Mutation breeding for *in planta* modification of amylose starch in cassava (*Manihot esculenta*, Crantz)

By

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Submitted in accordance with the requirements for the Philosophiae Doctor degree in the Department of Plant Sciences: Plant Breeding, in the Faculty of Natural and Agricultural Sciences

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November 2010

University declaration

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Dedication

This piece of work is dedicated to my mother Awovi Azametsi, my father Yao Agbayizah, my wife Henrietta Amenorpe, my brothers: Ben, Alfred and Stephen Amenorpe; sisters: Beauty, Alice and Edith Amenorpe; and my children Eyram Gift Amenorpe and Eli Eliana Amenorpe for waiting anxiously and praying hopefully for God to bless my hands to touch anything and make them very fruitful. I touched this PhD and made it useful. I hope it would be very more useful for people who read it.

Acknowledgements

My first thanks goes to God whose place nobody is yet qualified to take in my life because He has established the following people on my way for my favour. My second thanks to my wife who gave me the peace of mind to persue this course and third to Prof. Maryke Labuschangne who had accepted me to be among her numerous PhD students she trained across Africa with her own financial support. Mrs. Sadie Geldenhuys for the exceptional financial and administrative roles she played to make the Plant Breeding Department above all other departments a place for training all foreign nationals at the University of the Free States as if they are in their own country. The department breathes because of these professionals. It is not possible to mention the names of all individuals, institutions and organisations who contributed to this piece of work, but I fully recognise and appreciate your valuable contributions. The ones listed below are just a few.

- The Government of Ghana, Ghana Atomic Energy Commission, for granting me a three year study leave, for studies at the University of the Free State in South Africa.
- The University of the Free State for their financial and material support.
- Professor Gernot Osthoff of the Food Science Department of the University of the Free State for co-supervision and entrusting me with Programmable Brookfield viscometer for the viscosity studies. He did come to work even on Saturdays just to assist me. He is more than a supervisor to me.
- Prof. P.W.J. van Wyk for the love of his job and technical support at the Centre of Microscopy of the Plant Sciences Department of the Free State University, where scanning electron microscopy studies of starch granules were carried out.
- Professor Jannie Swarts of Chemistry Department at the University of the Free State for Differential Scanning Calorimetry work in his laboratory and Chris C. Joubert for training me on how to use the instrument.
- Prof. L. Herselman, Dr. A. van Biljon and Prof. C. Van Deventer for their effective teachings and moral support.
- Prof. E.H.K. Akaho, The Director General of the Ghana Atomic Energy Commission for administrative support.
- Prof. Josephine Nketsia-Tabiri, Director, BNARI, Dr. H. M. Amoate, Deputy Director, BNARI, Dr. Ken Danso and staff of BNARI for their administrative support.
- Dr. Elizma Koen for her expertise, patience, determination and encouragement in the course of execution of the molecular work at the University of the Free State.
- Dr. Gabre Kemp of the Chemistry department for assisting me with high-performance size-exclusion chromatographic analysis of starch.

- Mrs. Joubert of the Geology department for assisting me with X- ray diffraction chromatographic analysis of starch.
- The green-house staff of the Department of Plant and Soil Sciences (Ghana) for helping with their limited resources in gamma irradiation, planting and harvesting of cassava under difficult weather conditions.
- My good friends from within and outside the departments; church mates, Oskar Elago, Comrade Trevor Chiweshe, Robert Kawuki, man of Jehovah Davies Mweta, Elizabeth Parkes, Katleho Senoko, Rosina Montsoh, Sarah Chalo, Obed Mwenye, for their prayers, cooperation and support. They were a source of encouragement and laughter when the going got tough.
- The Almighty God again because everything begins and ends with Him successfully.

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ISO	Isoamylase
TEM	Transmission electron microscopy
NH_3	Ammonia
ANOVA	Analysis of variance
AFM	Atomic force microscopy
Bp	Base pairs
CMD	Cassava mosaic disease
Cm	Centimetre
cM	Centimorgans
cps	Centipoises
CV	Coefficient of variation
Con	Concanavalin
CGIAR	Consultative Group on International Agricultural Research
cm^3	Cubic centimeter
Da	Daltons
DP	Degree of polymerization
DPn	Degree of polymerization by number
^oC	Degrees Celsius
ΔH_G	Enthalpy
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
DMSO	Dimethyl sulphoxide
DE	Disproportionating enzyme
db	Dry basis
DWB	Dry root weight basis
Te	Enset/ conclusion temperature
ΔH_G	Enthalpy of gelatinization
ELSD	Evaporative light scattering detector
EST	Expressed sequence tags
FAO	Food and Agricultural Organisation
GPC	Gel permeation chromatography
GD	Genetic dissimilarity
G	Genotype
GxE	Genotype by environment interaction
GOPOD	Glucose peroxidase
G	Gram
Gy	Gray
$g.g^{-1}$	Gram per gram
GBSSI	Granule bound starch synthase I
g	Gravity
GDP	Gross domestic product
ha	Hectare
HPLC	High performance liquid chromatography
HPSEC	High-performance size-exclusion chromatography

h	Hour
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
CIAT	International Centre for Tropical Agriculture
IITA	International Institute of Tropical Agriculture
IPGRI	International Plant Genetic Resources Institute
ISO	International Standards Organization
ISI	International Starch Institute
I_2	Iodine
J	Joule
J g^{-1}	Joule per gram
Kg	Kilogram
LCLS	Leaf central lobe shape
LSD	Least significant difference
LD_{50}	Lethal dose: The safe dose at which half of the planting material survive
L	Litre
ELSD-LT	Low temperature evaporative light scattering detector
M	Metre
M_1V_1	The first cycle of vegetative propagation
M_1V_2	The second cycle of vegetative propagation
µg	Microgram
µl	Microlitre
mg	Milligram
mg.kg^{-1}	Milligram per kilogram
mL	millilitre
mm	Millimetre
mM	Millimolar
Min	Minute
MC	Moisture content
M	Molar
MW	Molecular weight
MSG	Monosodium glutamate
MAP	Months after planting
Ng	Nanogram
Nm	Nanometre
Nat.	Control landrace
NS	Not significant
To	Onset temperature
PHI	Peak height index
Tp	Peak temperature
%	Percentage
KCl	Potassium chloride
KI	Potassium iodide
pH	Power of hydrogen
PASW	Predictive Analytics Software
PULL	Pullulanase

RVA	Rapid Visco-Analyzer
RC	Reducing capacity
SEM	Scanning electron microscopy
sec	Seconds
SSR	Simple sequence repeat
SEC	Size exclusion chromatography
NaOH	Sodium hydroxide
SB	Solubility
SADC	Southern Africa Development Community
SD	Standard deviation
SE	Standard error
SBE	Starch branching enzymes
SS	Starch synthases
SSA	Sub-Saharan Africa
Ssp	Subspecies
R	Temperature range
t	Ton
t/ha	ton per hectare
UV	Ultraviolet
U	Unit
UPGMA	Unweighted pair group method of arithmetic averages
v/v	Volume per volume
H_2O	Water
WAC	Water absorption capacity
w/v	Weight per volume
$W.g^{-1}$	Watt per gram

CHAPTER 1

GENERAL INTRODUCTION

Cassava (*Manihot esculenta*) belongs to the *Dicotyledonae* class, *Fructicosae* section and *Euphorbiaceae* family and it originated in the Americas (Jos, 1969). The nutritional value of leaf protein is equal in quality to the protein in egg (Nassar and Marques, 2006). The starchy root of cassava is a staple food for millions of people in the tropics and subtropics. If a major catastrophe should strike the cassava crop, there would be widespread famine in developing countries (IAEA, 2002; Scott *et al.*, 2000). The starch market is dominated by maize, potato and wheat starches (Fungulani and Maseko, 2001; Itaye, 2001; Munthali, 2001) but cassava is bound to make a large impact in the near future because it produces higher quality starch at a relatively cheaper rate. Cassava starch has high gel clarity, excellent thickening properties, a neutral flavour and desirable textural qualities (Blanchard, 1995). Modified cassava starch can compete with other starches for the production of alcohol, starch for sizing paper and textiles, glues, sweeteners, bio-degradable products, butanol and acetone, manufacturing of explosives, and coagulation of rubber latex (FAO, 2001). The demand for new cassava genotypes with a high starch content and starch granules suited for easy fermentation into bio-fuel is extremely high, and at this stage no country satisfies it.

The incorporation of dietary starch in food has gained importance as it is considered to be a good replacement for dietary fat. Consumption of starch in adequate amounts instead of fat has been associated with the prevention of some chronic diseases like coronary heart disease, cancer, and diverticulosis (Asp and Bjorck 1992; Kamal *et al.*, 2000; Lopez *et al.*, 2001; Topping and Clifton 2001). It is envisaged that there will be a considerable demand in the near future for modified starches. To meet the demand, many different chemical modifications of starch, including cross-linking, have been widely used to obtain desirable physico-chemical properties that are suitable for various food applications. Cross linking end products of chemical starch modification for food depends on many chemical origins such as monosodium phosphate, sodium tripolyphosphate, epichlorohydrin, phosphoryl chloride and vinyl chloride (Wu and Seib, 1990; Yeh and Yeh, 1993; Wattanchant *et al.*, 2003), but similar cross linking end-products of chemically modified starches with similar physico-chemical properties could be achieved by *ex situ* irradiation of starch, irrespective of the botanical source of the plant (maize, potato, wheat, or rice) (Raffi *et al.*, 1981). However, negative perception among consumers about safety of chemically modified starches and safety debates on the direct use of gamma rays for food products still persists.

With expanding genomic DNA sequence information from many plant species, increasing knowledge regarding the functional roles of specific genes in traits of agronomic importance, it is now possible to consider the modification of specific plant traits in a directed manner. One approach is to use transgenes to transfer a single or multiple genes of interest within or across species. Using this approach, scientists have been able to create rice producing provitamin A in the grain (Ye *et al.*, 2000). Transgenic approaches have been met with a high level of public disapproval and its use for food production is currently banned in many countries. This calls for an alternative non-transgenic approach for crop improvement.

Most cassava cultivars are monoecious and have a marked protogynous flower habit. A high degree of heterozygosity may therefore be expected. Many existing cassava cultivars are undoubtedly the derivatives of natural hybrids. The variability generated by crossing is so great that there is little chance for the selection of improved types among seeding progeny while at the same time retaining the general characteristics of the adapted cultivars from generation to generation. This renders seed based mutation of cassava arduous. This implies that starting irradiation with cassava stakes (stem cuttings with an average of five adventitious buds) may be a better alternative to seeds.

Ceballos *et al.* (2008) mentioned several advantages for the introduction of inbreeding in cassava. One of them is that self-pollinations help to identify recessive mutations such as waxy starch in cassava. The best approach to commercially exploit the recessive mutant and overcome inbreeding depression is to make crosses with elite clones to produce an " F_1 generation" and then make crosses among the F_1 plants to produce an "F_2 generation" which offers the advantage of very limited amount of inbreeding depression, which is relatively high in the case of S_1 genotype. Although self-pollinations are useful for removing undesirable genes regardless of their association with high or low amylose content, a long breeding cycle is required to develop and commercially exploit the recessive mutant, which further render seed based mutation of cassava arduous (Jennings and Iglesias, 2001). Thus, to improve one or two characteristics in a cassava, mutation breeding may be a better method of approach (Brock, 1970; Moh, 1976).

