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Optimisation of regeneration systems for a range of Cassava (Manihot Esculenta Crantz) cultivars suitable for growth in South Africa and transformation with SACMV N-REP gene

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2006-11-13T12:08:37Z
http://hdl.handle.net/10539/1640

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Chapter 1

GENERAL INTRODUCTION

Makwarela M and Rey MEC (2006) Cassava Biotechnology, a southern African perspective. Biotechnology and Molecular Biology Reviews 1:1-10

1.1 Cassava (*Manihot esculenta* Crantz)

Cassava (*Manihot esculenta* Crantz) is a vegetatively propagated root crop used as a staple throughout the tropics and subtropics. It is the fourth most important and cheapest staple food crop after rice, wheat and maize in developing countries, providing food for over 600 million people (Schöpke *et al*. 1993; Taylor *et al*. 2004). Cassava, otherwise known as tapioca, yucca, manioc or mandioca is an outcrossing, monecious member of the family Euphorbiaceae. Considered and allopolyploid (2n=36), it is a highly heterozygous, semi-woody, perennial shrub varying from 1-4 m in height depending on the cultivar, and produces between 3 and 36 storage roots per plant (Fig 1.1A). Storage roots on a fresh mass basis contain between 20% and 36% starch or approximately 77% on a dry mass basis (Cock 1982; Nweke 1995; Thro *et al*. 1999; Alves 2002; Gray 2003). Propagation takes place vegetatively usually via lignified stem cuttings. After planting, new roots are produced and axillary buds sprout to form the shoot system. Three to four months later, photosynthates produced by the established leaf canopy are diverted to root system where the excess energy is converted to starch and stored in the parenchyma of greatly thickened stage roots, generally referred to as tubers (Silvestre 1989; IITA 1990) (Fig 1.1B). It can also be grown in association with several other crops in most African countries, as discussed by Nweke (1994) (Fig 1.1C).

Although still a subject of debate, its centre of origin is generally believed to be southern border of the Amazon basin (Olsen and Schaal 1999; Allem 2002; Gray 2003). It was introduced into Africa in the Congo River delta by the Portuguese in the 15th century (Jones 1959), and was rapidly spread to many

agro-ecologies including East Africa through Madagascar and Zanzibar (Jennings 1976; Hahn *et al*. 1979; Hillocks 2002), and later to Asia (Gray 2003).

The varieties within climatically-determined limits of the sub-tropics of South Africa (SA) have come a long way from their tropical home. Cassava was introduced into Moçambique by the Portuguese in the 17th century, and was adopted as a food by the Tonga tribesmen, who later spread westwards into the eastern Transvaal (now Mpumalanga Province) and Swaziland and also south into northern Natal (Daphne 1980). Cultivation of cassava by neighbouring tribes took hold only gradually and it appears therefore that plantings in SA came mainly with the major tribal movements of the 1830s and 1860s (Daphne 1980). Cultivation continued to increase throughout the 20th century, most noticeably in Africa where colonial powers often encouraged its cultivation as a famine reserve.

Large scale cassava production in SA was impaired by a taste preference for maize, but in the late 1970s there was a renewed interest in cassava, and extensive yield trials were conducted throughout sub-tropical regions of Kwazulu-Natal Province and Northern Province (now called Limpopo Province) under a range of environments (Daphne 1980). At the beginning of the 21st century, Africa is the largest cassava producing region with 85.9 million tonnes fresh weight harvested from 10.3 million hectares, followed in production by Asia and Latin America and the Carrebbean (FAO 2002) (Table 1.1). According to figures supplied by FAO (2002) in Table 1.1, the rate of increase of production has been higher than any other crop in Africa over the past 15 years. Since 1990, this increase has been fuelled by rapid increases in the productivity following the

release of improved varieties in Nigeria (Nweke *et al*. 1994; Tshiunza *et al*. 1999). On a country scale Nigeria, Thailand, Indonesia and Brazil are the major cassava producers (Puonti-Kaerlas 1998) (Table 1.2). While aggregate production statistics on cassava are subject to large degrees of error, the figures shown in Table 1.2 give a general idea of the trend in cassava production in Africa.

Cassava is a reliable crop that tolerates well adverse environmental conditions, and is mainly grown by small or subsistence farmers in the poorer regions, where it provides up to 60% of the daily calorie intake among all the food crops (Cock 1985; Nassar 2002). Its success is based on its unusually adaptable and productive nature. Although greatest yields are obtained on loamy sandy soil under humid tropical conditions, it is highly tolerant of marginal or eroded soils and adapts easily to the acidic oxisols prevalent throughout much of tropics.

Cassava is widely consumed as a porridge, which is prepared from dried and pounded roots, but eaten in a very wide range of forms in different parts of the continent (DeVries 2002). Cassava is reported to be consumed in 28 different forms in Cameroon, alone (DeVries 2002; Kokora Nicole, pers. comm. 2002). In South Africa, there are a number of cassava-processing methods consisting of drying and pounding of the roots to produce porridge known as '*Xigema*', cooking of leaves to produce a condiment known as '*Mathapi*' (Diana Sikulane pers. comm. 2002) (Fig 1.2C). Cassava is also consumed as a snack food in various parts of the continent. Varieties used as snack food are 'sweet' types, low in cyanic acid, which can be boiled and eaten or even consumed raw (DeVries 2002). In certain regions, the leaves, which contain appreciable

quantities of protein and vitamins, are used as a major component of the diet to provide supplementary protein, vitamins and minerals to complement to the carbohydrate rich staple (Lacanster and Brooks 1983).



Fig 1.1 Cassava small-scale farming for subsistence by Mr. Flawana Masingi of Buyisonto, Bushbuckridge, in Mpumalanga Province. (A) Healthy cassava plants with no CMD symptoms (B) Flawana proudly showing off one of his cassava plants that has yielded a bounteous harvest in September 2003 (C) Lignified stem cuttings used for vegetative propagation and (D) Cassava (1) grown in association with *Ipomea batatas* (2)



Fig 1.2 Mrs. Daina Sekulane's subsistence cassava field approximately 600m north of Mr. Masingi's field A) SACMV severely infected plants B) Mrs. Sekulane sample of harvested roots from one plant in September 2003 C) Harvested leaves are pounded using mortar and pestle in order to eventually prepare mathapi and D) Mosaic symptoms in CMD plants

Table 1.1 Cassava production trends in selected regions.[a]

Region	Area Harvested (Ha x 10^6)			Growth (%/year)	
	1973-75	1983-85	1993-95	1973-75 to 1983-1985	1983-85 to 1993-95
Africa	7030	7518	10,158	9.7	3.1
Asia	2928	3730	3775	2.5	0.1
Latin America and the Carribbean	2722	2592	2593	-0.5	0.0

[a] In comparing cassava production figures with those of grain crops it should be borne in mind that cassava production figures are reported at 70% moisture content, while most grain crops are reported at approximately 15% moisture content.
Source: FAO (2002)

Table 1.2 Annual cassava production in major African cassava-producing countries. [a]

Country	Production (t $year^{-1}$)
Benin	2,377,339
Côte d'Ivoire	1,700,000
Ghana	7,226,900
Guinea	811,869
Nigeria	32,695,000
Kenya	910,000
Madagascar	2,404,000
Uganda	3,400,000
Angola	3,210,570
Congo, Democratic Republic of	17,100,000
Mozambique	5,639,000

[a] In comparing cassava production figures with those of grain crops it should be borne in mind that cassava production figures are reported at 70% moisture content, while most grain crops are reported at approximately 15% moisture content.
Source: FAO (2002)

1.2 Geminiviruses

The Geminiviradae family includes a large number of viruses that infect plants and produce in many cases very significant reductions in economically important crops of both monocotyledonous and dicotyledonous plants (Gutierrez 1999). They are basically, characterized by two distinctive features, namely a) the

morphology of the virion particle which is geminate, approximately 18-30 nm in size, and has the appearance of two quasi-icosahedral moieties with a total of 22 pentameric capsomers (Fig 1.3 1), and b) the nature of their genetic material consisting of one or two single-stranded DNA (ssDNA) molecules, 2.5-3.0 kb in length (Fig 1.3 2) (Fauquet 2001; Jeske *et* al. 2001; Zhang *et al*. 2001). They are classified into four genera: Mastrevirus, Curtovirus, Topovirus and Begomovirus (Rybicki *et al*. 2000). Mastreviruses and curtoviruses are transmitted by leafhoppers and possess a monopartite genome, begomoviruses are spread by whiteflies and most of them have a bipartite genome, called DNA A and DNA B. Sequence comparisons between the genera have led to the suggestion that curtoviruses have evolved from a recombination of ancient mastreviruses and begomoviruses (Stanley *et al*. 1986). Both DNA components of begomoviruses contain different sequences except for a 'common region' (CR) of ~200 bp.

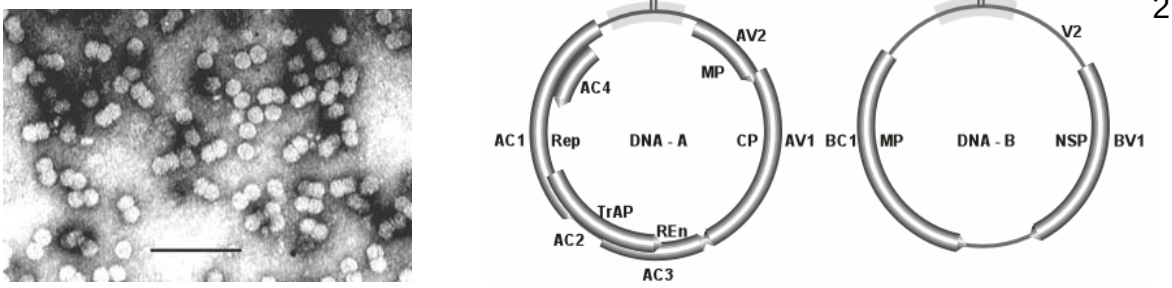


Figure 1.3 Typical geminiviruses consist of two quasi-isometric subunits with a characteristic 'waist' constriction and pointed ends (1) Geminivirus particles in electron microscopy of ACMV. The bar represents 100 nm. (Photo courtesy C.M. Fauquet (B) (2) Typical genomic organisation of begomoviruses. ORFs are denoted as being encoded either on the virion (V) or complementary (C) strand (Adapted from Virus Taxonomy, van Regenmortel *et al*. 2000)

The CR is nearly identical in both DNA-A and B of a single virus. It includes promoters and the origin of replication (Jeske *et al*. 2001). A small-hairpin-loop forming sequence in the CR is highly conserved among all geminiviruses (Hanley-Bowdoin *et al*. 1999). DNA-A generally contains one gene (CP/AV1) in the virus strand and three overlapping genes (AC1, AC2, and AC3) in the complementary strand. AV1 is the gene for virus coat protein and AC1 codes for the viral DNA replication (Rep), required for the replication of both genomic components (Etessami *et al*. 1991). The protein product of AC2 is a transcriptional activator (TrAP) for the virus-sense genes in both DNA-A and B components; and REn/AC3 is responsible for virus proliferation rates (Haley *et al*. 1992; Hong and Stanley 1995). The DNA-B carries two genes, BV1 in the sense and BC1 in the complementary sense strand required for cell to cell and long distance virus spread respectively and is probably responsible for the development of disease symptoms (Von Armin *et al*. 1993; Haley *et al*. 1995; Liu *et al*. 1997; Zhang 2000). The vector transmission and virus spread, as well as systemic infection of susceptible hosts need both genomic components (Von Armin *et al*. 1993; Haley *et al*. 1995). Geminiviruses replicate via double-stranded circular intermediates, which form minichromosomes within the nuclei of infected cells (Pilartz and Jeske 1992).

