor cassave. Deze transformatie methode is op het moment ontwikkeld voor één bepaald genotype. Het succesvol induceren van een embryogene suspensie cultuur bij andere cassave genotypen zal ook hier de weg tot transformatie openen. Echter het feit dat vanuit deze embryogene suspensies protoplasten te isoleren zijn die in staat zijn verder tot scheuten te ontwikkelen opent alternatieve wegen voor transformatie. Door protoplasten in combinatie met *Agrobacterium* cocultivatie, electroporatie of polyethyleen glycol (PEG) gemedieerde DNA overdracht toe te passen wordt enerzijds de efficiëntie aanmerkelijk verhoogd terwijl anderzijds de kans op chimerie, die bij particle bombardment van de embryogene suspensies groot is, verwaarloosbaar klein.
De hier beschreven transformatie methode brengt de veredeling van cassave met behulp van transgenen dichterbij.

RINGKASAN

Ubi kayu (*Manihot esculenta* Crantz) adalah tuberina yang tumbuh di daerah tropik serta mempunyai kegunaan yang unik. Ubi kayu adalah tanaman penghasil karbohidrat utama pada lahan yang marginal atau pada lahan yang pemeliharaannya minimum. Sampai pertengahan tahun 1960 ubi kayu masih merupkan makanan cadangan bagi masyarakat pedesaan di beberapa propinsi terutama di Jawa. Sekarang ubi kayu terutama digunakan sebagai tanaman pengganti jika tanaman utama gagal dipanen. Budidaya ubi kayu terutama ditujukan untuk industri tepung tapioka dan pakan ternak. Selain untuk bahan baku industri, ubi kayu yang berkadar HCN rendah dikonsumsi sebagai makanan penganan. Teknologi pengolahan lanjut karbohidart dari ubi kayu seperti untuk sirup glukosa dan monosodium glutamat di luar negeri, antara lain di Muangthai telah dimulai sejak tahun 1950. Kegunaan ubi kayu di bidang industri akhir-akhir ini semakin meningkat, di Jepang terutama dipakai untuk industri sirup dekstrosa, industri makanan, industri kertas dan industri kimia. Di Nigeria penelitian mengenai ubi kayu diarahkan untuk menghasilkan tapioka yang bisa dijadikan bahan pencampur tepung terigu menjadi "tepung komposit" untuk membuat roti. Program tepung komposit di beberapa negara berkembang di Afrika seperti di Nigeria, Uganda dan Zaire telah dimulai sejak tahun 1964, dengan tujuan mengurangi import tepung terigu yang mahal sehingga mampu menghemat keuangan negara. Dalam dekade ini industri makanan yang menggunakan tepung terigu sebagai bahan bakunya meningkat pesat di Indonesia. Jika rasio amilose-amilopektin dalam tepung tapioka dapat dimodifikasi maka penggunaan tepung terigu dapat dikurangi. Substitusi parsial tepung terigu dengan tepung tapioka yang dimodifikasi akan mengurangi impor gandum. Rekayasa genetik untuk merubah ratio amilose-amilopektin pada ubi kayu peluangnya terbuka dan menjadi kajian beberapa industriawan di negara anggota Masyarakat Ekonomi Eropa dan Anerika Serikat.

Agroindustri memerlukan kultivar yang cocok terutama untuk produksi tepung tapioka. Program pemuliaan ubi kayu pada tingkat internasional telah mengantisipasi tuntutan kebutuhan pasar yang situasional dengan teknologi DNA. Gen yang dikehendaki dapat di-transfer ke dalam genom dengan transformasi genetik. Transformasi gen perlu sebuah sistem regenerasi yang efisien dan dapat dibakukan. Beberapa sistem regenerasi pada ubi kayu melalui *somatik embriogenesis* yang diinduksi dengan auxin telah dikembangkan. Dalam sistem tersebut embryogenesis bisa dilakukan secara berulang-ulang sehingga merupakan siklus (*embriogenesis sekunder*).

Beberapa usaha untuk membuat sistem transformasi yang bisa dibakukan pada ubi kayu dengan menggunakan somatik embrio serta *Agrobacterium tumefaciens* sebagai mediatornya ternyata belum berhasil. Penggunaan bom partikel dengan target embrio matang juga tidak

berhasil mendapatkan transformasi gen yang stabil. Hasil maksimal dengan cara ini hanya embrio yang kimerik, berarti hanya sebagian kecil sel yang mengalami trasnformasi. Kimerik ini bisa dihindari jika regenerasi tanaman melalui protoplas berhasil. Oleh karena itu target pertama dari penelitian ini adalah mengembangkan sistem *somatik embriogenesis* dan regenerasi protoplas yang bisa dipakai target transformasi gen.