Natural cassava mutant with high free sugar properties was reported (Carvalho *et al.*, 2004) but artificial mutation is a faster, versatile and safe genetic technique that imitates nature in enhancing genetic traits for better adaptation of species. Mutagenesis and mutation breeding as a tool for crop improvement is based on the probability of altering genes by exposing their vegetative parts, cells, tissues, gametes, pollen or seeds to physical and chemical mutagens (Ali *et al.,* 2007). It continues to be a good option for breeding vegetative propagated crops (Ahloowalia, 1995; Sleper and Poehlman, 2006). It has been used extensively to improve several crops, without extensive hybridization and backcrossing (Maluszynski *et al.,* 2000; Ahloowalia, *et al.* 2004). It has led to the release of more than 3000 crop varieties from some 170 different plant species through direct intervention of the IAEA. These include plants with *in planta* modified cassava starch such as good cooking quality and high dry matter clone with low amylose (Asare & Safo-Kantanka, 1997), a superior high yielding triploid hybrid mutant cassava clone (Sreekumari *et al.*, 1999) and small granule high amylose mutant cassava clones (Ceballos *et al.*, 2008). In several induced mutant plants, morphological differences in storage organs along with quantitative and qualitative differences in starch biosynthesis have been identified and characterized (Smith and Martin, 1993; Coleman *et al.*, 1995). These varieties provide much needed food as well as millions of dollars in economic terms for farmers and consumers, in developing countries (IAEA, 2002). Induced mutations have played a major role in increasing world food security, since mutants released in food crops have contributed significantly to an increase in crop production in marginal areas (Kharkwa and Shu, 2008).

Cassava starch in its native form has limited food and industrial applications. There is a huge market demand for modified cassava starch. Many different transgenic and biotechnology approaches, and *ex situ* physical and chemical starch modifications have been widely used to obtain some desirable traits that are suitable for various food applications. But consumers are skeptical about the safety of the genetically modified foods and chemical residues. To meet the demand, gamma irradiation which is energetic electromagnetic wave which does not leave any residue, was used as a tool to induce *in planta* variation in amylose production. Since the functional properties of starch are largely influenced by the ratio of amylose and amylopectin, there is a need to breed for high amylose and low amylose (high amylopectin) mutants *in planta*. Any intermediate functional properties desired could be achieved by mixing high amylose and low amylose groups. Mutation is uncontrollable and could therefore lead to unexpected useful mutants which would be duly documented. Any novel starch would increase genetic diversity of cassava and expand existing markets. The *in planta* modified cassava starch could restore consumer confidence eroded by transgenes and chemically modified starches. The discovery of cassava mutants with modified properties would facilitate expansion of cassava products in the distant markets (Chiwona-Karltun, 2001) and create job and investment opportunities for local growers and processors. It is an opportunity to produce cassava amylose and amylopectin starch standards with more similar physical and biochemical properties to test material. The *in planta* modification would decrease the cost of imperative post-harvest modification, some of which are environmentally damaging (Slattery *et al.* 2000).

The objectives of the current study were to induce mutations in four elite cassava landraces, identify the four highest and four lowest amylose producing plants at the M_1V_2 stage and document any unexpected useful mutants which might be discovered along with these plants during morphological, starch and starch granules characterization of induced and control plants.

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CHAPTER 2

LITERATURE REVIEW

2.1 Cassava

Cassava (*Manihot esculenta* Crantz) belongs to the *Euphorbiaceae* family, sub-family *Crotonoideae*, the tribe *Manihotae,* and the genus *Manihot.* The genus comprises of 98 species and is believed to have arisen and diversified recently (Schaal *et al.*, 1994). It is a perennial shrub though cultivated in the tropics as annual food. It is the most widely cultivated species that belongs to the genus *Manihot* (Mkumbira, 2002). Those that bulk around 6 months after planting (MAP) are classified as early bulking cultivars whilst those species that produce fibre around 6 MAP but sizeable storage roots 18 MAP are late bulking (Amenorpe, 2002). Centers of diversity of cassava are Brazil (major) and Central America (minor). Portuguese brought cassava to Africa in the latter half of the 16th century from South America. It is grown widely in tropical regions of Africa and Nigeria is the leading producer of cassava in the world (Nassar, 2005). The cyanogenic glucoside has been used to place cassava cultivars into two major groups: bitter cultivars, in which the cyanogenic glucoside is distributed throughout the storage root, at levels higher than 100 mg/kg fresh root weight, and sweet/cool varieties, in which the cyanogenic glucoside, at low level, is confined mainly to the peel. The early-bulking local cultivars of sweet/cool varieties have cyanogenic potential of edible root flesh below the innocuous level of 50 mg HCN kg^{-1} and are therefore safer than the released late-bulking varieties, some of which exceed the safety margin (Amenorpe *et al.*, 2006a). The flesh of sweet/cool varieties is therefore relatively free of cyanogenic glucoside (Mkumbira, 2002; Nassar, 2005). Early literature on cassava described the genus as having two edible species, *Manihot utilissima* Phol and *Manihot aipi* Phol delineating cultivars with high and low cyanogenic glucoside concentration respectively. Cassava has recently been classified as being one species, *Manihot esculenta* Crantz (Onwueme, 1978). The most important cassava varieties imported from tropical America to Africa could not survive in Africa due to the devastating epidemic disease of the African cassava mosaic virus (ACMV) which is not present in the Americas (Briddon *et al.*, 1998).

Cytologically, cassava is usually diploid, with a chromosome number of 2n = 36 (Onwueme, 1978; Nassar, 2005). Sometimes, natural hybridization results in polyploids such as triploids (2n = 3x = 54 chromosomes) and tetraploids (2n = 4x = 72 chromosomes) (Mkumbira, 2002). The triploids and tetraploids differ from the diploids in plant vigour, leaf shape and bigger size. Triploid plants usually grow and yield better than tetraploid and diploid plants. Nassar (1978) and Nassar *et al*. (1996) reported some aneuploids for certain genotypes. Cassava is proposed to be an allotetraploid since there are extra nuclear chromosomes, which is high for a true diploid (Magoon *et al*. 1969). *Manihot* species are probably segmental allotetraploids derived from crossing between taxa whose haploid complements had six chromosomes in common but differed in the other three (Magoon *et al.*, 1969) and this was confirmed with biochemical markers (Jennings and Hershey, 1985; Charrier and Lefevre, 1987) however, it is now regarded as an old allotetraploids, therefore, behaves as a diploid species.

2.1.1 Importance of cassava

Cassava provides more dietary energy per hectare in terms of working hours than any other staple crop and is sixth among crops in global production (Akoroda, 1995; Fregene *et al.,* 2000; Nassar, 2005). The root of cassava stores about 80% carbohydrate as dietary energy (Scott *et al.,* 2000) ranking fourth after rice, sugarcane and maize. It provides half of the calorie needs of 800 million people in sub-Saharan Africa (SSA), Latin America and Asia (Shore, 2002). Over 70% of the Democratic Republic of Congo, 50% of Nigeria and 30-40% of eight other major producing countries eat cassava as staple (Anonymous, 2001). The leaves are available all year round as reliable source of vegetable, crude protein (17 to 32 % d.b.) (Hahn, 1988; Nassar and Marques, 2006), vitamins (A, B and C) and other minerals (FAO, 1993; Moyo *et al.*, 1998). Cassava is drought tolerant and grows in marginal areas where cereals and sugarcane cannot survive. These attributes project cassava as food security crop for farmers with limited access to agricultural inputs (Fregene *et al.,* 2000; Ugwu, 1996).

Native starch is a valuable ingredient for the food industry, being widely used as thickening, gelling, bulking, stabilising, texturising, moistening and anti-staling agents (Niba *et al.,* 2001; Singh *et al.,* 2003; Thomas and Atwell, 1999). Modified cassava starch is further processed for use in paper, textiles, pharmaceuticals, wood, packaging, ethanol, batteries and explosives industries (Whistler, 1984; Moorthy, 1994) as well as an important flavouring agent in Asian cooking (FAO, 2001). The unique properties of cassava starch might be useful for speciality markets such as adhesives, baby foods, non-allergenic products and food for hospitalised persons (Moorthy, 1994; Thomas and Atwell, 1999).

The globalization of economies and the increase in the price of mineral oil has opened new opportunities for cassava to become an attractive source of renewable raw material for different industries. In South East Asia, cassava is being exploited for ethanol production as automobile fuel (Sriroth *et al.*, 2000). About 280 litres (222 kg) of 96% pure ethanol can be produced from one ton of cassava with 30% starch content (FAO, 2009). In many cases, climate and availability become the determining factors for choosing a particular crop for bio-fuel production (Moore *et al.*, 1984; Fabiano *et al.*, 2001). This explains why the USA uses maize starch while Canadians, Australians and New Zealanders mostly use wheat starch, and Europeans use potato and maize starch. Tropical countries like Brazil and the East Indies (in Asia) use cassava and other crop starch (Radley, 1976; Jarowenko, 1977; Wurzburg, 1986).

2.1.2 Cassava production in Ghana

Over 80% of 24 plus million people in Ghana depend on staple crops such as cassava, maize, sorghum, rice, yam, plantain, pulses, and oilseeds (Bogetic *et al.*, 2007). Ghana's participation in the Africa Growth Opportunity Act (AGOA) has generated interest in the accelerated expansion of the textile and garment industries to meet its export quota offered by the USA. In line with this, a PSI (President's Special Initiative) has been launched to produce cassava starch for the local textile industry as well as for export. It is for this reason that starch levels are receiving considerable attention in cassava breeding programmes (Amenorpe, 2002). The average yield of cassava from 1990 to 2006 was 12 t/ha whilst achievable yield in other countries was 28 t/ha, thus the yield gap in Ghana was 16 Mt/ha (57.5%) (FAO, 2008). The national average yield of cassava, rice and maize from 1994 - 2005 was 12, 2 and 2 t/ha respectively. Using the baseline yield of 13 t/ha for the 2000 – 2006 period and the national production of

9426671 t, the projected targets for cassava in 2013 and 2015 will be 13522885 and 13522885 t respectively (MOFA, 2006).

2.1.3 Morphological characterisation

Over 250 local cassava accessions from the Western Region of Ghana showed that some cassava cultivars were called by the same name, although the cultivars were morphologically different in all aspects except for one or two characteristics. Contrarily, some cultivars bearing different names were found to be morphologically identical. This indicates that the traditional system of nomenclature is not foolproof and must be backed by morphological and genetic descriptive methods (Amenorpe *et al.*, 2006b). However, the identification of cassava genotypes using morphological characteristics was reported to be reliable (Soyode and Oyetunji, 2009) due to the presence of some morphological traits which does not vary with the environment and also typify the cultivar (Onwueme, 1978).

Cassava can be classified morphologically with the help of leaf lobe shape, root pulp colour and and external stem colour, which have a higher heritability than agronomic traits such as root length, number of roots per plant and root yield (Alves, 2002). Using the cassava morphological descriptors published by Gulick *et al.* (1983) and revised by Fukuda and Guevara (1998), the most commonly used traits for identification are: (i) apical leaf colour; (ii) apical leaf pubescence; (iii) central lobe shape; (iv) petiole colour; (v) stem cortex colour; (iv) stem external colour; (vii) phyllotaxies' length; (viii) root peduncule presence; (ix) root external colour; (x) root cortex colour; (xi) root pulp colour; (xii) root epidermis texture; and flowering (Alves, 2002; Benesi, 2002; Nassar, 2005). The use of morphological characteristics as the basis for species recognition and identification has permitted not only the development of a consistent taxonomy but also the generation of keys that allow for taxon identification (Dayrat, 2005). Many of the quantitative traits are difficult to analyse because they do not have the simple genetic control assumed in genetic models (Liu and Furnier, 1993) and are of little use (Tanksley *et al.*, 1989). Due to the influence of different environments on cassava morphology, morphological classification based on variable traits is difficult. Hence, phenotypic variance in cassava is more than genotypic variance for traits of agronomic importance like storage root weight (Mathura *et* *al.*, 1986). In addition to morphological characterization, molecular markers have been very useful in removing duplicates in germplasm collections and for genetic diversity studies (Fregene *et al*., 1994; Bonierbale *et al.,* 1997; Ocampo *et al.,* 1995). Due to large numbers involved in mutation breeding, reverse genetic studies called Targeting Induced Local Lesions In Genomes (TILLING) is most suitable for mutation confirmation of genes whose expressed sequence regions fall within a Li-Cor readable region (McCallum *et al.,* 2000).