The most important disease affecting cassava (*Manihot esculenta* Crantz) production in Africa is Cassava mosaic disease (CMD), or more recently as African cassava mosaic disease (ACMD), caused by several whitefly-transmitted begomoviruses. CMD was first described in 1894 in what is now Tanzania towards the end of the 19th Century (Warburg 1894) although its etiology

remained unclear for many years. It has since been recorded in all the main cassava growing areas of sub-Saharan Africa and on several islands including Madagascar, Mauritius, Bioko, São Tomé and Cape Verde (Thresh *et al*. 1998). Storey and colleagues (Storey 1936; 1938; Storey and Nichols 1938) were able to demonstrate the graft transmissibility of CMD, and confirmed earlier experiments by Hufferath and Ghesquière (1932) showing that a *Bemisia tabaci* whitefly species was the vector (Legg and Thresh 2003). They also recorded the occurrence and properties of mild and severe virus strains, and carried out the first epidemiological experiments recording seasonal differences in rates and spread (Legg and Thresh 2003). In the absence of any visible pathogen the disease was assumed to be caused by a virus, but no particles were detected until 1975 when sap inoculations from cassava to cassava and to the herbaceous host *Nicotiana clevelandii* Gray were successful (Bock 1975). However, there was initial initial uncertainty as to the role of the geminivirus that was isolated because it could be obtained from CMD-affected plants in western Kenya, western Tanzania and Uganda, but not from similarly diseased plants in coastal Kenya (Bock *et al*. 1978). For this reason it was at first not considered to be the cause of CMD and was referred to as cassava latent virus (Bock *et al*. 1977; 1981; Adejare and Coutts 1982; Sequiera and Harrison 1982; Thresh *et al*. 1998). The uncertainty was resolved when successful transmissions were made to *Nicotiana benthamiana* using sap from CMD-affected plants in both western and coastal Kenya (Bock and Woods 1983). The virus isolates obtained and characterised had geminate particles and were similar to those from CMD-affected cassava sampled elsewhere in Africa and also in India and caused

typical symptoms of the disease when returned from herbaceous hosts to cassava, thereby fulfilling Koch's Postulates (Thresh *et al*. 1998).

Subsequent studies have led to the recognition of several distinct but similar viruses: *African cassava mosaic virus* (ACMV); *East African cassava mosaic virus* (EACMV); *Indian cassava mosaic virus* (ICMV) (Hong *et al*. 1993; Swanson and Harrison 1994) and recently *South African cassava mosaic virus* (SACMV) (Berrie *et al*. 1998; 2001). What appears to be a hybrid recombinant between ACMV and EACMV has been reported in Uganda, Kenya, Tanzania and Democratic Republic of Congo and designated UgV (Calvert and Thresh 2002). The different viruses have very similar properties and they are all members of the newly created family: *Geminiviridae;* Genus: *Begomovirus* (Fauquet *et al*. 2003) (Table 1.3). Each of the cassava mosaic geminiviruses (CMGs) can cause CMD and there is evidence that virus combinations are more damaging than single infections alone, as reported in studies in Uganda and Cameroon (Harrison *et al*. 1997; Fondong *et al*. 2000; Pita *et al*. 2001). The symptoms of CMD occur as characteristic leaf mosaic patterns that affect discrete areas and they are determined at an early stage of leaf development. The chloric areas fail to expand fully so that stresses set up by unequal expansion of the lamina cause malformation and distortion. Severely affected leaves are reduced in size, misshapen and twisted, with yellow areas separated by areas of normal green colour. The plants are stunted and young leaves absciss (Storey and Nichols 1938; 1951; Calvert and Thresh 2002). The leaf chlorosis may be pale yellow or nearly white, or just discernibly paler than normal. The chlorotic areas are usually clearly demarcated and vary in size from the whole leaflet to small flecks or

spots. Leaflets may show a uniform mosaic pattern or the pattern is localised to a few areas which are often at the bases of the leaflets (Fig 1.2D). Distortion, reduction in leaflet size and general growth retardation, appear to be secondary effects associated with symptom severity. Symptoms vary from leaf, shoot to shoot and plant to plant, even for the same variety and virus strain in the same locality. Symptoms variation may be due to virus strain differences, host sensitivity, age of the plant and environmental factors such as soil fertility, availability of soil moisture, temperature and radiation. Cool temperatures usually enhance expression of symptoms while warm temperatures tend to do the opposite.

Table 1.3 The viruses of cassava (Calvert and Thresh 2002).

Africa
Cassava mosaic geminiviruses (*Geminiviridae: Begomovirus*)#
Cassava brown streak virus (*Potyviridae: Ipomovirus*)
Cassava Ivorian *Bacilliform* virus* (unassigned)
Cassava Kumi viruses A and B*
Cassava “Q” virus*
Cassava common mosaic virus* (*Potexvirus*)
South/Central America
Cassava common mosaic virus (*Potexvirus*)
Cassava virus X (*Potexvirus*)
Cassava vein mosaic virus (*Caulimoviridae*)
Cassava Colombian symptomless virus (*Potexvirus*)*
Cassava American latent virus (*Comoviridae: Nepovirus*)*
Cassava frogskin “virus”
Asia/Pacific
Cassava common mosaic virus* (*Potexvirus*)
Indian cassava mosaic virus (*Geminiviridae: Begomovirus*)
Sri Lankan cassava mosaic virus (*Geminiviridae: Begomovirus*)
Cassava green mottle virus* (*Comoviridae: Nepovirus*)

Viruses with names in italics are recognized species.
#The cassava mosaic geminiviruses have recently been reclassified. See Table 1.4.
*Viruses with localised distributions and not of economic importance.

Table 1.4 Cassava mosaic Geminivirus species and strains (Fauquet *et al*. 2003).

Species	**Accession number**	**Acronym**
African cassava mosaic virus (Cassava latent virus)		ACMV
African cassava mosaic virus – [Cameroon-DO2]	AF366902, AF112353	ACMV-[CM/DO2]
African cassava mosaic virus - [Cameroon]	AF112352, AF112353	ACMV-[CM]
African cassava mosaic virus – [Ghana]		ACMV-[GH]
African cassava mosaic virus – [Côte d'Ivoire]	AF259894, AF259895	ACMV-[IC]
African cassava mosaic virus – [Kenya]	J02057, J02058	ACMV-[KE]
African cassava mosaic virus – [Nigeria]	X17095, X17096	ACMV-[NG]
African cassava mosaic virus – [Nigeria-Ogo]	AJ427910, AJ427911	ACMV-[Nig-Ogo]
African cassava mosaic virus – [Uganda]	Z83252, Z83253	ACMV-[Ug]
African cassava mosaic virus – [Uganda Mild]	AF126800, AF26801	ACMV-UGMld
African cassava mosaic virus – [Uganda Severe]	AF126802, AF126803	ACMV-UGSvr
East African cassava mosaic Cameroon virus		EACMCV
East African cassava mosaic Cameroon virus – Cameroon	AF112354, AF112355	EACMCV-CM
East African cassava mosaic Cameroon virus – Cameroon [Côte d'Ivoire]	AF259896, AF259897	EACMCV-CM[CI]
East African cassava mosaic Malawi virus		
East African cassava mosaic Malawi virus – [Malawi, EACMV-MW		EACMMV
East African cassava mosaic Malawi virus – Malawi [K]	AJ006460	EACMMV-MW[K]
East African cassava mosaic Malawi virus - Malawi [MH]	AJ006459	EACMMV-MW[MH]
East African cassava mosaic virus		EACMV
East African cassava mosaic virus –[Kenya – k2B]	Z83258	EACMV-[KE-k2B]
East African cassava mosaic virus – [Tanzania]	Z83256	EACMV-[TZ]
East African cassava mosaic virus – [Uganda1]	AF230375	EACMV-[UG1]
East African cassava mosaic virus – Uganda2 (Uganda variant)	Z83257	EACMV-UG2
East African cassava mosaic virus – Uganda2 Mild	AF126804	EACMV-UG2Mld
East African cassava mosaic virus – Uganda2 Severe	AF126806	EACMV-UG2Svr
East African cassava mosaic virus – Unganda3 Mild	AF126805	EACMV-UG3Mld
East African cassava mosaic virus – Uganda3 Severe	AF126807	EACMV-UG3Svr
East African cassava mosaic Zanzibar virus		EACMZV
East African cassava mosaic Zanzibar virus	AF422174, AF422175	EACMZV
South African cassava mosaic virus		SACMV
South African cassava mosaic virus	AF155807, AF155806	SACMV
South African cassava mosaic virus – [M12]	AJ422132	SACMV-[M12]

Calvert and Thresh 2002). Quantification of disease severity is central to the evaluation of gross host-pathogen interactions in plant pathology. Quantitative estimation of plant disease severity has been largely restricted to visual scoring of diseased tissues using either disease keys or standard area diagrams (Zodaks and Shein 1979; Kranz 1988). In recording experiments and screening for resistance to CMD, much use has been made of simple numerical scoring systems based on the extent and severity of the symptoms expressed. Scales of 0-4 or 1-5 (1 being no disease and 5 being severe mosaic and distortion of entire leaf) (Terry 1975; Ogbe *et al*. 2003) have been widely used to quantify differences due to variety, season and virus strains and to assess the relationship between symptom severity and yield loss (Calvert and Thresh 2002; Ogbe *et al*. 2003). Fauquet and Fargette (1990) used 0-5 scale, where, 0= healthy; 1= faint mosaic; 2= yellow mosaic, malformation, 5% size reduction; 3= severe mosaic, distortion, reduced size; 4= severe mosaic mosaic, severe distortion, up to 50% size reduction, and 5= very severe mosaic, leaf reduced to veins, 50-80% size reduction. Such methods of symptom assessment for many plant diseases are either qualitative or semi-quantitative and often rely heavily on visual assessment by a single scorer. Various studies, however, have demonstrated the unreliability of the human eye for the objective determination of symptoms (Kranz 1988; Price *et al*. 1993; Parker *et al*. 1995; Martin and Rybicki 1998). These studies have found that data based on visual assessment of disease symptoms are inaccurate and contain biases correlated with the numbers, areas and shapes of disease lesions/symptoms. Symptom severity is associated with the magnitude of yield loss (Thresh *et al*. 1994b; Fargette *et al*.