Dalam Bab 2 dikemukan bahwa NAA mampu menginduksi *somatik embriogenesis sekunder*, tapi tidak untuk induksi primer. Induksi embriogenesis dengan NAA ternyata lebih responsif dan produktif serta waktu yang diperlukan lebih singkat dibanding dengan induksi dengan 2,4-D. Tapi frekuensi siklus embriogenesis yang tinggi seperti di atas lima siklus, induksi NAA ini dapat menyebabkan embrio berakar (adventitious root), pada 2,4-D fenomenon ini tidak dijumpai.

Induksi *embriogenesis sekunder* dengan NAA hampir bersifat genotipe independen, semua genotipe yang diuji bisa memproduksi embrio. Populasi eksplant yang tinggi atau memperpendek siklus kultur, cenderung menghasilkan embrio yang premature, baru berkembang menjadi embrio matang jika ditransfer ke medium MS ditambah 0.1 mg/l BAP. Pada prinsipnya somatik embrio adalah klon, sehingga dapat dipakai untuk perbanyakan bibit secara masal.

Penelitian protoplas pada ubi kayu umumnya menggunakan daun muda atau batang sebagai donor protoplas. Dalam Bab 3 disajikan hasil penelitian dengan menggunakan mesofil daun dan *somatik embrio* sebagai donor protoplas. Beberapa komposisi pengatur tumbuh dicoba pada berbagai stadia perkembangan protoplas, ternyata tidak berhasil menginduksi regenerasi tanaman. Kalus yang berwarna hijau dan kadang-kadang berakar dapat diinduksi, baik dari mesofil daun maupun dari *somatik embrio*. Induksi akar berhubungan dengan adanya NAA pada medium A (induksi pembelahan sel). Kegagalan regenerasi diduga akibat inkompetensi protoplas atau komposisi media tidak tepat atau akibat kombinasi keduanya.

Sistem *somatik embriogenesis* yang paling baru telah diketemukan yaitu kalus *embriogenik* remah (friable embryogenic callus=FEC). System ini berbeda dengan *somatik embriogenesis* yang menggunakan eksplant berasal dari jaringan yang telah berdeferensiasi dan mengalami organogenesis. FEC bentuknya selalu "pre-globular embryo" berdiameter maksimal 1 mm, tingkat proliferasinya tinggi serta berasal dari lapisan permukaan sel.

Upaya untuk memperoleh system regenerasi tanaman dari protoplas diulang dengan mengunakan FEC sebagai donor protoplas. Metoda isolasi dan kultur protoplas dalam Bab 3 diaplikasikan lagi pada FEC (Bab 4). Perubahan hanya pada media pertama yaitu

menggunakan medium TM2G. Kultur protoplas tahap pertama dalam medium TM2G perlu waktu 3 sampai 4 minggu untuk pembelahan sel dan induksi mikro kalus, seterusnya setelah dikulturkan selama dua bulan, protoplast berkembang menjadi FEC dengan kualitas yang tinggi. Setelah ditransfer ke media MS yang dimodifikasi (medium pematangan), FEC tumbuh menjadi embrio.

Perbanyakan FEC dilakukan dengan pertama dikulturkan pada media Gresshoff dan Doy 4% (GD4) + 2 mg/l Picloram selama 21 hari, seterusnya transfer ke GD2 + 10 mg/l Picloram. Kualitas FEC yang baik bisa dipakai untuk inisiasi kultur suspensi sel. Induksi tunas dilakukan dengan cara mengkulturkan embrio pada medium MS2 + 1 mg/l BAP. Tunas akan tumbuh setelah dikulturkan minimal selama empat minggu.

FEC adalah *proembrio* yang tumbuh dari sel tunggal di permukaan embrio yang telah ada, jadi dia merupakan "daughter cell" dari *proembrio*, oleh karena itu cocok untuk dijadikan target transfer gen dengan bom partikel. Genotipe TMS60444 dibom dengan *plasmid* konstruksi pJit100 atau pJit64 yang mengandung gen Luciferase berasal dari serangga Kunang-kunang (Bab 5). Parameter-parameter seperti jarak sel target, momentum peluru pembawa DNA ternyata tidak determinatif. Kualitas bahan awal (FEC) lebih menentukan keberhasilan transformasi gen. Oleh karena itu perhatian harus diutamakan agar kultur FEC dengan kualitas yang prima dapat dicapai. Kultur FEC selama 6 minggu pada medium cair SH6 berpengaruh positif untuk transformasi transien, tapi untuk transformasi LUC yang stabil pengaruhnya tidak konsisten.