2.2 Starch

The sugar produced during the day by green leaves and stems are stored as small starch granules in chloroplasts, called transitory starch which are hydrolyzed and translocated to the amyloplasts (sink) at night. The specific shape and size of granules depend on botanical origin and the amyloplast (Davis *et al.,* 2003). Well extracted cassava starch settles between 30 – 60 min and gives a good yield which is free from colour, proteins and fats (Moorthy, 1994). High starch content is an important component of root quality of cassava (Jennings and Hershey, 1985). Cassava storage roots contain 20-40% and 73.7- 84.9% of starch on fresh and dry weight bases (Amenorpe *et al.,* 2007), with higher potential of producing clear starch than other tuber crops (Singh *et al.*, 2005). The starch granule is solid with a density of approximately 1.5 g/ml (Hoover, 2001). Starch is made up of macro molecules called amylose and amylopectin.

2.2.1 Starch molecules

2.2.1.1 Amylose

Amylose is the minor fraction of the starch granule and represents 20-30% of the polysaccharide content which varies with botanical source (Wurzburg, 1986; Tester *et al.*, 2004). Maize, wheat, potato, sweet potato and cassava have average amylose contents of 28%, 26%, 20%, 18% and 17%, respectively (Balagopalan, 1988; Onwueme, 1978; Young, 1984). Wickramasinghe *et al*. (2009) reported higher levels of amylose in cassava starches (25.4-28.8%) than in starches from sweetpotato (16.6-23.5%) grown in Sri Lanka. Amylose influences both the rheological and the viscoelastic characteristics, including gelatinization and retrogradation (Zeng *et al.*, 1997).

Swelling power is reported to increase with longer amylopectin chains and lower amylose content (Sasaki and Matsuki, 1998). Amylose in the presence of lipids forms insoluble complexes limiting the swelling power of starch (Tester and Karkalas, 1996). Oda *et al*. (1980) demonstrated that reduced amylose content leads to a reduction in both the gel temperature and the temperature at the maximum viscosity. The amylose content had a low positive correlation with cassava starches and an insignificant correlation with flours (Pérez, 2000).

Amylose molecules consist of single chains of 500-20000 α-(1; 4)-D-glucose units (>99% bonds) that coils into a helical structure (Figure 2.1). Dependent on source, less than 1% α-1- 6 branches and linked phosphate groups are recorded but these have little influence on the molecule's behaviour (Ral *et al.*, 2008; Buléon *et al.*, 1998). Amylose can form an extended shape (hydrodynamic radius 7-22 nm) but generally tends to wind up into a rather stiff left-handed single helix or form even stiffer parallel left-handed double helical junction zones. The helix is a coil of six glucose units in each complete leftwise turn, thus forming a compact storage molecule. Single helical amylose has hydrogen bonding with O-2 and O-6 atoms on the outside surface of the helix with only the ring oxygen pointing inwards. Hydrogen bonding between aligned chains causes retrogradation and releases some of the bound water (syneresis). The aligned chains may then form double stranded crystallites that are resistant to amylases. These possess extensive inter- and intra-strand hydrogen bonding, resulting in a fairly hydrophobic structure of low solubility. Single helix amylose behaves similarly to the cyclodextrins by possessing a relatively hydrophobic inner surface that holds a spiral of water molecules, which are relatively easily lost to be replaced by hydrophobic lipid or aroma molecules. It is also responsible for the characteristic binding of amylose to chains of charged iodine molecules where each turn of the helix holds about two iodine atoms and a blue colour is produced due to donor acceptor interaction between water and the electron deficient polyiodides (Orlando, 2003). Mestres *et al.* (1996) and Marques *et al.* (2006) reported that the blue complex is rather formed between linear regions of amylose chains and polyiodide ions in aqueous solutions allowing the determination of amylose content.

2.2.1.2 Amylopectin

Amylopectin is the major fraction of the starch granule and it is made up of large molecules ranging in size (∼10000-100000 glucosyl residues) and contains around 5% non-random α-1→6 branching of the 95% amylose - type α-(1→4)-D-glucose structure (Ral *et al.*, 2008; Buléon *et al.*, 1998) (Figure 2.2 a, b). The α-1→6 branching is determined by branching enzymes that cleave each chain with up to 30 glucose residues. The molecular weight of amylopectin is between 1 $x10^7$ -1x 10^9 Daltons (1Da= 1gram/mole) (Thomas and Atwell, 1999). The degree of polymerization (DP) of amylopectin is between 9600 and 15900. The average degree of polymerisation is 1450 for maize amylopectin, 1300 for cassava amylopectin and 2000 for potato amylopectin (Jarowenko, 1977; Wurzburg, 1986). Cassava starch contains on average 79% amylopectin and a trace quantity of lipids (<1%) (Hoover, 2001). Differences in amelopectin directly affect granule swelling (onset of viscosity), peak temperature, and peak viscosity, shear thinning during pasting, and gel firmness during storage of corn starches (Bahnassey and Breene, 1994; Doublier *et al.*, 1987).

2.2.3 Starch granule structure

Although most authors seem to agree that the production of amylose and amylopectin is under enzymatic control, there are different concepts on how amylose and amylopectin molecules are packed into a granular structure. In 1969, Nikuni described the starch granule as having only one amylopectin macromolecule concentrically packed with only one reducing group at the hilum (as in Fig. 2.2b). It has branched chains which are organized into clusters. The smallest cluster was made of amylopectin side chains and its amorphous lamellae region was calculated to be ~7 X 10 nm in size (Yamaguchi, *et al.*, 1979). Gallant *et al.* (1997) and Duprat *et al.* (1980) refuted Nikuni's concept of representing only one reducing group at the hilum, and only one amylopectin macromolecule for the whole granule to be incorrect. Again the clusters were not concentrically packed in the granules as proposed by Nikuni (1969). French (1984) and Lineback (1986) however, acknowledged the representation of the starch layers as constituted by groups of clusters made of short chains as an ideal key for further models. Gallant *et al.* (1992) therefore proposed that amylopectin packed itself into blocklets level of structures. De-branching studies of starch lend further support to the idea that a blocklets structure exists (Hizukuri, 1986). Using HPLC, Hizukuri (1985) demonstrated that B chains of amylopectin can participate in more than one crystalline amylopectin side chain cluster. He therefore proposed a revised model of amylopectin structure. With agreement and inputs from scientific communities, the revised model is that a granule of starch has a unit of amylopectin molecule which contains the main chain (α-1, 4-linked glucose) and a branched chain (α-1, 4-linked glucose). The branch point between the main chain and a branched chain has α-1, 6 glucose linkages, the narrow space occupied by the branch point is called amorphous lamellae (Robin *et al.*, 1974).

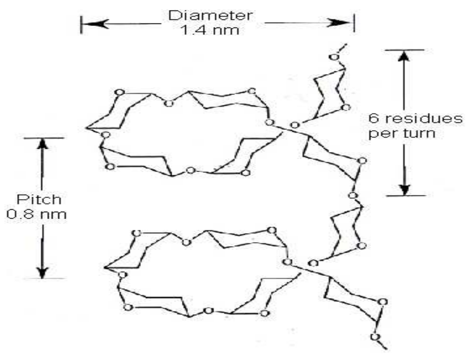
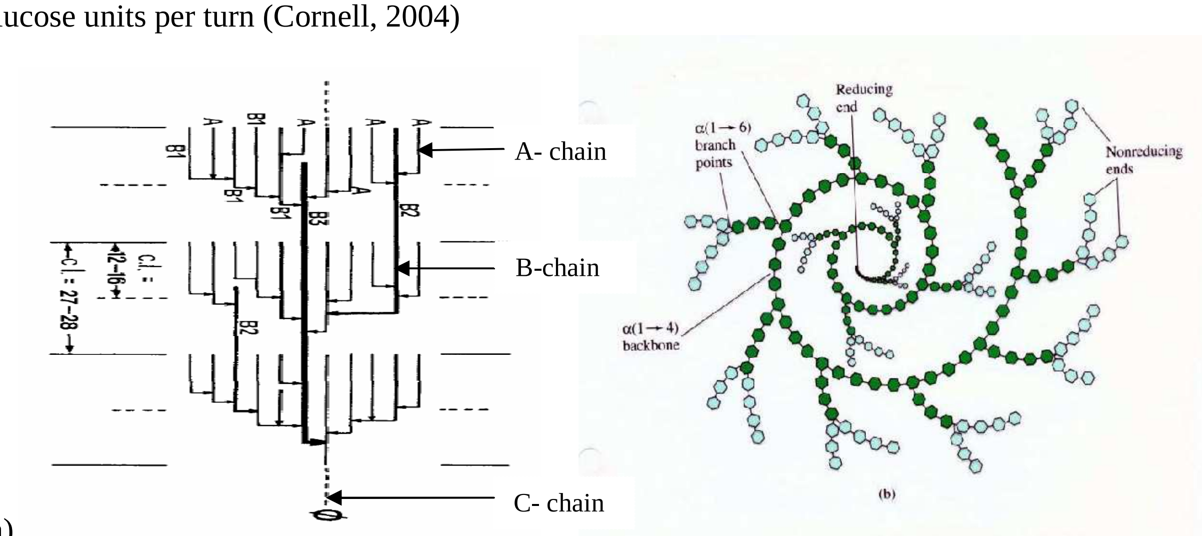


Figure 2.1. Helical conformation of amylose. A left-handed helix containing six anhydrous glucose units per turn (Cornell, 2004)



(a)

Figure 2.2. The cluster structure of amylopectin chains. The solid lines (a) showed the side view chain of α-1,4 linked glucose units with arrows indicating α-1,6 linkage points. The central “c” chain carried only one reducing end (ø). The external unbranched A chains flanked the branched long chains of B1, B2 and B3 that respectively linked cluster 1, 2 and 3 together giving it more strength (Hizukuri, 1986). Diagram (b) shows the upper view of amylopectin chain with one reducing end and non-reducing ends in continuously and concentrically arranged pattern (Nikuni,1969).

The ordered inter-connection between branched chains above and below amorphous lamellae resulted in the formation of side chain clusters (Nikuni, 1969). The branched chains are classified by their location in reference to the central C-chain and the cluster length (CL) they cover. The 'outermost' unbranched terminal A-chain is short and covers only one cluster length. A-chains generally consist of between 13-23 residues (glucose units). There are slightly more A-chains than B-chains. Hizukuri (1986) earlier classified the B chains according to the number of side chain clusters in which they participated. Thus, Bl chains participate in one cluster, B2 and B3 chains extend into two or three clusters, respectively, while B4 chains link four or more clusters (as in Fig. 2.2a). The "middle" B-chain consists of two main types: the long and the short B-chains. The long B chains are chains connecting two or more length of clusters together (about 23-35 residues) to give strength and elasticity to granules (Chiotelli and Meste, 2002). This difference in starch-granule rigidity was probably the cause of varietal difference in cooked rice texture (Reddy et al., 1994). The longer B-chain is characteristic of starches with the B crystal pattern as found in potato whilst the shorter chains are similar in length to the terminal A-chains as found in the A- crystal pattern of cereals and cassava (Hizukuri, 1987).