1996) and some yield loss models are derived from relationships between disease severity and disease incidence.

CMGs are disseminated in the stem cuttings used routinely for vegetative propagation. They are also transmitted by the whitefly, *Bemisia tabaci* Gennadius. Dissemination in stem cuttings can lead to the introduction of CMD new areas and accounts for the occurrence of the disease in areas where there is little or no spread by the whitely vector (Calvert and Thresh 2002). The distribution of immigrant whiteflies and of plants newly affected by CMD is influenced by the direction of the prevailing wind and by the effects of wind turbulence around and within stands. The incidence of whiteflies and CMD tend to be greatest at the crop margins, especially along the windward and leeward edges and environmental gradients have been observed when whitefly populations decrease with increasing distance from the field boundaries (Fargette *et al*. 1985; Colvin *et al*. 1998; Calvert and Thresh 2002). Incidence is also increased by breaks or discontinuities in the crop canopy which facilitate the alighting and establishment of viruliferous vectors (Fargette *et al*. 1985; Calvert and Thresh 2002).

Obvious benefits are realised by decreasing the losses caused by CMD and this can be achieved by a reduction in the incidence and/or severity of the disease (Calvert and Thresh 2002). Various approaches to control are possible, however, the main attention has been given to the use of resistant varieties (Fargette *et al*. 1998a) and phytosanitation, involving the use of CMD-free planting material and the removal (rouging) of any additional diseased plants that occur (Thresh *et al*. 1998b; Legg and Thresh 2003). Farmers occasionally use

insecticides in attempts to restrict the spread of CMD by controlling the whitefly vector. However, the use of insecticides on cassava or other tropical crops has received little attention from researchers in Africa and this approach is unlikely to be effective. It is also inappropriate considering the costs that are involved and risks to farmers, consumers and the environment (Calvert and Thresh 2002).

Losses as indicated by evidence are more qualitative than quantitative. Trials to assess the effect of CMD on cassava yield have provided differing results ranging from virtually no loss to almost total loss (Thresh *et al*. 1994b). Results from such experiments depend on a series of factors, some of the most important of which are susceptibility of the variety, the stage of crop growth at which infection occurred, the severity of the virus or virus mixture causing the infection, and the abiotic environmental conditions (Fargette *et al*. 1988; Fauquet and Fargette 1990). In conducting trials with symptomless and diseased plants of different varieties, Bock (1994) observed no differences in root weight but recognised extensive areas of necrosis on tubers of diseased plants, which eventually rotted as a result of secondary invasion by micro-organisms. The findings of Bock (1994) were consistent with those of Nichols (1950) who reported losses due mainly to reduced quality of tubers. Disease surveys in Tanzania, based on above-ground symptoms showed a mean incidence of 9% in parts of the country where the disease is known to occur. Disease incidence in parts of the South-east Coastal Region was 24% (Legg and Raya 1998). Using FAO production estimates, CMD incidence and the 30-40% range of yield loss used by Thresh *et al*. (1997), estimates of “lost” production have been calculated for 16 of the main cassava-producing countries in Africa (Table 1.5). For the

remaining 10% of production for which incidence figures are not available, the average incidence of 50% for the 16 countries has been used. Based on these assumptions, the losses attributable to CMD are 19-27 million tonnes, based on the current (CMD affected) production total of 97 million tonnes (FAO 2003).

1.3 The transmission of cassava mosaic viruses by the whitefly *B. tabaci*

The putative virus assumed to cause CMD in Africa was one of the first pathogens to be transmitted experimentally by whiteflies (Calvert and Thresh 2002). *B. tabaci* adults have a 'rod shaped' appearance, as the wings are held upright and parallel to the body, commonly exposing the abdomen (Sseruwagi *et al*. 2004). Studies began in the 1920s when it became evident that the virus was spreading naturally and that whiteflies were only sap-feeding insects on cassava likely to be vectors. The first transmissions were reported from Congo using adults of species referred to as *Bemisia mosaicivecta* (Ghesquiré 1932) which was later stated to be a misprint for *B. mosaicivectura* (Storey and Nichols 1938). Later experiments on the mode of transmission were carried out in Nigeria (Chant 1958), Ivory Coast (Dubern 1994) and Kenya (Seif 1981) using *B.tabaci* Gennadius. The virus is tained by adult whiteflies for at least 9 days (Calvert and Thresh 2002). It is known to persist during moulting, but it is not transmitted transovarially (Dubern 1994). Nymphs can transmit but they are not of epidemiological importance since they are not mobile. Recently studies have shown that the epidemiology of CMGs is associated with more than one geographical haplotype (Berry *et al*. 2004), although the exact relationship between the whiteflies, viruses, cassava hosts and disease severity has not been clearly established.

Table 1.5 Surveys of the incidence of cassava mosaic disease (CMD) in 18 African countries.

Country	Organization (Reference)	Year	CMD Incidence (%)	Production 2002 m/t	Estimated loss (30-40%)
Uganda	NARO (1)	1990-1992	57		
Uganda	NARO (2)	1994	65		
Uganda	NAROMITA (14)	1997	68	5.27	1.4-2.0
Chad	USAID (3)	1992	40	0.31	0.04-0.06
Malawi	NARS (4)	1992	21		
Malawi	NARS/IITA (15)	1998	42	1.54	0.22-0.31
Tanzania	NARS/IITA (5)	1993	26		
Tanzania	NARS/IITA (16)	1998	34	5.65	0.64-0.089
Ghana	ESCaPP (6)	1993-1994	72		
Ghana	NARS/IITA (17)	1998	71	8.97	2.43-3.56
Benin	ESCaPP (6)	1994	53		
Benin	NARS/IITA (18)	1998	36	2.45	0.30-0.41
Cameroon	ESCaPP (6)	1994	67		
Cameroon	NARS/IITA (19)	1998	62	1.70	0.39-0.56
Nigeria	IITA (7)	1994	55		
Nigeria	ESCaPP (6)	1994	82		
Nigeria	NARS/IITA (20)	1998	56	33.56	6.78-9.69
Zambia	NARS/SARRNET	1995-1996	41	0.95	0.13-0.19
Zanzibar	(12)	1998	71	NA	
South Africa	NARS/NRI (8)	1998	31	<0.01	
Madagascar	NARS (9)	1998	47	2.23	0.37-0.52
Mozambique	NARS/IITA (21)	1999-2000	20	5.36	0.34-0.47
Rwanda	NARS/NRI (10/12)	2001	30	0.69	0.07-0.09
DRC	NARS/IITA (22)	2002	60	14.93	3.28-4.71
Congo Rep.	NARS/IITA (23)	2002	79	0.85	0.26-0.39
Guinea-Conakry	NARS/IITA (24)	2003	63	1.00	0.23-0.34
Kenya	NARS/IITA	1993	20		
(Western)	KARI/NRI (13)	1996	56		
(Western)	KARI/NRI (13)	1998	84		
(Western)	KARIESARC (13)	2000	58		
Kenya (Coastal)	NARS/NRI (11)	1998	51	0.95	0.17-0.24
Kenya	NARS/IITA (25)		Est.50	10.60	1.87-2.65
*Others			50	97.01	18.87-27.05
Total					

Adapted from Legg and Thresh (2003)

1.4 Genetic engineering of cassava

Biotechnology is fast proving to be a valuable tool for genetic improvement of plants. However, the prerequisites for efficient exploitation of biotechnology are the development of reliable transformation systems which are an interdependent relationship between tissue culture regeneration methods, transformation and selection methods of transgenic plants. Genetic engineering is a powerful tool that complements traditional breeding and can extend the genetic pool of useful gene sources beyond the species (Fregene and Puonti-Kaerlas 2002). Transgene technology also offers the advantage of precisely transferring single or even quantitative traits without the problems of linkage encountered in traditional breeding.

In cassava improvement programs, the limiting factors in production and utilization are among others, increased resistance to viral and bacterial pathogens and insect pests, the production of novel compounds for value-added products from cassava, improved starch characteristics and rapid post-harvest deterioration, a ubiquitous problem which limits marketability of the crop and hinders the development of medium to large scale commercial exploitation. Additional problems still unresolved by traditional breeding are lack of high quality planting material, low protein content of cassava products and the presence of cyanogenic compounds in the tubers are also considered limiting factors (Puonti-Kaerlas 1998; Taylor *et al*. 2000).

Conventional breeding for the agronomic improvement of cassava is frustrated by the crop's inherent heterozygous nature, inbreeding depression and the polygenic and recessive nature of many desirable traits. Therefore, genetic

engineering has been identified as a powerful tool to overcome these limitations. Traditional breeding of cassava is difficult as few natural resistance genes have been found in sexually compatible germplasm. The allotetraploid nature of cassava that leads to polymorphisms after crossing, its high outcrossing nature and its low fertility linked to inbreeding depression restrict the use of traditional breeding (Puonti-Kaerlas 1998).

Genetic engineering presents an alternative to traditional plant breeding. Using the techniques of molecular biology, a single gene that codes for a desired trait, such as insect resistance, increased protein content, or tolerance to drought is isolated and then combined with a promoter sequence that will allow the gene to be expressed. This combination of genes is then introduced directly into the plant genome (Chrispeels and Sadava 2003). To improve cassava by genetic engineering, an essential prerequisite is the development of an efficient regeneration and transformation procedure.

1.4.1 Cassava *in vitro* regeneration

Plant cells are generally considered to be totipotent, thus being able to regenerate whole plants from single cells *in vitro*. The ability to regenerate *in vitro* is however, often limited to certain tissues and developmental stages, and the requirements for transformation and regeneration competence may not always be compatible. Furthermore, a method for efficient transfer and stable integration of the transgenes into plant genomic DNA is essential for transformation, as well as means for identifying and selecting transformed cells (Fregene and Pounti-Kaerlas 2002).

The main constraint is usually is usually not the delivery of foreign DNA to the regenerable cells, but the recovery of the transformed cells. Finally, the introduced genes must be correctly expressed in the primary transgenic plants and transmitted stably to their progeny (Zhang 2000). As cassava is vegetatively propagated, the transgenes can be fixed already at the level of primary transgenic plants, and stable inheritance is of concern only when the transgenic plants ate to be incorporated in breeding programs.

Plant regeneration through tissue culture can be accomplished using one of the three methods, namely, meristem culture, somatic embryogenesis and organogenesis. Fig 1.4 illustrates how all three methods are used in regeneration and recovery of transgenic cassava plants. Of the different explants used for regeneration, meristems are the tissue of choice as they represent 'growth centres' of plants (Fregene and Pounti-Kaerlas 2002; Zhang 2001; Murashige 1974). Therefore, this system is easy, fast and relatively genotype-independent. Applications of this system include germplasm preservation, micropropagation, transformation and eliminating virus or disease from plant materials (Kartha 1974; Kartha and Gamborg 1975; Roca, 1984; Ng *et* al. 1990). In cassava, meristems can be induced to form multiple shoots on cytokinin-containing medium. Most of the shoots are derived from pre-existing axillary meristems, but also *de novo* formation of new meristems and shoots occurs (Konan *et al*. 1994a; 1995; 1997). Transient and stable expression of both GUS and luciferase have been demonstrated in meristems and meristem-derived somatic embryos and multiple shoot clusters after particle bombardment (Puonti-Kaerlas *et al*. 1995; 1997a).