Seleksi FEC yang telah mengalami transformasi ("transformant") baik yang transien maupun stabil dilakukan dengan mengukur aktifitas gen luciferase (LUC). Aktivasi gen LUC oleh luciferin pada sel mengakibatkan transmisi sejumlah photon yang dapat dimonitor dengan camera CD super sensitive VIM dan diproses dengan penghitung citra Argus-50 bikinan Hamamatsu Phototonic Systems. Embrio yang positif mengandung LUC dikulturkan dalam medium cair atau padat SH6 + 10 mg/l Picloram. Dalam waktu 8 minggu "transformant" FEC yang dikulturkan dalam medium cair meningkat hampir lima kali sedangkan dalam medium padat tiga kali.

Sebelum induksi tunas, embrio "transformant" yang telah matang diperbanyak dengan cara *somatik embriogenesis* seperti diuraikan pada Bab 2. Embrio menjadi matang (hypocotil dan cotyledon nampak jelas) setelah dikulturkan dalam medium MS yang dimodifikasi. Analisa molekul untuk mengetahui status *transgenik* tanaman, yaitu dengan PCR menggunakan primer yang mengandung luciferase (LUC1 dan LUC2), serta Southern Blot Analysis menggunakan EcoRV atau BgIII sebagai enzym pemotong DNA.

Pada Bab 6 daya kecambah embrio yang diinduksi dengan NAA dan 2,4-D dibandingkan. Dengan metoda *desikasi* ternyata embrio yang diinduksi NAA berkecambah lebih tinggi dari embrio yang diinduksi 2,4-D. Agar embrio berdaya kecambah tinggi diperlukan cytokinin. Konsentrasi cytokinin rendah (0.1 mg/l BAP), dan diinkubasikan dalam ruang gelap hasilnya setara dengan konsentrasi tinggi (1 mg/l BAP) dan dikulturkan di tempat terang. Tapi morfologi tunas dan waktu berkecambah dari kedua lingkungan di atas berbeda.

Thesis ini merupakan sebuah terobosan utama dalam mebuat cara-cara transformassi gen yang bisa dibakukan melaui bom partikel pada ubi kayu. Seterusnya juga akan membuka alternatif untuk transformasi gen dengan menggunakan regenerasi protoplas melaui *elektroporasi* dan PEG.