There is only one "central" C- chain with a single reducing group (Nikuni, 1969) and thus makes amylopectin difficult to break down (good for storage). Within the cluster, the adjacent side-chains inter-coil to form denser double helices, which are packed in a highly ordered manner called crystalline lamellae. The crystalline lamellae alternate with the amorphous lamellae. The repeated structure has a periodicity of 9-10 nm (Oostergetel and van Bruggen, 1989; Jenkins *et al.*, 1993; Smith, 1999). The alternating crystalline lamellae (amylopectin side chain clusters, on average 6 nm length) and amorphous lamellae (amylopectin branching zone, on average 4 nm length) are concentrically arranged within the granule to give semi-crystalline zones several hundreds of nanometers wide. One semicrystalline zone and one amorphous zone (in which the organization of amylopectin is much less ordered) are called a growth ring and the collection of growth rings forms a granule (Smith, 1999).

Gallant *et al.* (1992; 1997) proposed that both the inside and surface of granules are made of tiny egg like shells but their packing differ in both crystalline and amorphous regions. The surface of granules is hard and crystalline because they are made of tightly packed bigger and harder crystalline shells. Beneath the hard surface layer of the crystalline region towards the inside of the granules lies a region of loosely packed smaller and softer semi-crystalline shells. Several of both regions alternate towards the hilum and are concentrically arranged around the hilum. The hilum is at times located off the centre of the granule. The shells are thinner towards the granule surface due to increasing surface areas that are added to by the constant growth.

Each tiny egg-like shell is called a “blocklet” or “building block”. Within the blocklet lies stacks of blocklet lamellae on each other interspersed with narrow amorphous lamellae where the branched points of amylopectin side chains occur with much less organization of the amylopectin chains. In the crystalline lamellae the side-chains of amylopectin form double helixes which interact to form the dense crystalline nature of a cluster. Blanshard (1987) reported that amylose-lipid (and protein) feature in the organization of the amylopectin chains. The crystal structures of the starch polymers therefore indicate a more compact structure of A - type of granules than B - type of granules (Gallant *et al.,* 1992; Imberty *et al.,* 1987; Imberty and Pérez, 1988).

Gallant *et al.* (1997) proposed a third structure involving amorphous radial channels that open at the surface of granules as pores. The pores are located where two or more hard shells meet at the surface. The pores and channels communicate with various soft and hard regions of the granules and the outside of the granules. They are not straight or at right angles to the regions but are believed to be serpentine and radially distributed. Depending on the abundance of these pores and channels, the starch granules can resist or accept acid or enzyme hydrolysis. The surface pores and interior channels are believed to be naturally occurring features of the starch granule structure, with the pores being the external openings of the interior channels (Fannon *et al.,* 1993). Consequently, the radial amorphous channels (in association with blocklet size) play a role in starch resistance to enzyme attack (Fannon *et al.* 1993; Gallant *et al.,* 1997). If a resistant starch (for example amylomaize) has more of these channels lined with porous amorphous layers, it would reduce its resistivity to hydrolysis. On the contrary, if a normal starch (starch with a normal level of amylose) has very few of these, it would close entry to acid and enzymes and therefore, resist their hydrolysis. In conclusion, high crystallinity and high amylopectin levels are not enough to explain resistance of starch and its application in bio-ethanol, food and other industries, but abundance of channels and pores are also major contributing factors. Furthermore, the location of amylose within the granules may influence local crystallinity and resistance.

It is now known that significant enrichment of amylose exists towards the granule surface in many starches, including wheat and potato (Geddes *et al.*, 1965; Morrison and Gadan, 1987), which may be responsible for increased resistance towards the granule surface. This is collaborated by the fact that starch granules of the B crystalline type are progressively exo-eroded during α-amylolysis (or during sprouting of the tuber), without the formation of corrosion channels. This implies that more semi crystalline layers are located at the surface and crystalline layers within the granules, hence exo-erosion of the amorphous portion of the semi-crystalline layer with enzyme. For acid hydrolysis, the corrosion of both crystalline and amorphous lamellae would take place at the same time, leaving less evidence of resistant blocklets when hydrolysis is prolonged. New methods of probing the organisation of polymers within the granule such as using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM), X- ray and neutron scatter have suggested new layers of complexity, including the organisation of amylopectin into blocklets instead of clusters (Gallant *et al.*, 1997), superhelices (Oostergetel and van Bruggen, 1993) and there are differences in structure between the core and the periphery of the granule (Bogracheva *et al.*, 1996; Bulbon *et al*., 1998; Jane and Shen, 1993).

2.3 Granule crystallinity

2.3.1 Granule crystallinity types

Amylopectin is solely responsible for crystallinity of granules (Manners and Rowe, 1969; Nikuni, 1969; Hizukuri, 1986). Based on X- ray diffraction spectroscopy of crystalline shells, cereal starches and also small starch granules of some tropical tubers such as cocoyam exhibits the A - type pattern (monoclinic lattice) (Imberty *et al.*, 1987). Potato starch and certain other tropical tuber starches which are morphologically similar with respect to their granule shapes and sizes, as well as some amylose-rich starch granules such as some tubers, legume, root, banana, stem starch, amylomaize, barley and wrinkled pea have a B-crystalline pattern (hexagonal lattice) (Imberty and Pérez, 1988; Gallant *et al.*, 1992). An extensive review by Tian *et al.*, (1991), indicated that sweet-potato varieties show different crystalline patterns (A and C or mixtures). Starch granules from other tropical tubers, peas, beans and seeds as well as most legume starches possess the C pattern (Gallant *et al.*, 1992).

The internal structure of starch granules from pea embryos are known to contain two different sorts of amylopectin crystallites, A and B (hence the term C - type starch), which reflect two different types of packing of the double helices within clusters (Bogracheva *et al.*, 1996; Bulbon *et al.*, 1998). Whether A or B crystallites are formed is thought to be related to the average chain lengths of chains within amylopectin clusters: A- type crystallites are formed from shorter chains whilst B crystallites are formed from longer chains. Both studies report that the inner part of the pea starch granule is enriched in B and the outer part in A – type crystallites. Thus, longer chain lengths of amylopectin are located in the inner and shorter chains of amylopectin are located in the outer part of the pea starch granule. The implication is that chain lengths of amylopectin in the inner and outer part of the granule are different. This difference could result from the changes during embryo development in relative activities of different isoforms of starch synthase and SBE (Burton *et al.*, 1995) and also from modification of amylopectin within the granule matrix. Although pea starch is unusual in containing both A- and B- type crystallites, there is also evidence from potato of differences in polymer structure and composition between the core and the periphery of the granule (Jane and Shen, 1993). The C_A - type structure is an intermediate C-structure between A- and C - type whilst the C_B - type structure is an intermediate C-structure between the C- and B - type. Sweetpotato starch shows variable X- ray patterns between the C and A (Moorthy, 2002; Hoover, 2001). Finally, due to amylose complexation, a V pattern (often associated with the A, B or C patterns) can appear after gelatinization, although such patterns are also reported to exist in native starches (Gallant *et al.*, 1992).

Both A- (monoclinic) and B-(hexagonal) type crystal lattices possess double helical structures but differ in packing density of the double helices in the unit cell. A 10 nm length of amylopectin side chain of A-type crystalline unit cell has 9 -17 double helical polymer strands whilst B-type crystalline unit cell of the same length has only 11 double helical strands confirming that B-type starch is on average more sparsely packed than A-type starch (Manners, 1989). The B-type has a column of four water molecules in the center whereas there is no space in the A- type for the water molecule. It can be deduced that the crystallinity of B-starch can be modified by increasing or decreasing the water molecules where in A-starches there is little or no room for such manipulations. That implies that B - type crystal lattices can expand more during cooking than A - types. The C - type X- ray pattern is an intermediate structure between A- and B - types (Ong *et al.,* 1994; van Soest *et al.*, 1996). This implies that the A type is the most compact crystalline structure followed by C and B types.

2.3.2 Identification of the crystallinity types of granules

By X- ray diffraction spectroscopy, A - type starches show peaks at 15°, 17°, 18° and 22° 2θ angles while the B - type has four main reflection intensities at 5.5°, 17°, 22° and 24 2θ angles. The B - type X- ray pattern of starch is usually characterized by the position and relative peak intensity in the range of 2θ = 5 – 6°, while the absence of the peak of 2θ = 5 – 6° is characteristic of A - type starch. The C - type X- ray pattern reflects at 5.5°, 17.0°, 18.0°, 20.0° and 23.5° 2θ, which is believed to be a superimposition of the A- and B - type patterns (Zobel *et al.*, 1988a). Cassava starch possesses A, C, or a mixed pattern with three major peaks at 2θ = 15.3º, 17.1º and 23.5º. The native cassava starch exhibits an A - type X- ray pattern, with characteristic peaks at 9.6, 10.9, 14.6, 16.5, 17.6 and 22.6° (2 θ) (Garcia *et al.,* 1996) or with strong peaks at about 15°, 17°, 18°, and 23° (*2θ*) (Zobel, 1988b; Veregin *et al.,* 1986; Defloor *et al.,* 1998a; b). Cassava starch showed a C_A - type crystal structure with a maximum at 16.6° (*2θ*) (Kasemsuwan *et al.,* 1998; Ong *et al.,* 1994).

2.3.3 Variations in crystalline types of starch

Variations in crystalline nature of starches from the same crops have been attributed to variety, sample preparation, growth conditions and maturity of the plant at the time of harvest (Noda *et al.,* 1995; Sugimoto *et al.,* 1987). In a hybridization experiment of the same variety, some progenies of cassava showed B-type instead of the A- or C-type reported for cassava

(Srichuwong *et al.,* 2005). Such differences could most likely be attributed to the amylopectin molecular properties because the crystalline region is an ordered arrangement of double helical amylopectin structures (Tukomane *et al.,* 2007; Rodríguez-Sandoval *et al.,* 2005). Also native maize is a cereal that has A-type crystallinity; however, due to mutation, high amylose starches such as from amylo-maize and barley have B-type crystallinity. Similarly, low amylose starch (waxy maize mutant) showed high A - type crystal structure.

2.3.4 Variation in the degree of crystallinity

The degree of starch granule crystallinity ranges from 15-45% (Zobel, 1988b) or 17-51% (Tang *et al.,* 2006). Nuwamanya *et al.* (2010) reported cassava progenies with consistently lower crystallinity (28.9 - 37.4%) than their respective parents (38.0 - 40.4%). A higher degree of crystallinity results in a higher gelatinization temperature (Defloor *et al.,* 1998b) and higher peak viscosities. Processing may affect the degree of crystallinity and crystal pattern as it was shown that the crystallinity of the milled cassava flour (17.5%) was lower than that of pure starch (>35.0%) (Rodríguez-Sandoval *et al.,* 2005). The thermoforming of high amylose maize and barley starches produces the thermoplastic starch (TPS) that gelatinized to create V - type crystal structures due to retrogradation (Frost *et al.,* 2009).