Somatic embryogenesis is the production of embryo-like structures from somatic cells. The somatic embryo is a bipolar structure which is independent and not vascularly attached to the tissue of origin (Ammirato 1987). This system is now the most commonly used regeneration method in cassava. In cassava, somatic embryogeneis is restricted to meristematic and embryonic tissues. Somatic embryos can only be induced on a limited number of explants such as cotyledons of zygotic embryos (Stamp and Henshaw 1982; Konan *et* al. 1994a; 1994b), immature leaf lobes, (Stamp and Henshaw 1987a; Szabados *et al*. 1987; Matthews *et al*. 1993; Raemakers 1993; Raemakers *et al*. 1993a; Li *et al*. 1995; 1996; 1998a; Taylor *et al*. 1996; Puonti-Kaerlas 1997a; 1997b). Other tissues include meristems and shoot tips (Szabados *et al*. 1987; Narayanaswami *et al*. 1995; Frey 1996; Puonti-Kaerlas 1998), anthers (Mukherjee 1995) and immature inflorescences (Woodward and Puonti-Kaerlas 1998). Primary somatic embryos can be induced to produce secondary somatic embryos by further sub-culturing on auxin-containing medium (Stamp and Henshaw 1987b). By constant subculturing of somatic embryos, a cyclic embryogenesis system can be established either in liquid or solid medium, where the embryos rarely pass the 'torpedo' stage, until transferred to germination medium (Fig 1.4).

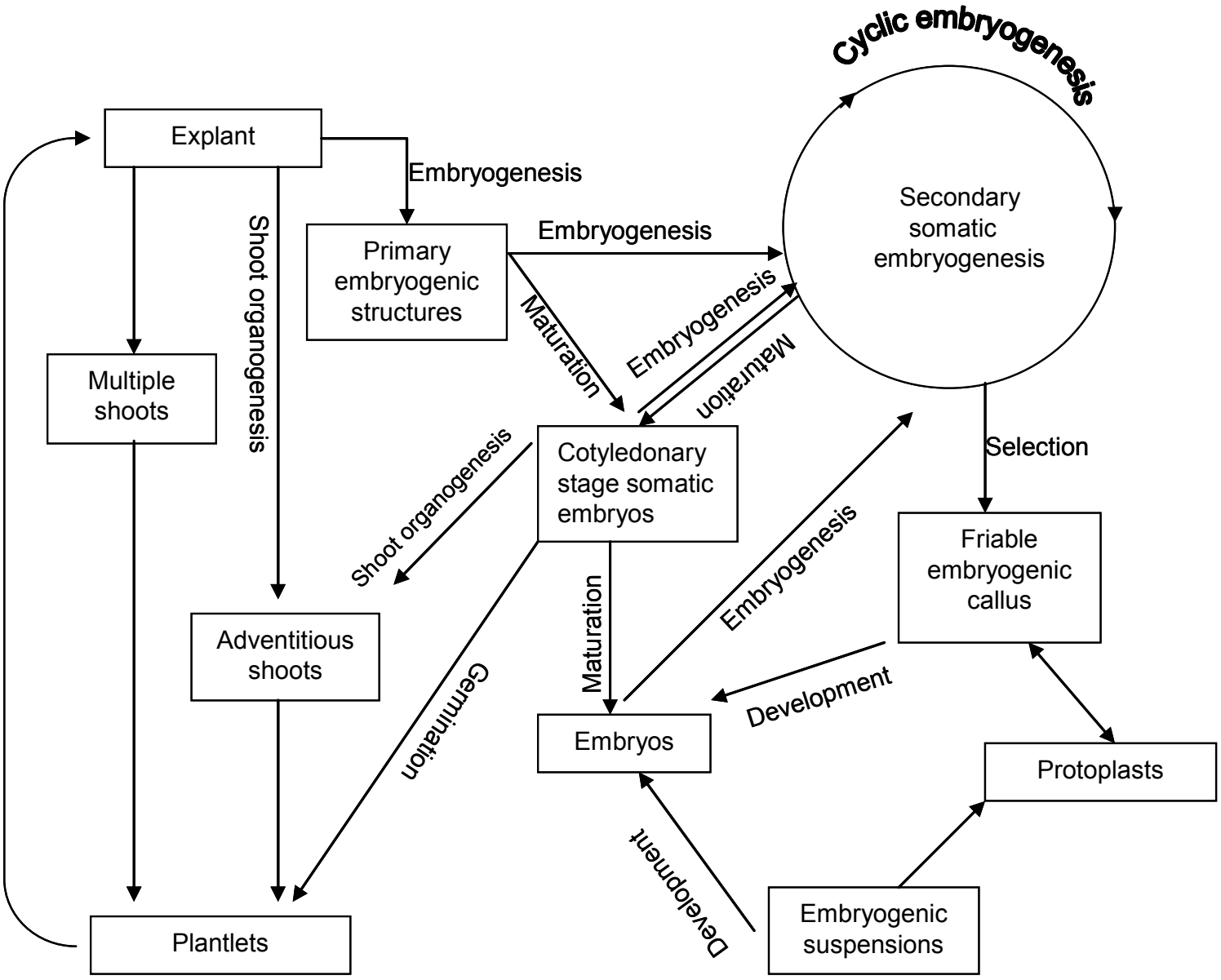


Figure 1.4 Schematic representation of different regeneration steps in cassava
Adapted from Zhang (2000)

Cassava has proven to be recalcitrant to plant regeneration from protoplasts. Cassava protoplast isolation and culture have been performed by various laboratories (Mabanza and Jonard 1983; Mabanza 1983; Szabados *et al*. 1987b; Villegas *et al*. 1988; Nzoghe 1989), but regeneration was observed only in one instance (Shahin and Shepard 1980) and has not been repeated. Recently, protoplasts isolated from FECs and embryogenic suspensions of cassava cv. TMS60444 were found to divide and develop readily into callus after culture in a medium supplemeted with 0.5 mg/l NAA and 1 mg/l zeatin (Sofiari *et al*. 1998). After 2 months of culture, about 60% of the callus had a friable embryogenic nature. One disadvantage of this system is the long span (≥20 weeks) from explant to suspension culture and to regenerated plantlets which may result in somaclonal variation and loss of regeneration capacity, the most common problem associated with suspension cultures. Another limiting factor is the production of FEC which is strongly genotype-dependent (Zhang 2000).

A method for regeneration of cassava plants through somatic embryogenesis has been available since 1982 (Stamp and Henshaw 1982). However, the use of embryogenic structures generated by this culture system as target tissues for genetic transformation via *Agrobacterium* (Calderón 1988; Schöpke *et al*. 1993; 1997) and electroporation (Luong *et al*. 1995) has yielded at best only chimeric embryos. Taylor *et al*. (1996) developed an alternative regeneration system in which clusters of embryogenic cells are suspended in liquid medium. These suspension cells are far more suitable for genetic transformation protocols with regard to accessibility or regenerable cells and selection procedures.

1.4.2 Cassava genetic transformation

Plant transformation is performed using a wide range of tools such as *Agrobacterium* Ti plasmid vectors, microprojectile bombardment, microinjection, chemical (PEG) treatment of protoplasts and electroporation of protoplasts (Veluthambi *et al*. 2003). Of the above-mentioned methods for delivering foreign DNA into plant cells, the most used are *Agrobacterium*-mediated gene transfer and particle bombardment (Fregene and Puonti-Kaerlas 2002; Veluthambi *et al*. 2003).

1.4.2.a *Agrobacterium*-mediated gene transfer

The naturally evolved unique ability of *Agrobacterium tumefaciens* to precisely transfer defined DNA sequences to plant cells has been very effectively utilized in the design of a range of Ti plasmid-based vectors. *A*. *tumefaciens* is a soil-borne, Gram-negative bacterium first described in 1943 (Braun 1943). It is responsible for the crown gall disease of many plants. The bacterium infects through a wound in the stem of the plant, and a tumour develops at the junction of root and stem. The agent responsible for crown gall formation is not the bacterium itself, but a plasmid known as the Ti plasmid (Liu *et al*. 1992). Ti plasmids are large ranging in size from 140 to 235 kb. During infection, a small portion of Ti plasmid DNA, is transferred to the plant cell nucleus where it becomes covalently inserted into the nuclear DNA. In this manner, the T-DNA becomes stably maintained in the genome of transformed cells. T-DNA carries the genes responsible for tumour formation and for synthesis of unusual amino acid derivatives known as opines. The genes responsible for transfer of T-DNA are also contained on the Ti plasmid and are called virulence genes (*vir* genes)

(Chen *et al*. 1991). *Agrobacterium* infection requires wounded plant tissue because *vir* genes results in the transfer of T-DNA that are absolutely required for its transfer and integration into the plant genome are the border regions. These are short repeat sequences of 25 base pairs. Any DNA sequence inserted between the border repeats will be transferred to and integrated into the plant genome. *Agrobacterium*-based DNA transfer system offers many unique advantages in plant transformation including a) The simplicity of *Agrobacterium* gene transfer makes it a 'poor man's vector b) a precise transfer and integration of DNA sequences with defined ends c) a linked transfer of genes of interest with the transformation marker d) the higher frequency of stable transformation with many single copy insertions e) reasonably low incidence of transgene silencing and lastly f) the ability to transfer large (>150 kb) T-DNA.

Agrobacterium system historically was the first successful plant transformation system marking the breakthrough in plant genetic engineering in 1983 (Chawla 2000). As stable transformation frequencies are low, the use of different marker genes is necessary to allow the identification and to be susceptible to *Agrobacterium* (Calderon-Urrea 1988; de Vetten *et al*. 2003). Pathogenecity of different *Agrobacterium* strains is highly variable and genotype dependent (Chavarriaga-Aquirre *et al*. 1993; Sarria *et al*. 1993; Li *et al*. 1996; Puonti-Kaerlas *et al*. 1997b). Cotyledons from somatic embryos of cassava cv. MCol22 have been the target for *Agrobacterium*-mediated transformation of cassava (Li *et al*. 1996). Their study showed that regeneration of transgenic shoots was achieved via organogenesis from somatic embryo cotyledon explants after co-cultivation with *A. tumefaciens* and selection on hygromycin or geneticin.

Agrobacterium strains LBA4404(pTOK233) and LBA4404(pBin9GusInt) gave the highest transient transformation rates. Schöpke *et al*. (1993a) reported the use of plasmids for transformation that contained an *uid*A-intron gene controlled by different versions of the cauliflower mosaic virus (CaMV) 35S promoter and the *htp* or *npt*II genes as selectable markers. Optimal transient expression of *uid*A was observed when cotyledon pieces were co-cultivated for 4 days with *Agrobacterium* strain LBA4404. In another study by González *et al*. (1998), *Agrobacterium*-mediated transformation was applied to introduce the *uid*A-intron and *npt*II genes into cassava tissue derived from embryogenic suspension cultures.