ملخص

الكسافا (*Manihot esculenta* Crantz) هى محصول استوائى درنى له خصائص مميزة ويزرع خصيصا للاستفادة من جذوره النشوية. ويعتبر محصولا هاما فى اندونيسيا حيث يعرف باسم(ubi kayu) او (singkong). فقد كانت الكسافا حتى منتصف الستينات هي محصول الغذاء الرئيسي في المناطق الريفية في بعض محافظات اندونيسيا اما الان فهى من المحاصيل الثانوية او البديلة عندما لا تنجح المحاصيل الاخرى. حتى فى الاراضى المهملة اوالمحروثة قليلا فان الكسافا تعتبر ذات كفائة عالية لانتاج المواد الكربوهيدراتية. يستخدم جزء كبير من هذا المحصول لغذاء الحيوان وفى الصناعات المعتمدة على النشا كما انة يصدر لليابان والسوق الاوروبية المشتركة. الطلب المحلى على الكاسافا الطازجة للاستخدام الادامى والصناعات الزراعية فى ازدياد. لم يكن ممكنا التوسع فى زراعة الكسافا نظرا لوجود مشاكل كثيرة احداها هى قلة الانتاج. فبالرغم من ان انتاجية اكثر من ٩٠ طن للهكتار تم تسجيلها الا ان الانتاج عادة لا يزيد عن ١٢ طن للهكتار. ويرجع هذا الى حد ما الى عدم التسميد خلال فترة النمو. ايضا الحساسية لجميع الامراض والافات وذ لك لان المزارع ينتج بنفسة النقاوى اللازمة للزراعة بالتكاثر الخضرى. ويعتبر مرض الندوة البكتيرية للكسافا من الامراض الخطيرة فى المناطق الرطبة كما فى غرب جافا و سومطرة اما فى المناطق الجافة فالاكاروس الاحمر يهدد الحصاد. اصناف الكسافا التى تحتوى على نسبة منخفضة من السيانيد مطلوبة للاستخدام الادمى اما الصناعة فتحتاج خاصة للاصناف ذات الانتاجية العالية من النشا و التى يسهل حصادها اليا. تواجة برامج التربية لمحصول الكسافا على المستوى العالمى مواقف صعبة. و ترجع صعوبتها الى انها محصول الفقراء وتحتاج كثير من الوقت. ولذلك اجرى القليل من الابحاث على هذا المحصول الهام. ولان الكسافا تتكاثر خضريا فان التلقيح الخلطى بين السلالات لم يعط نتائج مرضية. لقد بدأت فى الثمانينات عدة برامج تربية ضمن برامج المعهد المركزى للمحاصيل الغذائية المتخصص فى الابحاث الزراعية والتنمية فى اندونيسيا لانتاج اصناف محسنة. حيث ان برنامج التربية ياخذ ١٠-١٥ سنة من بدء التربية و حتى ادخال الصنف الى السوق لذلك فمن المهم لمربى النبات استخدام جميع الوسائل المتاحة لانتاج صنف محسن. فيمكن باستخدام الزراعة النسيجية (in vitro) انتاج اعداد كبيرة من النباتات المحسنة. كما ان التحول الجينى (Genetics transformation) (الذى عن طريقة يمكن اضافة جينات معينة للكسافا) فتح المجال لاضافة او تغيير صفات ليس من الممكن تغييرها بسهولة عن طريق برامج تربية النبات العادية.
هذة الرسالة تبحث عدة اوجة من التكشف و التحول الوراثى فى الكسافا وتهدف الىتكوين اجراء روتينى للتحول الجينى فى الكسافا.

يلقى الفصل الاول نظرة عامة على العوامل المختلفة المتحكمة فى نجاح عملية التغيير الجينى. علاوة على ذالك تم مناقشة الطرق المختلفة لنمو وتكشف الانسجة (regeneration) و عدم صلاحيتها للاستخدام فى عملية التغيير الجينى للكسافا اذ انة يحتاج الى طريقة اكثار ذات كفائة عالية والتى يمكن استخدامها كنظام لنقل الجينات . نظام الاكثار الذى تم تطويرة سابقا يتم من خلال الاجنة الخضرية (Somatic Embryoes) فى دورات متتالية. لقد باءت المحاولات الرامية الى دمج هذا مع نظام التحول الجينى بالفشل. تم باستخدام هذة الطريقة انتاج الاجنة المتغيرة جزئيا. احد اسباب ذلك هو طريقة انتاج الاجنة. فباستخدام مادة ٢-٤-د (2,4-D) تتكون البراعم الجديدة من طبقة عمقة فى الانسجة المستخدمة وباستخدام اوكسين آخر (حامض النفثالين اسيتك(ن.ا.أ) (NAA)) لم يكن ممكناً انتاج اجنة خضرية اولية ولكن عندما استخدمت الاجنة الخضرية الثانوية او المنتجة من دورات هذا الاوكسين زاد تكوين الاجنة الجديدة من طبقة تحت البشرة . وكذلك تطبيق التحول الجينى لم يؤد الى تغير كامل للاجنة (الفصل الثانى). استخدام ن أ أ (NAA) كان لة تاثيرة الأيجابي الواضح على سرعة وكمية الأجنة المتحصلة عليها.

كما ذكرفى الفصل الاول فان طريقة الاجنة الخضرية اثبتت انها الطريقة التى يمكن استخدامها فى انتاج الكسافا خضريا فى المعمل بكفائة عالية. فى الفصل الثالث تم وصف عدة تجارب لتطوير نظام الاكثار عن طريق البروتوبلاست (Protoplast) (خلايا بلا جدر). استخدمت فى الدراسات السابقة الاوراق والسيقان كمصدر لانتاج البروتوبلاست. نتائج استخدام الاوراق وبصفة خاصة الاجنة الخضرية لانتاج البروتوبلاست تم وصفها ومقارنتها. السبب وراء ذلك هو الافتراض ان البروتوبلاست المعزولة من الاجنة الخضرية ربما يكون لها قدرة

اعلى على التكشف عند مقارنتها بالمعزولة من الاوراق. تم اختيار عدة مركبات من منظمات النمو في مراحل مختلفة من الزراعة ولكن لم تعط نتيجة. من ناحية اخرى فالبروتوبلاست المعزولة من اجنة خضرية او انسجة الأوراق (الميزوفيل) من عدة اصناف اعطت كالوس اخضر. لوحظ انتاج الجذور في عدة حالات ولكن لم تتكون أى افرع. الفشل في الحصول على نباتات من البروتوبلاست قد يرجع لعدم قدرة البروتوبلاست على النمو والتكشف او لعدم صلاحية البيئة او العاملين معا.