2.3.5 Application of granular crystallinity in pharmaceutical industries

In the pharmaceutical industry, starch is compressed together with chemicals into tablets. Acid modified starch is sometimes used because the amylose component in the amorphous regions is already removed (Sanguanpong *et al.,* 2003; Chun, 1997). The resulting small-particle dextrin left after partial acid hydrolysis is structurally changed, including size, shape, crystallinity and double helical domains and leads to altered hydration and physicochemical properties of individual granules (Sanguanpong *et al.,* 2003). The relative crystallinity of native and acid-modified tapioca starches are 39.53 and 57.8% respectively (Atichokudomchai *et al.,* 2001). The erosion of the amorphous regions by acid hydrolysis may result in a reduced hindrance for the double helical chains of amylopectin to approach each other. The native and acid-modified tapioca starches were compressed into tablets using various compression forces. Thus, when applying the compaction force to the starch granules, the crystalline regions could be forced to become closely packed, thereby improving the intermolecular forces, i.e., van de Waals forces and hydrogen bonding which leads to a more ordered rearrangement within the starch granules. As the percentage relative crystallinity increases, the crushing strength of the tablet is also increased. There is a strong relationship between percentage relative crystallinity and crushing strength of the tablets, with a correlation factor (r^2) of 0.95–0.99 (Atichokudomchai *et al.*, 2001). This implies acid-modified cassava starch could potentially be used as tablet filler in direct compression processes. It is known that water has a significant effect on molecular properties of starch and the presence or absence of residual water can lead to helical formation of the linear chains and an increase in crystallinity (Bogracheva *et al.*, 2001).

2.3.6 The effect of semi-crystalline regions on bulk crystallinity of granules

If the amylopectin polymer is predominantly responsible for granule crystallinity, why is it that the total amylopectin is more than the degree of crystallinity? The degree of granule crystallinity in normal starch ranges between 15-45% (Zobel, 1988b) or 17-51% (Tang *et al.*, 2006). It is smaller than the total amylopectin composition (~ 75 %) of granules. More than 50% and up to 85% of amylopectin is used in crystallinity, depending on botanical origin. The high level of double helical order in the granule is also significantly higher than the level in granule crystallinity (Gidley and Bociek, 1985). About 80-90% of the total number of chains in an amylopectin molecule is involved in forming the side chain clusters, whilst the remaining 10-20% of chains forms the inter-cluster connections (Manners, 1989). This leads to the conclusion that amylopectin is located both in the crystalline and semi-crystalline shells (Gallant *et al.*, 1997). In the semi-crystalline shells, amylopectin is therefore predominantly in the double helical form but contributes very little to crystallinity, possibly due to increased interaction with amylose. The increased interaction between amylose and amylopectin in these regions causes their decreased crystallinity (Zobel, 1988b; Morrison *et al.*, 1994; Jenkins and Donald, 1995). If such a hypothesis is true, it is possible that the presence of amylose may influence blocklet size, since the crystalline blocklet size is generally found to be smaller in the amorphous shells of the granule (corresponding to the lower degree of crystallinity). These conclusions fit the observation that the semi-crystalline shells of the granule contain smaller blocklets (20-50 nm in diameter), which indicates a less crystalline organization. Baldwin (1995), using AFM, also confirmed that the blocklet sizes were different in the alternate hard and soft layers. In the semi-crystalline shells of wheat starch, the blocklet diameter is small (25 nm), whereas in the hard, crystalline shells the blocklet diameter is larger, 80-120 nm.

2.4 Starch granule morphological classification

The physical granular structure is relevant when considering the mechanism of starch degradation, as many glucan (amylose and amylopectin) metabolizing enzymes are substrate specific and therefore appear to be unable to act upon most intact granules (Buléon *et al.*, 1998).

2.4.1 Shape of starch granules

Based on shape of single granules, A, B and C types can be observed with the aid of light microscopy (LM) and scanning electron microscopy (SEM). Native cassava starch granules were reported to be round or oval with a flat surface on one side containing a conical pit which extends into a well called an eccentric hilum (Moorthy, 1994) or truncated or kettledrum (Moorthy, 1994; Thomas and Atwell, 1999). Normal wheat granules mostly have a lenticular shape called A - type whilst sorghum starch is dominated with the spherical shape called the B - type. The polygonal shape is found in oats and millets, while the irregular shape is found in amylomaize. The C type is a tiny irregular shape found among A and B types in some species.

2.4.2 Size of starch granules

The size of cassava starch granules are classified as small (5-10 μm), medium (10-25 μm) and large (>25 μm) (Lindeboom *et al.*, 2004). The diameter for cassava starch granules ranges from 4-35µm (Onwueme, 1978; Thomas and Atwell, 1999); 5–40 µm (Moorthy, 1994); 4-24 µm (Grace, 1971), 8 - 22 µm with an average of 15 µm (Garcia *et al.*, 1996; Sriroth *et al.*, 1999a). Ceballos *et al.* (2008) reported the first cassava mutant with reduced starch granule size (5.80µm) which is about a third the normal size (10-25µm). The starch granules vary considerably in size, from a diameter of 2 µm in the pollen starch of amylomaize to 175 µm for canna starch (Banks and Greenwood, 1975). "Simple" starch granules are very individual in structure and content (snowflake phenomena) and vary from 2-100 μm in size. A simple granule contains one nucleus. A "compound" starch granule has several nuclei and a rigid matrix structure. A single granule will gelatinise over a range of ~2°C but a sample of complex starch grits or particles will have a 4-10°C gelatinization range. Starches with large granules also display higher swelling power but lower solubility than starches with smaller sized granules (Kaur *et al.,* 2002). Physicochemical properties, such as percentage light transmittance, amylose content, swelling power and water-binding capacity are significantly correlated with the average granule size of starches extracted from different plant sources (Zhou *et al.,* 1998).

Starch granule size is an important factor in several industrial processes including the production of thin films (Lim *et al.*, 1992), paper coatings, cosmetic products (Ellis *et al*., 1998), and carbonless paper (Nachtergaele and Van Nuffel, 1989). Furthermore, starch granule size is important in the brewing process. A significant portion of the small B - type granules from barley is not completely gelatinized in the mash and the undegraded residue causes mechanical problems during subsequent processing, e.g. filtration (Tillett and Bryce, 1993). There are significant additional processing costs required for the isolation of large or small granules. Thus, the *in planta* production of starch with predominantly large or small granules would be very desirable. In addition, small granules may have problems for commercial exploitation related to the difficulties in purification (Davis *et al.,* 2003; Lindeboom *et al.,* 2004).

There is a strong correlation between mealiness, the size of the starch granules and dry matter content. Plants with mealy tubers have large starch granules, while non mealy types have small granules (Safo-Kantanka and Owusu-Nipah, 1992). There are differences in functional properties of small and large starch granules which suggest that the granule size distribution is an important parameter in the food industry. Small granules have higher affinity for water at room temperature. It seems that there is a less ordered arrangement of the polysaccharide chains in the smaller granules compared to the larger ones (Chiotelli and Meste, 2002).

Based on the size distribution of granules, cassava starch is composed of a monomodal population of granules (Garcia *et al.,* 1996) as found in maize and rice or bi-modal distribution of granules as found in wheat, barley and rye. Starch granule size is reduced during dry seasons and increased during the raining season, when starch granules are hydrolyzed along the middle stele root of the storage root into sugar to support the growth of new leaves (Amenorpe, 2002). Cassava granules take about 6 months to grow to the full size and the size remains constant thereafter. The size of starch granules is influenced by storage root size. Starch granules are significantly smaller in small storage root than in larger ones (Moorthy and Ramanujam, 1986). Small starch granules (5-10 μm) gelatinize at a higher temperature than larger ones (Baruch, 1985; Geddes *et al.* 1965).

2.4.3 Starch granules with holes

The rate and efficiency of physical, chemical and biochemical starch modifications depend on the botanical origin, as well as surface structures of granules. The presence of pores or channels penetrating the granule and hilium are natural morphological features of starch granules (Fannon *et al.*, 1992; Huber and BeMiller, 2000) and are not artifacts produced by isolating and drying of starch. Pores are found along the equatorial groove of large granules of wheat, rye, corn, sorghum, millet and barley starches but not on other starches (rice, oat, potato, tapioca, arrowroot and canna) (Fannon *et al*., 1992). However, using very high definition Atomic Force Microscopy (non-contact method), Juszczak *et al.* (2003) observed pores or protrusions both in the case of potato and tapioca starch. It was observed that tapioca granules are smoother than potato granules with regions of relatively flat surface without visible structures, pores and protrusions. It is proposed that the pores affect the pattern of attack by amylases and some chemical reagents (Fannon *et al.*, 1992). The size and shape of surface pores or protrusions or depressions is dependent on the botanical origin of the starch (Juszczak *et al.*, 2003). The presence of pores and channels in starch granules would also have an influence on its chemical reactivity (Huber and BeMiller, 2000). The granules with holes would facilitate hydrolysis and fermentation of starch into bio-ethanol. Pores or holes on granules increased the surface area to volume ratio to the granules and this is suitable for more effective absorption of odour onto its surface and is suitable for inclusion in perfume, deodorant and talcum powder (Peter, 1999).

2.4.4 Protrusions on the surface of starch granules

Potato starch shows a B-crystalline pattern with a range from 200-500 nm larger blocklets and a depth of about 10 microns stacked on top of one another at the granule surfaces. Small protrusions of ca 10-50 nm were also observed at the surfaces of native wheat starch granules. Larger spherical protrusions (20 and 500 nm) were obvious at the surface of native potato starch granules. Taking into account the sizes of these structures, it became evident that ‘blocklets’ and ‘protrusions’ were the same structures (Thompson *et al.,* 1994; Baldwin 1995).

2.5 Bases of starch biosynthesis and mutation in plants

2.5.1 Granule bou*n*d starch synthase (GBSS)

Granule bound starch synthase I (GBSSI) belongs to the class of starch synthases (SS). The class is sub-divided into three groups based on their location in the plastids: those bound exclusively to the granule (Fig.2.3); those with exclusive or nearly exclusive activity in the soluble phase and ones present in both the granule-bound and soluble phase. The fractions are further classified into four subclasses based upon cDNA and amino acid sequences, i.e. GBSS (60 kDa), SSI (57 kDa), SSII (77 kDa) and SSIII (110-140 kDa) (Harn *et al.,* 1998). The GBSS I was regarded as the sole enzyme in amylose synthesis at first (Leloir *et al.,* 1961; Visser *et al.*, 1991) but later, gramineae waxy mutants were shown to accumulate normal starch granules in tissues such as pericarp, leaf, stem and root indicating that other gene(s) control amylose production. An isoform of GBSSI, designated GBSSII from waxy wheat was later discovered (Nakamura *et al.*, 1998), while in pea leaves it was designated GBSSIb (Edwards *et al.,* 2002). Indeed, both isoforms were demonstrated to be involved in the synthesis of amylose. In cassava the preferential expression of GBSSI is in storage roots whereas that for GBSSII is in source leaves (Munyikwa *et al.,* 1997). The expression pattern was very much dependent on the age of the plants. In 180 days after planting (DAP), GBSSI expression was high in leaves and almost undetectable in storage roots and in 360 DAP, GBSSII expression was low in all organs examined, including leaves (Baguma *et al.*, 2003).

2.5.2 The synthesis of amylopectin by the soluble starch synthases (SS)

The SSIII contributes to amylopectin branch-length distribution (Edwards *et al.,* 1999) but the specific role of SSI and SSIIb is not yet clear (Fig. 2.3). An antisense inhibition of SSII and SSIII singly (Edwards *et al.,* 1999; Lloyd *et al.*, 1999) or in combination (Lloyd *et al.*, 1999) resulted in a significant shift from longer to shorter chains of amylopectin in potato tuber. The loss of SSII (in dicots) or SSIIa (in monocots) would result in reduced starch content, reduced amylopectin chain length distribution, deformation of the starch granules, altered physicochemical properties of starch and perturbed crystallisation (Edwards *et al.,* 1999).