1.4.2.b Particle bombardment

Although the success of *Agrobacterium* vectors was paramount, the technique continues to have problems and limitations. Resultantly, its inability to infect monocots inspired researchers to develop an alternative delivery system. Particle bombardment is a procedure in which microscopic gold or tungsten particles coated with genetically engineered DNA are explosively accelerated into plant cells. This technique has become the second most widely used vehicle for plant genetic transformation after *Agrobacterium*-mediated transformation system (Gray and Finer 1993). Several distinct particle guns have been used including the Biolistic PDS 1000/He (Kirkert 1993), which is the only commercially available device. The most attractive of the non-commercial devices is the particle inflow gun (PIG; Finer *et al*. 1992), which is based on a flowing helium device described by Takeuchi *et al*. (1992), since it can be fabricated from a steel plate with readily available parts and offers performance on par with the Biolistic PDS 1000/He

(Brown *et al*. 1994). Despite its crude nature, this technique requires careful preparation, administration and quite often tissue regeneration. Target cells in the front line are usually destroyed, but projectiles normally penetrate those cells just behind without killing them. Some of those cells that would have survived the bombardment, incorporate the DNA from the microprojectiles into the genome and begin to express the gene product of the foreign DNA.

As already mentioned in section 1.4.2.a above, the use of different marker genes is necessary to allow the identification as stable transformation frequencies are low. The most commonly used visual markers are GUS-encoded by the *uid*A gene (Jefferson *et al*. 1986; Jefferson 1987), the luciferase genes from the firefly *Phonitus pyralis* (Ow *et al*. 1986) and soft coral *Renilla reniformis* (Mayerhofer *et al*. 1995) and green fluorescent protein (GFP; Chalfie *et al*. 1994). The most selectable marker genes encode resistance to aminoglycoside antibiotics (*npt*II; Bevan *et al*. 1983; Fraley *et al*. 1983; Herrera-Estrella *et al*. 1983), hygromycin (*htp*; van den Elzen 1985; Waldron *et al*. 1985) and phosphinotrin (*pat* and *bar;* Murakami *et al*. 1986; De Bock *et al*. 1987; Thompson *et al*. 1987; Wohllenben *et al*. 1988).

The effectiveness of microprojectile mediated system was shortly demonstrated successfully by scientists after discovery in transforming monocots, the first of which was Black Mexican Sweet corn (Fromm *et al*. 1990; Grodon-Kamm *et al*. 1990). Raemakers *et al*. (1996) investigated the effect of different bombardment and culture parameters on transient an stable expression of the firefly luciferase gene (*luc*) after particle bombardment of cassava embryogenic suspension cultures. Continuous selection and subculture of light-

emitting tissue eventually resulted in cultures consisting totally of transformed tissue. Differentiation and maturation of somatic embryos occurred on an MS-based medium supplemented with a complex mixture of organic components in addition to 4.14 µM picloram and 0.43 µM adenine sulfate. Different promoters (35S, e35S, 4Oe35S, UBQ1) fused to the *uid*A gene were bombarded into cassava leaves of cv. Señorita with a pneumatic particle gun (Franche *et al*. 1991; Schöpke *et al*. 1993b) in order to study their efficiency in cassava tissue. Transient gene expression was measured 24h after bombardment with fluorometric GUS assay using methylumbelliferone glucorinide (MUG) as a substrate. Higher activities measured with MUG assays corresponded to larger diameters of blue spots. Puonti-Kaerlas *et al*. (1997) used a particle inflow gun to investigate the efficiency of shoot meristem transformation in cassava. After bombardment with a particle preparation containing a range of sizes,particles were found to have lodged in first and second cell layers and even deeper. Using the *uid*A gene resulted in 50% of bombarded meristems showing 2-8 blue spots per meristem after GUS assays. Bombardment with the *luc* gene allowed for gene expression screening in living tissues and thus eliminating non-expressing plants. In a different study, Schöpke *et al*. (1997b) established and optimized conditions for particle bombardment of tissue derived from embryogenic suspension cultures of cassava cv. TMS60444. The optimal conditions bombardment parameters were found to be 1100 psi bombardment pressure, 1.0 µm particle size, two bombardments/sample, and an osmotic treatment with 0.1 M sorbitol and 0.1 M mannitol. When used in combination, these treatments

resulted in an average number of 1350 blue spots/cm^2 of bombarded sample of embryogenic-derived tissue.

Positive selection is a new concept for selection of transgenic plant cells developed recently (Joersbo and Okkels 1996; Haldrup *et al*. 1998; Joersbo *et al*. 1998). The transgenic cells are selected by addition of a compound which is converted by the transformed cells into a compound inducing a positive response, for example, growth or shoot formation. Non-transgenic cells stay alive without shoot formation, which means that neighbouring cells are not exposed to toxic selections from dying cells. Simultaneously, cells containing the transgene can utilize a component in the medium which results in growth or differentiation and non-transformed cells remain unaffected, therefore having no detrimental effect in transgenic cells (Zhang 2000).

Calderón (1988) was the first to describe transformed callus lines of cassava. He infected leaf pieces, stem pieces, and embryogenic callus with *Agrobacterium* containing plasmids with the coding sequences for neomycin phosphotransferase II (*npt II*), phosphinotricin acetyltransferase (*bar*), or β-glucuronidase (*uid A)*. Southern blot analysis with one callus line demonstrated the stable integration of T-DNA into the cassava genome.

The first reports on successful regeneration of transgenic cassava plants have been published only in the second half of the 1990s (Li *et al*. 1996; Raemakers *et al*. 1996; Schöpke *et al*. 1996). The current status of cassava transformation is summarized in Table 1.6.

Table 1.6 A summary of methods used in genetic engineering cassava programs.

Target tissue	Regeneration mode	Gene transfer system	Selection	Transgenic tissue	Analysis	Reference
Somatic embryos	Somatic embryogenesis	Eletroporation	-	Chimeric embryos	Transient GUS expression	Luong *et al*. (1995)
Somatic cotyledons	Shoot organogenesis	*Agrobacterium*	Hygromycin geneticin	Transgenic plants	Southern, Northern	Li *et al*. (1996)
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	Paramomycin	Transgenic plants	Southern	Schöpke *et al*. (1996)
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	Luciferase	Transgenic plants	Southern	Raemakers *et al*. (1996) Schöpke *et al*. (1997a)
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	-	Chimeric suspenions	Transient gene expression	Munyikwa *et al*. (1998a)
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	Luciferase and phosphinotricin	Transgenic plants	Southern, Northern	González *et al*. (1998)
Embryogenic suspension	Somatic embryogenesis	*Agrobacterium*	Paramomycin	Transgenic plants	Southern	Sarria *et al*. (2000)
Somatic cotyledons	Somatic embryogenesis	*Agrobacterium*	Basta	Transgenic plants	Southern	Zhang *et al*. (2000)
Somatic cotyledons	Shoot organogenesis	Particle bombardment	Hygromycin	Transgenic plants	Southern,RT-PCR	Zhang and Puonti-Kaerlas (2002)
Somatic cotyledons	Shoot organogenesis	Particle bombardment	Mannose, hygromycin	Transgenic plants	Southern, Northern, RT-PCR	Zhang and Puonti-Kaerlas (2002)
Embryogenic suspension	Somatic embryogenesis				RT-PCR	Zhang *et al*. (2001)
Embryogenic suspension	Shoot organogenesis, Somatic embrygenesis	*Agrobacterium*	Mannose, hygromycin	Transgenic plants	Southern, Northern, RT-PCR	Zhang *et al*. (2001)

Adapted from Fregene and Puonti-Kaerlas 2002.

1.4.3 Mechanisms of genetic engineering for virus resistance

Plant virus diseases cause severe constraints on the productivity of a wide range of economically important crops worldwide (Dasgupta *et al*. 2003). The application of genetic transformation for increased resistance to the major cassava viruses is a major priority. Strategies for the management of viral diseases normally include control of vector population using insecticides, use of virus-free propagating material, appropriate cultural practices and use of resistant cultivars. However, each of the above methods has its own drawback.

Sanford and Johnson (1985), working with bacteriophages described the concept of pathogen-derived resistance (PDR), and later demonstrated by Abel *et al*. (1986). PDR resistance strategies have proved effective in other crops (Beachy 1997) and are being developed against both the major viral diseases of cassava, namely, cassava common mosaic disease (CsCMD) and African cassava mosaic disease (ACMD).

Many viral host resistance genes have now been isolated and are used in transgenic plants to provide protection against viral infection. In a number of crops, transgenics resistant to an infective virus have been developed by introducing a sequence of the vial genome in the target crop by genetic transformation (Dasgupta *et al*. 2003). Virus-resistant transgenics have been developed in many crops by introducing either viral coat protein (CP) or replicase (Rep) gene encoding sequences. Resistance obtained by using CP is conventionally called coat protein mediated resistance (CPMR). In this case, resistance has been shown to be due to an inherent plant response, known as post-transcriptional gene silencing (PTGS).

Because of the essential nature of the viral movement protein (MP) for intercellular movement of plant viruses, movement problem sequence has also been used for achieving viral resistance (Okeese and Pinto 2003). Other pathogen-derived approaches include the use of satellite RNA and defective-interfering viral genomic components.

1.4.3.a Coat protein-mediated resistance

The use of viral CP as a transgene for producing virus-resistant plants is one of the most spectacular successes achieved in plant biotechnology. Early experiments demonstrated that plants transformed with the CP gene were more resistant when high levels of the viral capsid protein were expressed, confirming the importance of the actual protein in resistance (Powell-Abel *et al*. 1986). There was correlation between the levels of intact, functional coat protein and the degree of protection (Dasgupta *et al*. 2003; Kees and Pinto 2003). The resistance, however, could be overcome with high concentrations of virus inoculum unencapsidated viral RNA. Transgenic potato, expressing the CP of *Potato virus x* (PVX) also showed resistance against PVX (Hemenway *et al*. 1998; Okuno *et al*. 1993; Powell *et al*. 1990).

Fauquet *et al*. (1993) employed *Agrobacterium*-mediated transformation of *N. benthamiana*, which can be infected by both ACMV and CsCMV to study the expression of the viral CPs and their ability to provide protection against the respective viruses. Plants transgenic for the ACMV-CP gene were shown to contain low levels of mRNA corresponding to the coding sequence of the ACMV-CP gene. Accumulation of the CP was detectable by western blots, but it was relatively low.

Challenge of the CP positive plants with ACMV resulted in some degree of resistance at a virus concentration of 20-100 ng/ml. On the other hand, plants transgenic for CsCMV-CP gene accumulated the CP to levels of up to 2% of total protein and some plant lines showed a very high resistance to infection with CsCMV.