في الفصل الرابع -بالنسبة للكسافا – تم استخدام نظام جديد لزراعة الاجنة الخضرية ويسمى الكالوس الجنيني سهل التفتيت او المزروعة المعلقة (اف.اى.سى) (F.E.C) (Friable embryogenic callus) تم تطوير هذا النظام بواسطة د.ن ثيلور من جامعة بث في المملكة المتحدة. يختلف هذا النظام في عدة نواحي عن النظام السابق من زراعة الاجنة الخضرية . ال اف.اى.سى (F.E.C) يحدث دائما في المرحلة الجنينية (قبل الكروى) في حجم اقل من ١ مم وله درجة مسامية عالية وهو يتكون من الخلايا السطحية لهذه البراعم (في اغلب الأحيان) من خلية واحدة. بذلت الجهود لأنشاء نظام اكثار البروتوبلاست باستخدام اف.اى.سى كمصدر لانتاج البروتوبلاست. خطوات عزل البروتوبلاست من الأجنة الخضرية (الفصل ٣) استخدمت في المزرعة المعلقة الناتجة من ال اف.اى.سى. تم عمل تعديلات في مكونات البيئة المستخدمة في المرحلة الأولى لزراعة البروتوبلاست. مرحلة الزراعة الأولى احتاجت على الأقل ٣ الى ٤ اسابيع لاحداث انقسام الخلايا ونمو الكالوسات المصغره .(Microcalli) بلغت نسبه الكالوس المفتت ٦٠٪ بعد شهرين من الزراعة. لأكتمال نمو الأجنة المفتتة ال اف.اى.سى ثم نقلة الى بيئة اخري لمدة شهرين. اخيرا ظهرت الأفرع بعد زراعة الأجنة المكتملة النمو على بيئة (ام.اس٢) (MS 2) المضاف اليها املجرام للتر من البنزيل امينو بيورين (بى .ا.بى) (BAP) بعد ٤ اسابيع على الأقل على بيئة الأنبات ولكن ٣٠٪ فقط من البراعم المكتملة النمو اعطى انبات

نظرا لتكوين البراعم على سطح الوحدة الجنينية و من خلية واحدة في ال (اف.اى.سى) فقد ا ستخدمت هذه البراعم كبداية لعملية نقل الجين المباشر باستخدام القذف الجزيئى (Particle bombardment) ففى (الفصل الخامس) استخدم ال (اف.اى.سى) من نبات الكسافا رقم (٦٠٤٤ تى.ام.اس)(TMS 6044) لقذفة ب (د.ن.ا) (DNA) محتوى على جين اللوسيفيرازا (Luciferase) من ذبابة النار. هذا الجين – عادة لا يوجد في النبات – قادر على اصدار ضوء يمكن اكتشافة بجهاز لومينو ميتر (Luminometer) (كاميرا حساسة جدا للضوء) قياسات جهاز القذف مثل المسافة بين الخلية الهدف والجزيئات الحاملة للجينات ايضا السرعة وقوة القذف لم يتم تسجيلها. نوعية المواد الاولية المستخدمة اساسية لنجاح عملية النقل الجيئى. فقد وجد ان ال اف.اى.سى المزروعة لمدة اسبوع على الاقل في محلول البيئة اس.اتش ٦ (SH6) تعتبراحسن انسجة يمكن استخدامها كهدف لعملية النقل الجيئى . اختيار ال اف.اى.اس الثابتة النقل الجيئى والغير ثابتة كان يعتمد على نشاط جين اللوسيفيرازا لذبابة النار. الاجنة المفتتة المحتوية على بقعة واحدة بنشاط اللوسيفيرازا تم زراعتها على بيئة صلبة او سائلة. بعد ٨ اسابيع من النقل الجيئى اف.اى.سى المزروعة على بيئة سائلة زادت حوالى ٥ اضعاف والتى على بيئة صلبة حوالى ٣ اضعاف. الاجنة المفتتة من مزارع محتوية على حوالى١٪ انسجة محولة تم زراعتها لأكتمال النمو. الاجنة الناضجة الخضرية المحولة تم اكثارها بدورات زراعة الاجنة الخضرية قبل تكوين افرع. طبيعةالتحويل الجيئى لمزارع اف.اس.سى وكذلك النباتات النامية تم التاكد منها بطريقة البى.سى.ار(PCR) وتحليل ساوثرن بلوط (Southern blot) باستخدام جين اللوسيفيرازا كبروب.(Probe)