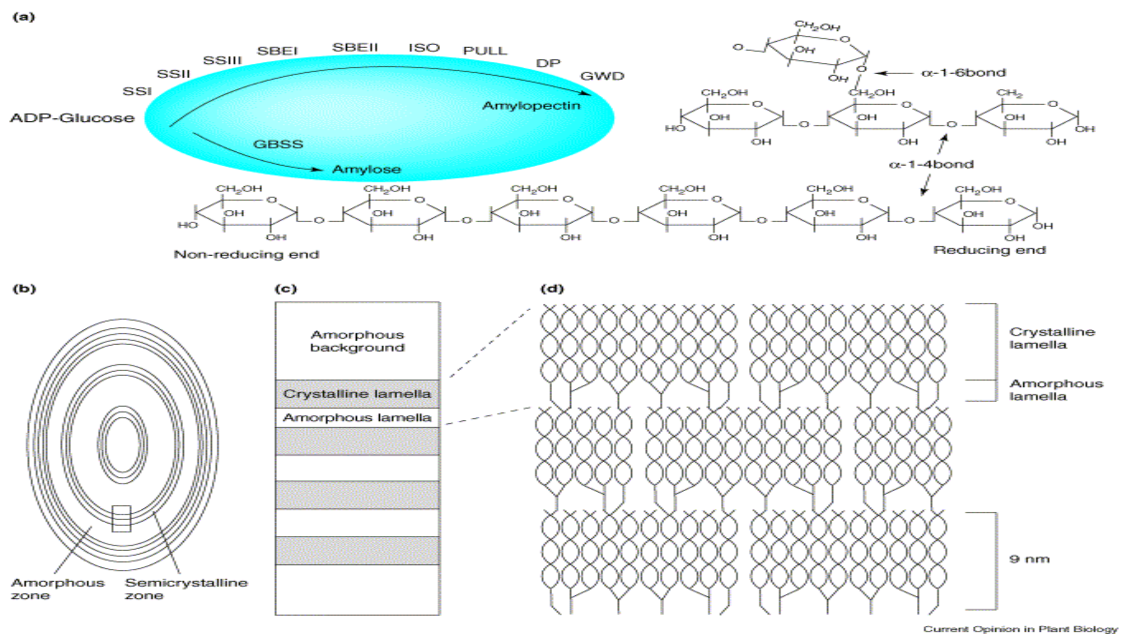
2.5.3 The role of starch branching enzymes (SBE or Q-enzyme)

Starch branching enzymes (SBE, 40 – 152 kDa) are involved in amylopectin synthesis (Fig. 2.3). They catalyse the breakdown of α-1,4 bonds and building of α-1,6 glucosidic bonds between the cleaved chain and a hydroxyl group on C6 of a glucosyl moiety of an α-1,4 glucan template. They are a member of the α-amylase family which possesses a catalytic $(\beta/\alpha)_8$-barrel domain (Jespersen *et al.,* 1993; Svenson, 1994). The domain encompasses specific active sites that arise from the inter-connecting β-loops providing for substrate binding and catalytic activity. To date, two classes [referred to as A (SBEII) and B (SBEI)] have been identified based on amino acid sequences and *in vitro* catalytic properties of purified enzymes (Burton *et al.,* 1995).

2.5.4 The concept of amylopectin synthesis from amylose

The hydrolysis of sucrose transported from leaves and stems into glucose-1-phosphate is catalyzed by sucrose synthase and uridine diphosphate (UDP) glucose pyrophosphorylase. Adenosine diphosphate (ADP) glucose pyrophosphorylase catalyses the reaction between glucose-1-phosphate and adenosine triphosphate (ATP) to form ADP-glucose (the first step in starch formation). ADP-glucose is transported into amyloplasts in the roots by an adenylate transport protein and used as substrate of starch biosynthesis. In the root, granule-bound starch synthase (GBSS) as well as isoenzymes of SSS catalyse the removal of glucose from ADP-glucose. The glucose units are linked together by α-1, 4 glycosidic bonds to form an initial linear amylose chain. The GBSS elongate amylose chains by adding more glucose to non-reducing ends of the α-1-4-linkage chains, thus coupling an extra glucose residue to this chain with the simultaneous release of ADP (Martin and Smith, 1995; Preiss and Sivak, 1996). Nine branching enzymes located at the surface of the starch granule (Fig. 2.3) transform amylose into amylopectin by regularly cleaving α-1,4 glycosidic bonds of amylose into amylose fragments and linking the amylose fragments by α-1,6 glycosidic bonds. Amylose and amylopectin chains are elongated by starch synthases (Jobling, 2004). When the production and elongation of amylose is more than the conversion into amylopectin, excess amylose accumulate in granules resulting in lenthel (non-waxy phenotype) starch. This occurs when GBSS is highly active and its product (amylose) cannot be entirely transformed into amylopectin by the starch branching enzymes.

In waxy mutants, under the mutation inhibition to the activity of GBSS, all the amylose synthesized by SSS iso-enzymes of low activity is transformed into amylopectin by starch branching enzymes (SBE). There is then no excess amylose, thus only amylopectin is accumulated in the roots and the root phenotype is waxy. Waxy genes are recessive and they are designated as *wx,* because they are allelic on cytogenetic level (James *et al.*, 2003; Tsai 1974). Therefore, mutation of the waxy locus *wx* (Shure *et al.*, 1983) which encodes the GBSS protein creates a starch that has no amylose. Contrarily, the high-amylose phenotype is caused by a mutation in the gene that encodes SBE IIb, which is also known as 'amylose extender (ae), and their production artificially requires anti-sensing or down regulation of its expression in storage roots (Jobling *et al.*, 1999).



Source: Jobling (2004).

Figure 2.3. Starch biosynthetic enzymes and granule structure. (a) Schematic view of enzymes involved in the biosynthesis of amylopectin and amylose, together with the molecular structure of these glucans. The GBSS is shown within the granule as it is the only enzyme that is exclusively bound to the starch granule and is responsible for the synthesis of amylose and its extension. Amylopectin biosynthesis occurs at the surface of the granule through the activities of the indicated enzymes. (b) Schematic view of the structure of a starch granule showing alternating amorphous and semi-crystalline zones. Pairs of these zones constitute a growth ring. The amorphous zones are thought to contain amylopectin in a less-ordered state together with amylose. The granule is approximately 50 µm in length. (c) Enlargement of a semi-crystalline growth ring, showing the arrangement of the alternating crystalline and amorphous lamellae. (d) Each crystalline lamella consists of clusters of parallel α1-4-linked glucan chains packed together in a helical conformation. Amorphous lamellae are located at the α-1-6-branch points. Disproportionating enzyme (DE), Isoamylase (ISO) and pullulanase (PULL) also play a part (Jobling, 2004).

2.5.5 The synthesis of amylose from amylopectin concept

Another school of thought claims that amylopectin is rather synthesized first and amylose later from fragments of amylopectin. Monille *et al*. (1996) reported a debranching enzyme that hydrolyzes specifically α-1,6 glycosidic bonds of amylopectin and transforms amylopectin into amylose. Defects in GBSSI strongly reduce amylopectin synthesis (Maddelein *et al.* 1994). One or two functional GBSSI genes in wheat produce waxy wheat with intermediate levels of amylose. There are waxy starches reported in maize and rice that are composed almost entirely of amylopectin with little or no amylose (Park *et al.*, 2009). The presence of waxy mutants with a full granule structure containing amylopectin with wild - type crystalline organization (Buléon *et* *al.*, 1997) confirms the fact that amylose is not required for the biogenesis of normal granule structures. *In vitro* studies of amylose synthesis (Ponstein *et al.*, 1991; Denyer *et al.*, 1996), establish that GBSSI incorporates glucose both in amylopectin and amylose. Van de Wal *et al.* (1998) studied amylose synthesis *in vitro* from purified *Chlamydomonas reinhardtii* starch granules and reported that initially the major GBSSI extends glucans available on amylopectin. Amylose synthesis occurs thereafter at rates approaching or exceeding those of net polysaccharide synthesis. These results suggested that amylose originates from cleavage of a pre-existing external amylopectin chain, and such transfer of chains from amylopectin to amylose was directly evidenced from pulse-chase experiments. Moreover, high molecular mass branched amylose synthesis preceded that of the low molecular mass, suggesting that chain termination occurs consequently to glucan cleavage. Short pulses of synthesis followed by incubation in buffer with or without ADP-Glc prove that transfer requires the presence of the glucosyl-nucleotide. These observations make a compelling case for amylopectin acting as the *in vivo* primer for amylose synthesis. These further prove that extension is followed by cleavage.

2.5.6 The anti-sense gene orientation breeding

The use of antisense genes could partially block starch synthesis and result in accumulation of the precursor sugars. Raemakers *et al.* (2003) cloned the GBSS gene from cassava in the antisense orientation, and were able to recover 50 transgenic plants. Subsequently, two of these plants were able to produce amylose-free starch (known as waxy starch) in their storage roots. Munyikwa *et al.* (1998) reported the cloning of cassava c-DNAs encoding AGPase, GBSS and branching enzyme (BEI and BEII), involved in the formation of branched molecules in amylopectin. Over accumulation of glycogen was achieved by transforming cells with a plasmid containing the glycogen biosynthesis genes (glg*C*) encoding AGPase, which is the rate-limiting step in cassava starch biosynthesis. Researchers at Ohio State University incorporated a modified version of the glgC gene from *E. coli* into cassava and succeeded in increasing AGPase activity by over 65% compared to controls (Ihemere *et al.,* 2003).

According to Salehuzzaman *et al.* (1993), sequencing of the cloned cDNA showed that it has 74% similarity with potato GBSS and 60-72% similarity with GBSS from other plant species.

The cDNA encodes a 608 amino acid protein of which 78 amino acids form a chloroplast/amyloplast transit peptide of 8.37 kDa. The mature protein has a predicted molecular mass of 58.61 kDa (530 amino acids). Comparison of the GBSS proteins of various plant species and glycogen synthase of bacteria showed extensive identity among the mature form of plant GBSS proteins, in which the monocots and dicots form two separate branches in the evolutionary tree. From analysis of the genomic DNA of allotetraploid cassava, it is shown that GBSS is a low-copy-number gene. GBSS transcript is synthesized in a number of different organs, but most abundantly in tubers. Potato plants were transformed with the cassava GBSS cDNA in antisense orientation fused between the potato GBSS promoter and the nopaline synthase terminator. The expression of the endogenous GBSS gene in these transgenic potato plants was partially or completely inhibited. Complete inhibition of GBSS activity by the cassava antisense gene resulted in the absence of GBSS protein and amylose giving rise to almost complete amylose-free potato starch. This shows that heterologous genes can be used to achieve antisense effects in other plant species.

2.5.7 Comparison of normal starch with novel (high and low amylose) starches

The differences in characteristics between waxy starch and normal starches are: waxy starches have short-chain amylopectin (A or B1 - types), no or very little amylose, higher gelatinization temperature, resist water absorption, resist expansion during cooking (stable granules), forms a stable clearer paste that slowly turns into a clear gel that resists syneresis (separation of liquid from the gel) whereas normal starches have long-chain amylopectin (B2-B4), normal content of amylose, lower gelatinization temperature, absorb more water than waxy starch, expand more during cooking (less stable granules), forms a less stable cloudy paste that turns turbid and easily undergoes syneresis (Jobling, 2004). The application of waxy starch cuts across most industries while normal starch is mostly limited to food industries.