Further evidence of recombination under high selection pressure in laboratory experiments was obtained for *African cassava mosaic virus* (ACMV), when a CP deletion mutant that incluces mild systemic symptoms was inoculated to *Nicotiana benthamiana* transformed with three different constructs comprising the CP coding sequence and the viral intergenic region (common region; Gallitelli and Accotto 2001). Recombinants with wild type virus properties were recovered from plants transformed with constructs containing also the intergenic region, but not from those transformed with CP coding sequence alone. This led to suggest that the intergenic region can be a prime target for recombination (Frischmuth and Stanley 1998).

1.4.3.b Replicase-mediated resistance

The application of plant viral replicase (or polymerase) genes for the transformation of host plants, which leads to the generation of plant lines resistant to the donor virus, is termed replicase-mediated resistance. It has been shown to be effective in several cases (Carr and Zaitlin 1993; Palukaitis and Zaitlin 1997). In geminiviruses, only one virus-encoded protein is indespensible for replication of the viral DNA (Elmer *et al*. 1988). This protein of about 41 kDa is encoded by ORF C1 (also called AC1 or AL1) in all whitefly-transmitted geminiviruses and due to its similarities with rolling circle DNA replication initiator proteins of some prokaryotic plasmids (Koonin and Ilyina 1992), has been called Rep protein (Laufs *et al*. 1995; Stanley 1995). Rep

has a multifunctional role during viral replication (Lazarowitz *et al*. 1992) and also represses its own expression (Sunter *et al*. 1993; Eagle *et al*. 1994). Hong and Stanley (1996) found that integration and expression of the ACMV AC1 (Rep) gene driven by the enhanced 35S promoter, imparted elevated resistance to infection by this virus, while Sangaré *et al*. showed expression of mutated AC1 gene to delay symptom apparition and severity and to reduce virus movement (Sangaré *et al*. 1999).

In addition, truncated versions of the replication-associated protein of geminiviruses are also able to provide protection against viral infection. Transgenic *N*. *benthamiana* plants expressing the N-terminal 210 amino acids of *Tomato yellow leaf curl Sardinia virus* C1, could delay virus accumulation after inoculation (Chatterjii *et al*. 2001). Resistance was associated with defective amounts of viral mRNA and protein at the time of infection and correlated with a substantial reduction of viral DNA replication. Similarly, transgenic *N*. *benthamiana* expressing N-terminal 160 amino acids of the replication-associated C1 protein of *Tomato leaf curl New Delhi virus* inhibited homologous viral DNA accumulation (Chatterjii *et al*. 2001).

1.4.3.c RNA-mediated resistance

In addition to the sequences representing diverse functional viral proteins, defective or truncated versions of these genes, either expressed in sense or antisense can confer resistance. These types of resistance have been found to operate completely at the RNA level and are reffered to as RNA-mediated virus resistance (de Haan *et al*. 1992; Lindbo and Dougherty 1992; van der Vlugt *et al*. 1992).

Movement proteins (MP) are essential for cell-to-cell movement of plant viruses. These proteins have been shown to modify the gating function of plasmodesmata, thereby allowing the virus particles or their nucleoprotein derivatives to spread to adjacent cells (Dasgupta *et al*. 2003). The conferred resistance is believed to be based on the competition between wild-type virus-encoded MP and the preformed dysfunctional MP to bind the plasmodesmatal sites (Lapidot *et al*. 1993; Malyshenko *et al*. 1993). In addition, their resistance was seen to effective against distantly related or unrelated viruses, for example, resistance against TMV could be achieved in tobacco using the MP derived *Brome mosaic virus*, thereby suggesting functional conservation of MP among several proteins (Cooper *et al*. 1995). The cell-to-cell movement and the nuclear shuttle protein genes (MP and NSP; respectively) also have been used to confer resistance to begomoviruses. The ToMoV and *Bean dwarf mosaic virus* (BDMV) MP and NSP genes have been used to transform tobacco and tomato by Duan *et al*. (1997) and Hou *et al*. (2000), respectively. Some resistance to ToMoV was obtained for constructs containing the MP sequence in the first case, and for NSP and MP constructs in the latter study. The resistance obtained by Hou *et al*. (2000) was expressed as only a delay in the appearance of the ToMoV symptoms.

A recent report by Freistas-Astúna *et al*. (2001) demonstrated that tobacco plants transformed with ToMoV MP gene behaved biologically as if the resistance was RNA-mediated (recovery phenotype), but exhibited some characteristics at the molecular level that are typical of protein-mediated resistance (low, but detectable levels of MP mRNA and protein after challenge with ToMoV).

Some viral infections, in particular those involving tombusviruses and carmoviruses, are associated with the accumulation of defective interfering (DI) RNAs (Keese and Pinto 2003). These RNAs contain sequences essential for their replication by the helper virus, but have incomplete coding regions. They often reduce accumulation of the helper virus and may result in amelioration of symptoms. With the development of infectious full-length viral clones, artificial DI RNAs and DNAs can now be generated and tested. In a study conducted by Rubio *et al*. (1999), *N*. *benthamiana* was transformed with a DNA cassette designed to transcribe DI RNA from *Tomato bushy stunt virus* (TBSV). Self-cleaving sequences were added to the termini so that transcripts were competent to be replicated by the helper virus. Subsequent viral challenge showed resistance to TBSV and closely related tombusviruses. In another study by Frischmuth and Stanley (1993), incorporation of subgenomic DNA A and B conferred resistance to ACMV in *N*. *benthamiana*.

It has been reported that gene silencing can be induced by plant virus infections in absence of any known homology of the viral genome to host genes and this silencing may occur at the transcriptional or post-transcriptional level (Covey *et al*. 1997; Ratcliff *et al*. 1997). Therefore it seems possible that plants can naturally escape virus infection in a post-transcriptional manner.

1.5 Objectives and thesis plan

Successful application of transgenic technologies in cassava will not depend not only on technical advances, but also on successful transfer of knowledge, tools and expertise to the countries in which cassava has an important socioeconomic role. To develop virus control and other improved traits such as starch quality in South African cassava cultivars, the aims of this thesis aimed were to

1) transfer the reliable regeneration system already developed at ILTAB to local South African cultivars
2) establish a routine transformation system for transfer of SACMV resistance genes to cassava.

To date, reliable regeneration and transformation techniques have been successfully utilized by only three laboratories in the world, namely i) International Laboratory for Tropical Agricultural Biotechnology (ILTAB), St. Louis, USA ii) Laboratory of Plant Breeding, Wageningen, The Netherlands and iii) Institute of Plant Sciences, ETH Zentrum, Zürich.

A basic requirement for any transformation programme that proposes to develop improved plants is the availability of a reliable regeneration system. Towards this end, Chapter 2 attempted to induce direct organogenesis from cassava tuber tissue. Because only root induction was realised and shoot induction proved to be elusive, the results of this study provided an essential guide for designing new experiments which were later conducted at ILTAB. This laboratory had already developed a reproducible genetic transformation protocol for the West African

cassava cultivar TMS60444. This cultivar is considered an excellent model system due to its susceptibility to ACMD and rapid storage root formation in the greenhouse.

In Chapter 3, aimed at developing a method of testing for SACMV resistance in the laboratory before transgenic cassava plants are released to field conditions. This was achieved by using the already developed SACMV dimers A and B in our laboratory by Berrie *et al*. 2001.

Chapter 4 aimed at transferring the above-mentioned ILTAB system to local South African cultivars T200, T400, P4-4 and P4-10. This was achieved by investigating the ability of each of the above cultivars to induce organised embryogenic structures and eventual regeneration of matured embryos. In chapter 5, tobacco *N*. *benthamiana* was transformed with full-length replicase gene of SACMV and later studies on challenging with the same virus revealed that gene didn't express. Tobacco was employed as a model plant species to allow the rapid determination of the efficacy of the AC1 replicase gene in controlling SACMV infections. Consequently, a new N-terminus truncation (621 bp) of the AC1 gene (N-rep) from SACMV was produced. This fragment was eventually cloned into pCAMBIA2301 transformation vector which also contains GUS visual marker gene driven by the 35S promoter. The newly prepared N-Rep gene in the pCAMBIA2301 vector was then used to transform cassava cvs. TMS60444 and T200 fec tissue by particle bombardment. *Agrobacterium* co-cultivation was used to transform tobacco leaf disks.

Chapter 6 is the general discussion and conclusions of work undertaken in this thesis.

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Chapter 2

ATTEMPTS TO INDUCE DIRECT ORGANOGENESIS FROM TUBER DISKS OF CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

Presented in a modified form as a Poster to Cassava Biotechnology Network IV International Scientific Meeting – CBN. 3-7 November 1998, Brazil

Makwarela M, Rey MEC (1998) Direct organogenesis from tuber discs of cassava (*Manihot esculenta* Crantz). Brazilian Cassava Journal 17:54

2.1 INTRODUCTION

Cassava (*Manihot esculenta* Crantz), also known as yucca, tapioca and manioc is a perennial shrub of the Euphorbiaceae family, native to Brazil (Nestel 1980; Cock 1985). It is cultivated throughout the lowland tropics of Southeast Asia, South America and Africa for its starchy-tuberized roots (Stamp and Henshaw 1987). Over 153 million tons of cassava roots are harvested annually in the tropical regions of the world and is the major source of calories for subsistence-farmers and people living in sub-Saharan Africa (McMahon *et al*. 1995). Cassava is vegetatively propagated, and is particularly prone to virus diseases, especially African Cassava Mosaic Disease (ACMD) (Bock and Woods 1983; Gibson *et al*. 1996). Cassava was introduced into Mozambique by the Portuguese in the 17th century and was brought into South Africa (SA) by Tonga tribesmen, who later spread into the eastern parts of Mpumalanga in SA and south into northern Kwazulu-Natal. The Zulu tribesmen call it *indumbula* (Daphne 1980). Large scale cassava production in SA was hindered by a taste preference for maize, but in the late 1970s there was a renewed interest in cassava, and extensive yield trials were conducted throughout sub-tropical regions of Kwazulu-Natal and Limpopo Provinces under a range of environments (Daphne 1980). There has recently been an enormous upsurge of interest in cassava in SA for industrial applications such as the extraction of starch for animal feeds and paper manufacture, and as a potential food security crop for marginalised farmers. However, due to serious losses in cassava yields by begomoviruses, such as *South African cassava mosaic virus* (SACMV) (Berrie *et*

al. 1998), there is an urgent need to create new virus resistant cultivars (through clonical breeding). Regeneration of virus resistant transgenic plants is an interesting method allowing to reach that objective more rapidly.