يعتمد نجاح طريقة التحويل الجيئى على الصنف الذى يستخدم. ومن ناحية اخرى تبذل مجهودات كبيرة لجعل هذة الطريقة مناسبة ايضا لباقى الاصناف لتقليل الوقت اللازم للحصول على افرع محولة جينيا وكذلك لزيادة الكفائة. عنق الزجاجة الهام للوصول لهذة الاغراض هو تكشف الاجنة من مزارع ال اف.اى.سى وتكوين الافرع من الاجنة المكتملة النمو عن طريق تحسين الانبات.

ولأن الاجنة الخضرية المحولة هى فى الواقع من اصل واحد بطبيعتها. لذالك يمكن استخدامها لأنتاج نباتات على نطاق واسع ولكن لابد من نظام انبات كفء. من اجل ذالك فان قدرة ال ن.ا.ا (NAA) و ٢ - ٤ - د (2,4-D) للحصول على افرع تم ذكرها فى الفصل السادس. بعد مرحلة تقطيع الاجنة ن.ا.ا (NAA) اعطى انبات اكثر من ٢-٤-د (2,4 D)السيتوكاينين (Cytokinin) كان هاما لزيادة نسبة الانبات. فبوضع الاجنةالمقطعة فى حضانات فى الظلام مع تركيز منخفض من السيتوكاينين (او ١,. ملجرام للستر بى.ا.بى (BAP)) أو فى الضوء مع تركيز عالى(١ ملجرام للتر بي.ا.بي (BAP)) اعطى نفس التاثير على الانبات الا انة كانت هناك اختلافات فى الشكل الظاهرى للافرع ومدة انبات الاجنة. اذا كان تركيز ال بى.ا.بى (BAP)منخفض (اقل من ١ و. ملجرام للتر) ينمو الجنين الى فرع واحد اما فى حالة التركيز المرتفع (١,٥ ملجرام للتر او اكثر) تكونت عدة افرع من جنين واحد.

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Curriculum vitae

Eri Sofiari (Abullah Faqih), has no original date of birth that has ever been recorded. He was born during an fierce civil war in the middle of 1950s in a remote area of Tasikmalaya, West Java, Indonesia. He registered for The Primary School at Garut in 1959 and completed in 1963. Finally, he has an official date of birth of 1 May, 1950 given by his School Master.

The academic study started in 1971 at Tanjungsari Academy of Agriculture and he got the B.Sc degree in Farming Systems in 1974. He worked at Lembang Research Institute for Horticulture (LEHRI), Bandung, since 1974. First he joined the Seed Technology Group in ATA (Assistance Technical Agriculture) project 111 from the Netherlands, in the sub section of Plant Breeding, from 1974 until 1980. In 1982 he completed the degree of Stratum one (S1) in Agronomy from University of Padjadjaran, Bandung. From 1982 until 1985 he was a coordinator for the production of breeder and extension seed for the National Vegetables Seed Production, and as a seed inspector for Seed Potato Production Project. In 1985 he continued the study of stratum two (S2) programme at the Graduate School, University of Padjadjaran, and finished in 1987. From 1988 until 1991 he joined the Hot Pepper Research Group as a coordinator, and as a researcher in a programme of breeding for resistance to *Anthracnose* on Hot Pepper under the Project of ATA 139 financed by The Netherlands.

In April 1991 he attended the School of Plant Sciences at Reading University registered as an ad-hoc student for PhD programme in inter and intraspecific hybridization in *Capsicum spp.* with Dr. Barbara Pikergills. In September 1992 he decided to changed the study and moved to Wageningen Agricultural University. Since then, he has carried out the research programme on this thesis at the Cell Biology Laboratory of the Department of Plant Breeding under the supervision of Prof. Dr. Ir. E. Jacobsen and Dr. R.G.F. Visser.

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