In contrast, the high-amylose potato starch has a reduced swelling capacity, higher gelatinisation temperature and impacts the potato tubers with a ‘succulent’ texture with more free water (Jobling, 2004). In the food industry, the high gelling strength of these starches makes them especially useful for producing sweets. The film-forming ability of these starches keeps the coating on fried products crispy and reduces their fat uptake upon cooking. High-amylose starches can be processed into ‘resistant starch’, which has nutritional benefits (Bird *et al.*, 2000). Unlike normal starch, resistant starch is not digested in the small intestine but is fermented in the large intestine by gut bacteria, producing short-chain fatty acids such as butyrate that are beneficial for colon health. High-amylose starches are also used in adhesive products and in the production of corrugated board and paper. Very high resistance starches (above 60% amylose) do not swell when heated to 100°C (Schwall *et al.*, 2000). These potatoes soften during cooking, indicating that the swelling pressure generated by the intracellular starch granules has no role in this process, and the texture of the resulting potato is ‘succulent’ as they contain more free water than normal potatoes (Ormerod *et al.*, 2002).

2.5.8 Cracking in starch granules

Katayama *et al.* (2002) reported cracking of granules in a novel sweet potato (Kanto 116) and as a result it had pasting temperatures of 51.4 – 52.6°C, approximately 20°C lower than the control and parent starches. Onset, peak, and conclusion temperatures were 39.0°C, 46.9°C, 64.8°C respectively and energy of gelatinization (8.8 J/g) was much lower than the control and parent starches. However, starch and amylose contents of Kanto 116 were similar to that found in the control and the parents (Katayama *et al.*, 2002). It is widely accepted that soluble starch synthase and starch branching enzyme are responsible for amylopectin synthesis and two or more distinct isoforms of these enzymes exist in storage organs (Smith *et al.*, 1997). Studies of peas and potatoes showed that the loss or reduction of major isoforms of soluble starch synthase have resulted in changes in the amylopectin structure. Thus, Kanto 116 may lack these specific isoforms. The cracked granules were controlled by recessive genes since both parents showed normal granules (Katayama *et al.*, 2002).

2.5.9. Enzymatic basis of free-sugar cassava mutant

The ordered arrangement of side chains and the chain lengths of amylopectin render amylopectin both crystalline and insoluble (Imberty *et al.*, 1991; Gallant *et al.*, 1997). The soluble free-sugar clones are produced when enzymes responsible for amylopectin packing into organized crystalline structure are anti-sensed to produce distorted crystallinity which is soluble sugar. An α-(1-6) glucan hydrolases, i.e. a starch DBE, is necessary for the normal formation of crystalline starch granules. There are two types of DBE in plants, classified according to substrate specificity (Lee and Whelan, 1971; Doehlert and Knutson, 1991). Isoamylase - type DBEs cleave α-(1-6) branch linkages in amylopectin and glycogen into smaller units of α-(1-6)-linked maltotriose polymers, but do not hydrolyze α-1-4 bonds in pullulan (maltotriose). In contrast, pullulanase - type DBEs, also referred to as R-enzymes or limit-dextrinases (Manners, 1997), hydrolyze α-(1-6) linkages of pullulan (α-1,4- and α-1,6-glucan) into a smaller amylopectin, but have little or no activity toward glycogen (the storage polysaccharide found in zoological muscle). Sequence comparisons among plant and bacterial α-(1-6) glucan hydrolases indicate that the pullulanase - type and isoamylase - type DBEs have been conserved separately in evolution (Beatty *et al.*, 1999).

The mutation in DBE which results in the accumulation of soluble sugars and a water-soluble polysaccharide (WSP) is termed phytoglycogen (Morris and Morris, 1939). Phytoglycogen is similar to glycogen and it has approximately twice the frequency of branch linkages as amylopectin, a shorter average chain length, and a more uniform chain length distribution (Yun and Matheson, 1993). In *su1* kernels, the reduction in amylopectin content approximately matches the abundance of phytoglycogen, suggesting a diversion from biosynthesis of the normal insoluble branched glucan to the water soluble form. In maize and Arabidopsis the mutation responsible for this specific phenotype has been shown to directly reside in a gene that code for an isoamylase - type DBE (James *et al.*, 1995; Zeeman *et al.*, 1998). In cassava the natural free-sugar mutant (CAS36) was described by Carvalho *et al*. (2004) as lacking amylose and accumulating over 100 times more free sugar (mainly glucose) than commercial varieties. The natural free-sugar mutant cassava is amylose free whilst the cassava germplasm collection of Brazil from which it was discovered had a range of 18% - 25% amylose (Sriroth *et al.*, 1999b). The presence of a glycogen-like starch indicated a disruption in the starch synthesis pathway. Gene expression analysis of the gene coding for the major enzymes in this pathway indicates a lack of transcription of the gene encoding the branching enzyme, but not for the gene encoding the ADPG-pyrophosphorylase small subunit. The cultivation was abandoned because of its extreme low starch (2%) content (de Albuquerque, 1969) compared with 6.9 – 69.3% in the traditional cultivars. It accumulates additional sugars such as glucuronic acid, galacturonic acid and mannose. These sugars are components of the cross-linking glucan of the primary cell walls of the parenchyma cells (Reiter *et al.,* 1999). Therefore, their presence in the water-soluble fraction in only clone CAS36.1 indicates the degradation of the parenchyma cell walls during the process of their adjustment for the accumulation of high amounts of free glucose.

2.5.10 Enzymatic basis of small granule mutants

Ceballos *et al.* (2008) reported the first cassava mutant with reduced starch granule size (average 5.80 µm) which is about a third the normal size (10-25 µm), with a higher-than-normal amylose content (30.1% instead of 20.7-22.2%). It also had low paste clarity (cloudy instead of transparent gel), low peak viscosity (22 cP instead of 976 – 1080 cP; vPeak in 5% starch solution at 90°C using RVA), low swelling index (9.25 $g.g^{-1}$ instead of 21.77 – 27.92 $g.g^{-1}$) but higher solubility (36.8% db instead of 10.15-12.2% db) and volume fraction of the dispersed phase.

Small granules were caused by a lack of (Ceballos *et al.,* 2008) or a decrease in (Burton *et al.,* 2002; Bustos *et al.,* 2004) isoamylase activity which plays a role in amylopectin biosynthesis. The lack of ISA is always accompanied by an increase in the number of granules initiated, resulting in a decrease in starch granule size of cassava and other crops. As in other species, there are likely to be three types of the ISA genes in cassava: ISA1, ISA2, and ISA3. Of these, ISA1 and ISA2 are thought to be required for normal starch synthesis in several plant species, including potato (Bustos *et al.,* 2004), whereas ISA3 is thought to be involved in starch degradation (Wattebled *et al.,* 2005). Mutants with small starch granules lack either ISA1 or ISA2 (Bustos *et al.,* 2004; Dinges *et al.,* 2001). It was noted that a lack of ISA in mutants is linked to a low starch content (e.g., maize sugary1 mutants) but not always in potato ISA transgenics. However it leads to an increase in soluble starch called phytoglycogen or water soluble polysaccharide contents.

In addition to ISA, disproportionating enzymes (DE), pullulanase and an α-glucan water dikinase related to amylopectin biosynthesis (Jobling *et al.,* 2004) are also potential site(s) of mutation. Small deformed granules have also been observed in mutants lacking SS (Morell *et al.,* 2003) or

SBE (Lloyd *et al.,* 1996). High-amylose content is characteristic of mutants lacking SBE or one of the isoforms of SS that are responsible for amylopectin synthesis. Contrary to expectation, the small granule mutant of cassava does not lack SBE, which is one of the common causes of a high-amylose phenotype in mutants of other species (Ceballos *et al.*, 2008). A penalty in root yield was reported in high-amylose varieties from other crops (Jobling *et al.,* 2004).

2.6.1 Gelatinization

Starch gelatinization is a process that involves heating starch in excess water to break down the hydrogen bonds holding the macro-molecules together in the granules and allowing free flow of linear amylose out of granules followed by eventual collapse of the empty granular structure mostly made up of amylopectin. The penetration of water into the semi-crystalline granules increases randomness in the general structure and heat causes the semi-crystalline regions to diffuse into the amorphous form and to allow water to enter the crystalline lamellae. The helical structure of amylose is also converted into a linear form that could easily leach out and form a viscous paste outside the granules (Tester and Karkalas, 2004). As more amylose is leaching out into the hot water, the viscosity outside the granules increases until the peak viscosity is reached (Hoover, 2001). At peak viscosity most of the linear chains of the amylose are still hanging onto their respective granules. The viscosity rapidly decreases when most of the hanging chains of amylose drop off from their respective granules and the extra energy supplied by heating after peak viscosity, begins to destroy the hydrogen bonds between the bound water molecules and the starch hydroxyl groups. On average the granules could swell up to 30 times their original size (swelling power). When the crystalline structure of the amylopectin keeping the ghost shape of the granule also breaks down and dissolves, gelatinization is completed. The viscosity is then reduced and the rheology-texture of the solution changes (McGrane *et al.,* 2004).

During cooling of a hot paste starch the viscosity increases (set back viscosity) due to re-associate of amylose chains into a coiled state and after several hours very stable amylose semi-crystallites are formed. The re-crystallization of amylopectin side chains into a more ordered semi-crystalline state, also referred to as retrogradation, is much slower but the original crystal stage is not achieved (Atwell *et al.*, 1988). In the absence of emulsifiers, amylose might also co-crystallize with amylopectin, hence contributing to starch retrogradation (Gudmundsson and Eliasson, 1990). Retrogradation of starch is an important property to be considered, especially when formulating food products, as it affects quality, acceptability and shelf-life of starch containing foods because during retrogradation precipitation, gelation, and changes in consistency and opacity occur (Biliaderis and Tonogai, 1991). Research reports show that native cassava starch granules loose their granular structure when heated in excess water at around 85°C (Fig. 2.7) to form a gel, and this loss is irreversible (Paes *et al.*, 2008). This is different from starch granules absorbing cold water and swelling to a limited extent below the gelatinization temperature (GT), which is reversible (Wurzburg, 1986). Retrogradation therefore refers to the reverse process of crystal formation upon cooling and those molecules which could not strongly re-associate to form solid crystals sets into a semi- solid phase called a gel (Vaclavik and Christian, 2008). Gelation therefore refers to a more ordered structure than in the hot fluid phase of the paste (Thomas and Atwell, 1999).

It was shown that potato amylose leaches at 62°C which fits the experimental molecular weight distribution (MWD) (Banks and Greenwood, 1968). However pea amylose fractions leach at five different temperatures between 62 and 85°C, which suggests a larger than normal MWD (Ring *et al.*, 1985). Different stages in the gelatinization of cassava starch were visualized by light microscopy (Fig. 2.4) while the starch suspension was heated at 4°C/min from 25 to 95°C in a hot-stage device (HSM). The granules showed different stages of expansion during heating. On average the expansion started around 60°C, was completely swollen around 70°C and gelatinization was completed between 80 and 85°C. For temperatures higher than 80°C, granules started to lose surface integrity and completely collapsed before 95°C was reached (Paes *et al.*, 2008). Atkin *et al.* (1998) defined the starch granule surface to be the outermost layer of the granule that surrounds the majority of the internal polymers; and the starch granule ghost to be the remnants of the envelope after structural collapse and leaching of the majority of the starch polymers, mainly (amylose).