Currently, the routine way of regeneration is by somatic embryogenesis since direct or indirect adventitious shoot formation is seldom observed (Shahin and Shephard 1980; Raemakers *et al*. 1993; Narayanaswamy 1994). Shoots have been regenerated from callus derived from stem segments (Tilquin 1979), mesophyll protoplasts (Shahin and Shephard, 1980), cotyledons (Stamp and Henshaw 1987; Guohua 1998) and immature leaf lobes of *in vitro* cloned plants (Taylor *et al*. 1996a). Recently, cassava embryo culture protocol using immature seeds was described (Fregene 1999). The capacity to undergo embryogenesis depends to a larger extent on the genotype of explants, such as physiological age, explant size, source of explant, media and physical conditions (Taylor *et al*. 1996b). However, plant regeneration from cassava tissues via somatic embryogenesis is limited because the observed frequency of plant regeneration is usually low (Guohua 1998), except using higher concentrations of BAP (Raemakers *et al*. 1995).

Successful tuber disk transformation and regeneration systems for potato, *Solanum tuberosum* var. Pentland Dell, have been reported in the literature (Sheerman and Bevan 1988). Prolific shoot regeneration (91%) was observed on MS medium supplemented with vitamins, 1 mgl^{-1} ZEA and 0.5 mgl^{-1} IAA. In addition, several studies have shown that antibiotics in some instances are able to stimulate growth and development of tissues grown *in vitro* (Holford and

Newbury 1992; Yepes and Adwinckle 1994). Preliminary experiments in our laboratory demonstrated some success in cassava root induction from tuber tissue and the aim of this study was to assess whether direct organogenesis was possible from cassava tubers, since a direct shoot regeneration system would avoid the need for a callus stage which is a source of somaclonal variation. In addition, the hypothesis that the antibiotic carbenicillin may act as a growth stimulator, in particular a potential shoot inducer, was tested.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of roots and tuber tissues

Cassava tubers and roots of a locally bred SA cultivar T200 were obtained from the Northern Province. Both tubers and roots were stored at 4°C in the dark until use. Only fresh tubers (Fig 2.1A) and roots were used for regeneration experiments. These were scrubbed under running water to remove adhering soil, then peeled and washed thoroughly in distilled water. After rinsing, tubers were cut transversely into sections of approximately 4 cm length while roots were peeled before surface sterilisation for 15 minutes in 10% sodium hypochlorite solution containing few drops of 'Tween 20'. They were then washed 4 times in sterile water. Sections, punched through in an orientation parallel to the long axis of the tuber cylinders, were sterilised for further 5 minutes in 10% sodium hypochlorite, rinsed three times in sterile deionised water and placed in liquid MS medium. For roots, thin slices were cut and disks 1-2 mm in width were cut from the tuber cylinders using a sterile 1 cm diameter cork borer and the end sections

were diskarded where the bleach had penetrated the tissue. A maximum of five explants per 90 x 15 mm plastic petri dish (Fig 2.1B) were cultured onto different callus induction media under light or dark conditions. For callus induction, a minimum of 75 explants per each dark treatment and a minimum of 100 explants per each light treatment were used. Each experiment was repeated two times and in some cases three times. One-Way Analysis of Variance (ANOVA) (SAS 1987) was used to assess differences in the recorded percentage mean values of callus production (number of explants showing callus per number of explants) for each treatment. Percentage mean values that did not share the same letter were recognised as being significantly different from each other.

2.2.2 Plant growth media

Callus induction media (CIM) and shoot induction media (SIM) contained MS salts and vitamins supplemented with 30 gl^{-1} sucrose, 8 gl^{-1} agar (unless specified), and a range of hormones. The pH of the medium was adjusted to 5.8 before the gelling agent was added. Media were autoclaved at 121ºC for 25 minutes. The following hormones were tested in all combinations for their effectiveness in direct organogenesis induction: IAA (0.01, 0.05, 0.1, 1 and 5 mgl^{-1}) and ZEA (0.01, 0.05, 0.1, 1 and 5 mgl^{-1}) (Table 2.1). All CIM media treatments had 500 μgml^{-1} carbenicillin as a potential organogenesis enhancing factor since preliminary experiments in our laboratory showed that the inclusion of carbenicillin at 500 μgml^{-1} helped in the induction of roots from cultures (data not shown). Cultures were maintained at 26 ± 2°C under a 16h photoperiod at 200 $\mu Em^{-2}s^{-1}$ photosynthetic photon flux density for four weeks. Percentage of

explants with callus and/or shoots/roots were recorded. Calli were transferred onto several SIM with different hormones either used singly or in combination and different additives such as $CuSO_4$ or $AgNO_3$ (Table 2.2).

2.2.3 Microscopy

After 4 weeks of callus initiation, squash preparations were made and callus tissue was examined under the light microscope and assessed for the production of embryogenic and non-embryogenic tissue. The various types of calli were recorded using a Nikon FM2 camera with a 50 mm Mikro Nikkor macro lens.

2.3 RESULTS AND DISCUSSION

2.3.1 Effects of cytokinin in combination with auxin on callus induction

In preliminary experiments, cassava cv. T200 tuber disk explants cultured on MS medium supplemented with 0.5 mgl^{-1} IAA plus 1 mgl^{-1} ZEA and 500 μgml^{-1} carbenicillin failed to give rise to shoots but only produced creamy-yellow callus that later gave rise to roots at a low frequency (0.2%) (data not shown). In contrast, studies conducted by Sheerman and Bevan (1988) with potato tubers indicated that a similar medium was markedly superior for callus and shoot regeneration. In their study, the varieties Desiree and Pentland Dell responded with 100% and 91% of disks giving rise to shoots respectively after 4 weeks. When shoots were excised and placed on rooting medium containing

Table 2.1 Effect of combining IAA and ZEA on callus induction from tuber explants of *Manihot esculenta* Crantz cv. T200 after 4 weeks of culture under light and dark incubations

ZEA (mgl^{-1})/IAA (0.01 mgl^{-1})	Explants showing callus under light (%)[a]	Explants showing callus in dark (%)[b]
0.01	100.0a	96.0a
0.1	100.0a	100.0a
0.5	100.0a	100.0a
1	100.0a	100.0a
5	100.0a	100.0a
ZEA (mgl^{-1})/IAA (0.1 mgl^{-1})	Explants showing callus under light (%)[a]	Explants showing callus in dark (%)[b]
0.01	86.7ab	100.0a
0.1	85.3b	96.0a
0.5	96.0a	100.0a
1	100.0a	100.0a
5	0.0	0.0
ZEA (mgl^{-1})/IAA (0.5 mgl^{-1})	Explants showing callus under light (%)[a]	Explants showing callus in dark (%)[b]
0.01	96.0a	92.3a
0.1	100.0a	100.0a
0.5	100.0a	92.3a
1	89.0a	80.0bc
5	89.0a	52.0c

The above two columns of data were analysed separately.

The same letter in the column denotes no significant difference at 5% level of probability

[a] Percentage values are number explants showing callus and one petri dish had 5 explants

[b] Percentage values are number explants showing callus and one petri dish had 5 explants

Table 2.2 SIM treatments used for the induction of shoot regeneration from *Manihot esculenta* Crantz cv T200 tuber disks

SIM treatment [a]	2,4-D	NAA	IAA	BAP	IBA	Kin	TDZ	ABA	GA_3	$AgNO_3$	$CuSO_4$
1a	0.5	-	-	-	-	2.0	-	-	-	-	-
b	1.0	-	-	-	-	0.1	-	-	-	-	-
c	3.0	-	-	-	-	1.0	-	-	-	-	-
d	0.5	-	-	-	-	0.1	-	-	-	-	-
2	-	1.0	-	-	-	1.0	-	-	-	-	-
3a	-	-	-	-	-	-	10	-	-	-	-
b	-	-	-	-	-	-	15	-	-	-	-
c	-	-	-	-	-	-	20	-	-	-	-
d	-	-	-	-	-	-	22	-	-	-	-
4a	-	-	-	2.0	-	-	-	-	-	-	-
b	-	-	-	5.0	-	-	-	-	-	-	-
c	-	-	-	10	-	-	-	-	-	-	-
d	-	-	-	15	-	-	-	-	-	-	-
e	-	-	-	20	-	-	-	-	-		-
5[b]a	-	-	0.5	-	-	-	-	2.0	-	-	-
b	-	-	0.5	-	-	-	-	5.0	-	-	-
c	-	-	0.5	-	-	-	-	10	-	-	-
6[b]a	-	-	0.5	-	-	-	-	-	-	-	2.0
b	-	-	0.5	-	-	-	-	-	-	-	5.0
c	-	-	0.5	-	-	-	-	-	-	-	10
7[b]a	-	-	0.5	-	-	-	-	2.0	-	-	-
b	-	-	0.5	-	-	-	-	5.0	-	-	-
c	-	-	0.5	-	-	-	-	10	-	-	-
8	-	-	-	0.25	-	-	-	-	0.1	-	-
9	-	-	0.2 5	0.25	-	-	-	-	0.01 4	-	-
10	-	-	-	1.0	0.5	-	-	--	-	-	0.5

[a] All SIM contained MS salts with vitamins, 30 gl^{-1} sucrose and 0.8% agar

[b] All SIM media contained MS salts with vitamins supplemented with 0.01 mgl^{-1}IAA, 0.01 mgl^{-1} ZEA and 500 μgl^{-1} carbenicillin

Hormone concentrations are represented in mgl^{-1}

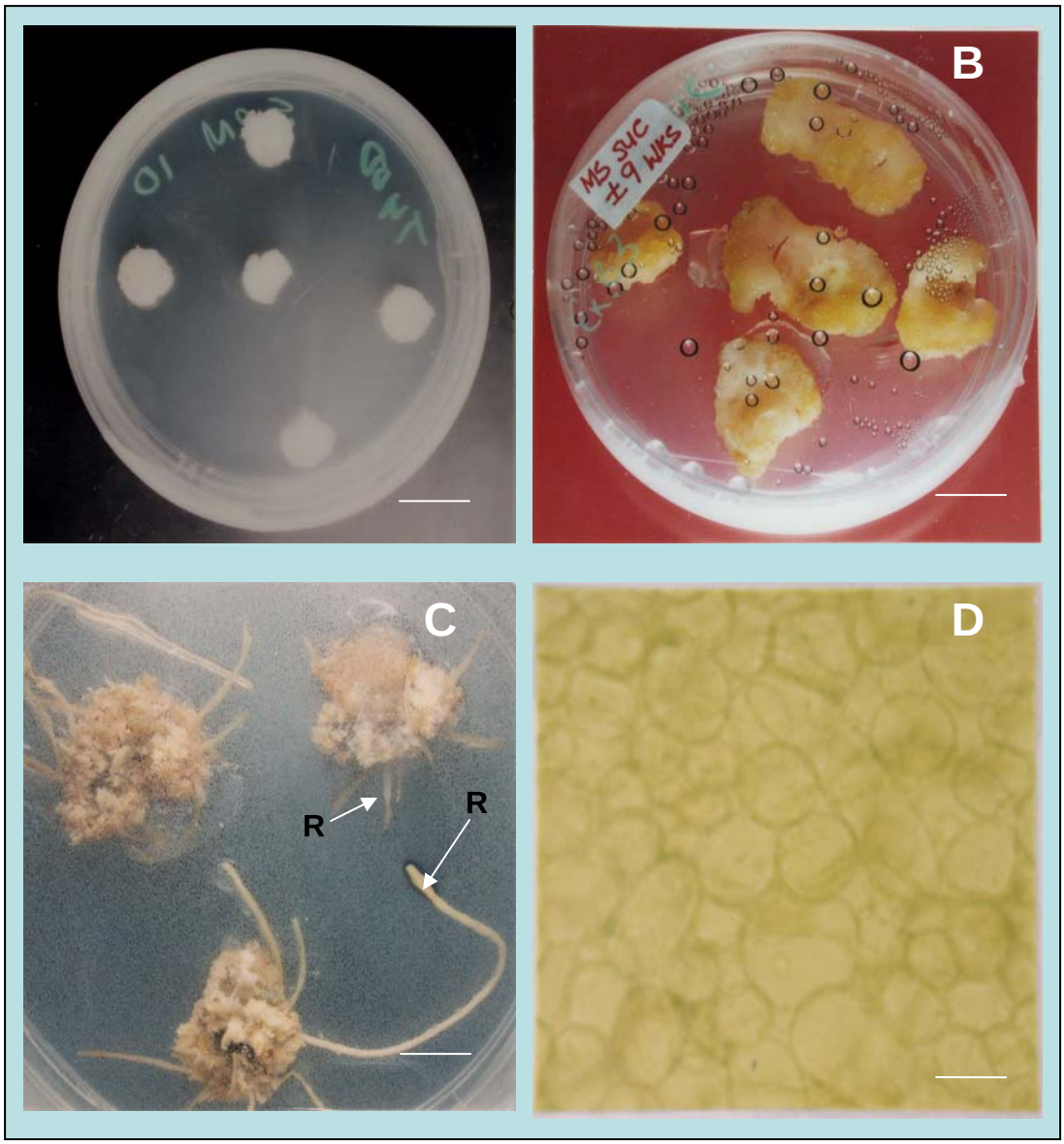


Fig 2.1A-D Callus and root induction in *Manihot esculenta* Crantz cv. T200 (bars **a** 2.3 mm, **b**,**c** 1.1 mm, **d** 0.8 mm). **A** tubers immediately after harvest. **B** tuber disks after 3 days of culture in IAA and ZEA supplemented medium. **C** creamy-yellow non-embryogeneic callus (*arrows*) induced in 0.01 mgl^{-1} IAA and 0.01 mgl^{-1} ZEA supplemented medium induced under light conditions. **D** prolific root formation (*arrowheads* **R**) developing in medium supplemented with 2 µM $CuSO_4$, 1 mgl^{-1} BAP and 0.5 mgl^{-1} IBA

carbenicillin and kanamycin, prolific root formation occurred in about 80% of the shoots, mostly from the nodes, with a few from the cut surface.

Consequently, in this study different hormone treatments were attempted in combination with carbenicillin as a potential organogenesis inducing factor, in an effort to promote shoot regeneration (Table 2.1). Our results clearly show that in cassava, auxins and cytokinins play no visible role in shoot differentiation from tuber disk explants since no shoot regeneration was obtained at any attempted growth regulator combinations, both under light and dark pre-treatments, and only callus was observed at certain hormone concentrations. Pre-treating tuber disk explants of T200 by growing them in MS medium with 0.01-5 mgl^{-1} IAA and 0.01-5 mgl^{-1} ZEA in either light or dark incubation resulted only in callus formation from most treatment combinations. Results after 5-7 days showed that there was no statistically significant difference between light versus dark treatments (Table 2.1). There was a tendency of explants to develop callus around the cut edges of the tuber disk explants in all treatments (Fig 2.1C). Although callus produced by tuber disk explants incubated in the dark showed no statistically significant difference to callus produced in the light, dark treatment was omitted from future work as calli produced were white and non-morphogenic. Increasing IAA concentration to 0.1 mgl^{-1} and 0.01-5 mgl^{-1} ZEA lowered the frequency of explants forming callus both under light and dark pre-treatments (Table 2.1). Treatments with high levels of both IAA and ZEA (1 and 5 mgl^{-1}) resulted in little to no callus formation (data not shown).

Hormonal balance is a key factor in regulating morphogenesis in cultured explants (Murashige 1974), and further/different hormone treatments may need to be investigated. Antibiotics, for example kanamycin or streptomycin, are commonly used as selectable agents for transformed plant tissue (Holford and Newbury 1992). In contrast to these effects, certain antibiotics have been shown to stimulate plant growth and development (Holford and Newbury 1992). No mechanism has been suggested for these effects however, and it has been assumed that the antibiotics mimic plant hormones. Conflicting reports exist in the literature as to the stimulatory effects of antibiotics on shoot or root regeneration. In their study, Holford and Newbury (1992) reported the production of callus by *Artirrhinum* hypocotyls to be stimulated by the addition of penicillin or carbenicillin at 250 and 500 μgml^{-1} to base medium. Shoot regeneration on medium supplemented with penicillin and carbenicillin at 500 μgml^{-1} also produced significant increases in callus production. Penicillin at 250 μgml^{-1} and carbenicillin 500 μgml^{-1} increased the average number of shoots produced per explant above control levels. Ampicillin had no effect on shoot production whilst both cefotaxime and cephalosporin significantly reduced shoot production below control levels. All antibiotics treatments reduced root production below control levels but had no effect on the proportion of explants producing callus, shoots or roots with the exception of cephalothin which also significantly reduced the number of explants producing roots. Holford and Newbury (1992) study also demonstrated that the significant increase of callus production was due to the breakdown of penicillin and carbenicillin to phenylacetic acid. In contrast, in

another study conducted by Yepes and Aldwinckle (1994), they showed that carbenicillin at a dose of 500 mgl^{-1} induced abundant callus formation in plant but inhibited shoot regeneration. In this study, our results appear to be more in line with Yepes and Adwinckle (1994) where callus production was enhanced by the addition of carbenicillin (Table 2.1), while root formation was only observed with SIM treatment 9 (MS supplemented with 2μM $CuSO_4$, 1 mgl^{-1} BAP and 0.5 mg^{-1} IBA) (Table 2.2). Shoot regeneration was never achieved under any conditions.

In the experiments conducted by Sheerman and Bevan (1988), thin slices of potato tuber tissue gave the most uniform and rapid shoot regeneration compared to leaf and stem explants. Shoots developed within 4 weeks from multiple points on the tuber disks. In their experiments, it was observed that tuber age was a critical parameter with young firm tubers giving the best results, while older and softer tubers with sprouts formed shoots only sporadically. In this present study, the cassava tubers approximately 8 months old at harvest and slices used were less than 2 mm thick, while less than 6 months old potato tubers were used by Sheerman and Bevan (1988). It is possible, but unlikely, that cassava tubers less than 8 months old may respond better to different shoot induction media.

2.3.2 Effects of auxins and cytokinins on tuber organogenesis response

Within 21-31 days of culture on the SIM containing 2μM $CuSO_4$, 1mgl^{-1} BAP and 0.5 mgl^{-1} IBA (Table 2.2) the creamy-yellow callus developed roots at a frequency of 60% without any sign of shoot production (Fig 2.1D). This cassava

callus line was confirmed to be of non-embryogenic nature by tissue squash preparations unlike friable callus reported by Taylor *et al*. (1996b) which is generally shoot forming. According to Taylor *et al*. (1996b), friable embryogenic callus is pale yellow, highly friable consisting of numerous spherical embryogenic units. Furthermore, Newell *et al*. (1995) reported that regeneration of shoots in potato occurred following development of embryogenic callus. All other SIMs containing auxins and cytokinins at various concentrations and additives such as ABA, $AgNO_3$ and $CuSO_4$ did not succeed in inducing shoot organogenesis (Table 2.2). In comparison, Otani *et al*. (1996) reported that in *Ipomoea batatas* (L.) Lam, a wild relative of sweet potato, shoot regeneration from leaf-calli was induced on the media containing more than 2 mgl^{-1} BAP and that 10 mgl^{-1} BAP was the most effective. The same authors reported that the addition of ABA or $AgNO_3$ was effective for enhancing shoot regeneration especially at 2.5 mgl^{-1} or 2 mgl^{-1} respectively. Despite the lack of $AgNO_3$ effect to induce or enhance shoot regeneration from cassava tuber tissue, the beneficial effect of $AgNO_3$ has been reported in other studies such as in pollen embryo formation from anther cultures of Brussels sprouts (Ockendon and McClenaghan 1993), shoot regeneration from callus cultures of *Nicotiana plumbeginifolia* (Purnhauser *et al*. 1987) and direct shoot regeneration from cotyledonary explants of chinese cabbage (Chi and Pua 1989). In those plant species the stimulation of morphogenesis occurred by the addition of 1.3 to 17 mgl^{-1} $AgNO_3$.

From the results obtained in this study, the pathway of regeneration via organogenesis using cassava tuber disk explants does not appear to follow the

regeneration pathway exhibited by potato tuber disks, cassava leaves or stems as a source of explants. This may be due to cassava tuber tissue lacking meristematic bud-like centres, found in potato, that give rise to shoots (Newell *et al*. 1995) and also because embryogenic callus was not inducible from cassava tuber tissue. This lack of response is also possibly due to a wrong choice of hormones.

2.3.3 Effect of root explants on organogenesis response

Since the physiological make-up of cassava tubers may differ from cassava roots or potato storage roots, cassava cv. T200 thin roots were also tested for their potential to induce both somatic embryogenesis and organogenesis. However, all tested combinations of auxins and cytokinins in CIM as used with the tuber disk explant, did not result in inducing callus or inducing organogenesis (data not shown). Surprisingly, little accurate information seems to exist in the literature on formation and development of cassava roots or tubers, although there are some few reports on cassava starch accumulation and storage (Hunt *et al*. 1977). Authors describe the cassava's first sign of storage root formation as generally manifested when cambial activity causes the stele to enlarge during the first month of growth of a plant regenerating from a stem cutting. This is followed by formation of cork cambium in the outer layers of the pericycle. Subsequently, bulking up continues with the production of parenchyma cells which store the bulk of starch grains. Hunt *et al*. (1977) findings are supported by the fact that cassava tuber deteriorates physiologically immediately after harvesting. Thus it

may be quite important to assess whether cassava roots or tubers are capable under any nutrient or hormonal treatments to become totipotent.

Addition of antibiotics in the regeneration medium has been shown to improve embryogenesis and regeneration potential of the tissue in a wide range of major crop plants including barley (Mathias and Mukasa 1988), carrot (Chang and Schmidt 1991) and pearl millet (Pius *et al*. 1993), but no investigations have been reported for tuber crops such as cassava. This research demonstrates that cassava tubers respond to carbenicillin as a root inducing factor. However, somatic embryogenesis from young leaves (Taylor *et al*. 1996a) and meristems (Puonti-Kaerlas 1996) remain the only systems at present that show potential for transformation and regeneration of cassava plants. A number of South African bred or commercially grown cassava cultivars are currently were optimized in this study (Chapter 4) for regeneration potential from young leaves.

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