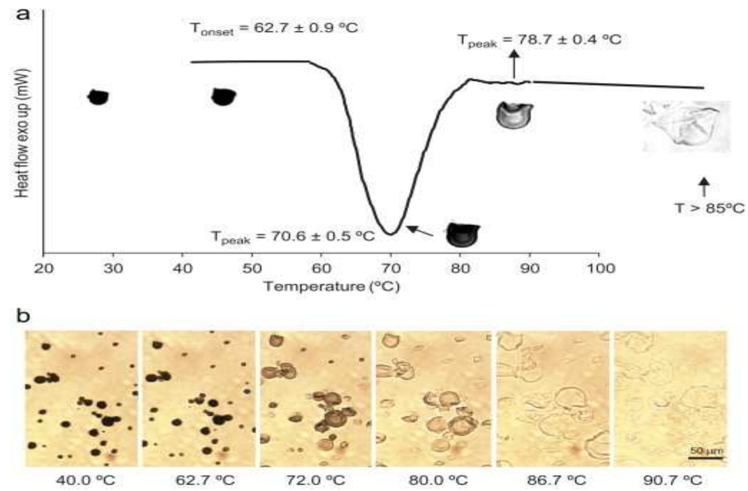


Fig. 2.4 Cassava starch granules lost its granular structure when heated in excess water around 85°C to form a gel (Paes *et al*, 2008).

The gelatenization is a phase transition that can be observed using Differential Scanning Calorimetry (DSC), and it is manifested as an endothermic peak in the DSC thermogram (Coral *et al.*, 2009). The characteristic temperatures of gelatinization are the onset (T_o), peak (T_p), conclusion (T_c) or and endset (T_e) gelatinization temperatures as measured by the Differential Scanning Calorimeter (DSC). Starches with higher levels of short chains had lower T_o, T_p and T_c or T_e temperatures, whereas higher contents of longer chains (DP 18–25 and DP 25–80) led to higher gelatinisation temperatures. Degree of polymerization (DP) is the total number of anhydroglucose residues present divided by the number of reducing ends). Peroni *et al.* (2006) reported cassava and sweetpotato starches having lower T_o temperatures of 61.5 and 62.8°C with corresponding enthalpy changes of 10.4 and 12.9 Jg^{-1} respectively.

On an industrial scale, energy input for gelatinization is a significant part of processing cost (Ellis *et al.*, 1998). This determines the temperature at which a starch paste takes on the desired processing qualities. Gelatinization for native cassava starch was shown to be between 58 and 70°C (Balagopalan, *et al.*, 1988) or 55°C and 85°C (Belitz *et al.*, 2004). Gelatinization temperature depends also on the amount of damaged starch granules. These will swell faster. Damaged starch can be produced, for example, during a milling process or when drying the starch cake in the starch plant (Cauvain and Young, 2001). Various methods of modifying starch, such as cross-linking or the addition of surfactants, such as potassium palmitate, which prevent the penetration of water into the starch granules, increase the gelatinization temperature (Moorthy, 1985). These modifications stabilize the viscosity of the paste, making it more resistant to damage by acids, heat, or shear forces, and thus enhancing the performance of starch during processing. A lower gelatinization temperature range is associated with reduced cooking quality (Balagopalan, *et al.*, 1988).

The gelatinization temperature during cooking of food depend on botanical origin of starch or flour, amount of water, pH, types and concentration of salt, sugar, fat and protein in the recipe and the heating technology used. Gelatinization temperatures of starch also depend on starch/water ratio as well as heating rate (Calzetta-Resio *et al.,* 2000). Although a starch: water ratio of 1:3 is used to reach the maximum value of enthalpy of gelatinization, excess water is essential for complete gelatinization of the starches (Resio and Suarez, 2001). Gelatinization temperature provides a qualitative measure of crystalline structure and is characteristic of genotype of the plant. Gelatinization temperature may also be influenced by environmental conditions. It is known to vary with amylose/amylopectin ratio, crystallinity, granule size, shape and distribution, and quantity of smaller components like phosphorus, lipids and proteins (Singh *et al.,* 2010; Goñi *et al.,* 2008; Pérez *et al.,* 2005). Gelatinisation enthalpies (ΔH) increased with degree of crystallinity (Gomand *et al.,* 2010).

Shorts chains of amylopectin are mostly found in the crystalline structure located on the external part of starch granules (Gidley and Bulpin, 1987). The presence of short chains with DP < 10, decreases the stability of the double helix in amylopectin molecules, which lowers the gelatinization temperature as well as enthalpy. A negative correlation exists between the amount of extremely short chains of amylopectin and gelatinization temperature. Conversely, the work of Sang-Ho and Jay-Lin (2002) on wheat and Albert *et al.* (2005) on cassava revealed the importance of long chains of amylopectin on pasting properties. They suggested that a higher proportion of longer chains (DP > 67) could be associated with amylose chain lengths forming longer helical structures and giving high gelatinization gel properties. Yamin *et al.* (1999) suggested that these double helical structures require a higher temperature to dissociate than shorter double helices. In agreement with this, Ral *et al.* (2008) reported negative correlations between amylopectin long chain with viscosity but failed to establish higher convincing correlations between amylose and viscosity. This is in agreement with Batey *et al*. (1997) who observed that when the amylose level is over 22%, the flour quality for viscous products decreased.

2.6.2 Viscosity

Viscosity is temperature dependent, decreases by heating and increases when the paste is cooled. Oladebeye *et al*. (2009) found that sweetpotato starch paste exhibited higher viscosity values than red cocoyam. Yuan *et al*. (2007) compared viscosity of starches from yam (*Dioscorea nipponica* Makino), cassava and potato using a Brabender viscograph. They observed lower peak viscosity, higher setback and lower breakdown viscosity for yam starch compared to potato and cassava starch. Differences in chemical composition account for the variations in paste viscosity of different starches. It is increased by esterification or etherification. High phosphate monoester content is known to increase paste viscosity while an increase in phospholipids results in lower paste viscosity (Singh *et al.,* 2003).

The common methods for measuring the pasting characteristics of starch are based on Brabender Visco-Amylograph (BVA), Rapid Visco-Analyser (RVA) and Brookfield digital viscometer instruments. The RVA and BVA measures real time changes in the viscosity of starch suspension with temperature and the results are plotted in an amylograph.

The root starches (e.g. cassava) contain very low amounts of phosphorous and lipid compounds which are covalently bound (Thomas and Atwell, 1999). Unlike cassava, potato contains an appreciable amount of bound phosphate ester groups which are bound to the C-6 position of glucose units of amylopectin molecules in potato starch. The number of phosphate groups in potato starch ranges from 1 phosphate group per 200 to 400 glucose units (Swinkels, 1985; Takahata *et al*., 1995). The phosphate group can be considered an ion-exchanging group (Swinkels, 1985). The phosphate substituent confers a polyelectrolyte property on the starch and the charged groups repel each other, forcing the molecules to disperse into aqueous solutions. Generally, potato starch shows a higher paste viscosity than other starches when compared at similar conditions. A higher phosphate content in potato starch results in a higher viscosity. The root and waxy starches also tend to have a higher paste viscosity (Swinkels, 1985). The larger proportion of amylopectin long-branch chains have been reported to increase viscosity of starch paste (Lu *et al.,* 2008; Charles *et al.,* 2005; Shibanuma *et al.,* 1996).

Peak viscosity and breakdown of starch increased with increasing tuber size (Christensen and Madsen, 1996). Liu *et al.*, (2003) also found that starch obtained from potatoes with a shorter growth time had higher gelatinization and pasting temperatures, lower peak viscosity and higher final viscosity (Christensen and Madsen, 1996).). Wickramasinghe *et al*. (2009) reported a positive relationship of peak viscosity and median particle size of the starch granule and a negative correlation with small granules. However, Tian *et al*. (1991) reported only a positively correlation with granule diameter.

According to Shuey and Tipples (1982) the viscosity at 95°C in relation to the peak viscosity reflects the extent of fragility of the swollen granules. In most cases, correlation between cooking quality scores and points on the amylograph were not statistically significant, suggesting that it is difficult to predict cooking quality using the amylograph, confirming previous reports (Asaoka *et al.,* 1992). The increase in viscosity when the paste is cooled to 50°C shows the extent of retrogradation of the starch paste. In all varieties, salt reduced retrogradation as compared to the control. Retrogradation leads to increased rigidity of the starch gel as a result of reassociation of the starch granules upon cooling, which leads to release of water called syneresis. Low amylose granules yields lower paste viscosity and they gel faster upon heating than granules with higher amylose content. Pasting temperature was negatively correlated to swelling power ($r = -0.518$, $p \leq 0.05$), peak and final viscosity ($r = -0.616$, -0.668, and -0.611, respectively, $p \leq 0.005$).

2.7 Starch modification

Starches in their native form have limited application in industries due to extreme conditions like pH, temperature and shear during processing. Native starches are modified to improve on their low shear stress resistance, thermal decomposition, high retrogradation, and syneresis (Fleche, 1985). The limited application of native cassava starches in industries can be enhanced through physical, and chemical modification or mutation breeding. Modification changes starch properties and greatly extends the range of starch applications beyond the native starches (Jobling, 2004).

2.7.1 Physical modification

There are several types of physical modifications: pregelatinization, heat moisture treatment and irradiation treatments. Pregelatinized starch is starch that has been gelatinized and then dried. Pregelatinization methods include roll drying, extrusion and spray drying. Pregelatinized starches are used as thickeners in foods that require minimal heating. Two types of heat-moisture treatments exist, i.e. heat-moisture and annealing. Heat-moisture treatment involves heating starch at temperatures above its gelatinization temperatures but with insufficient moisture levels (<35%) to cause gelatinization, while annealing involves heating starch in excess water (>60%) for prolonged periods below gelatinization temperatures (Vaclavik and Christian, 2008; Thomas and Atwell, 1999).

Both heat-moisture treatment and annealing have been shown to change functional properties of native starches. Heat-moisture treatment of potato and corn starches decreased granular crystallinity of potato and corn starch and increased enthalpy and onset temperature of the gelatinization but no significant differences in degree of retrogradation were observed (Lim *et al.*, 2001). Heat-moisture treatment of sago starch result in a change of the X- ray diffraction pattern from C - type to A - type, a decrease of rapid visco-analyzer peak and breakdown viscosity and an increase in final viscosity pasting temperature, T_o, T_p and T_c of gelatinization (Pukkahuta and Varavinit, 2007). Singh *et al*. (2005) also found a decrease in peak viscosity and an increase in cold paste viscosity following heat-moisture treatment of sweetpotato starch. However, there was a decrease in onset and gelatinization enthalpy. This indicates that different starches may respond differently to physical modification. Jayakody *et al*. (2009) found that starches from different cultivars of yam (*Dioscorea* spp) responded differently towards annealing. Annealing caused a decrease in crystallinity and increase in set-back viscosity in the Nattala cultivar but an increase in crystallinity and a decrease in setback viscosity in the Raja-ala cultivar. These differences were shown to have been influenced by the different chemical composition and molecular structure of the starches.

In irradiation treatment, there are *in situ* and *ex situ* starch modifications. The *in situ* modification is a genetic modification or breeding approach in which the planting material is irradiated with the physical mutagen to induce genotype(s) with an *in planta* modified useful starch for selection and the desirable plant is propagated thereafter. In the *ex situ* starch modifications, the native starch could be bought from the market and irradiated at various doses and the dose rate producing desirable characteristic is used commercially. The application of ionizing radiation (gamma and electron beam) is reported to generate free radicals that are capable of inducing molecular changes and fragmentation of starch (Ciésla *et al.,* 1991; Grant and D'Appolonia, 1991; Sabularse *et al.,* 1991; Sokhey and Hanna, 1993). During *ex situ* irradiation, the glycoside bonds are broken and new macromolecules with smaller chains are created in starch granules (Raffi *et al.,* 1981a; b; Hayashi and Aoki, 1985). The crystalline content of granules decrease as a result of distortion in the semi crystalline and crystalline regions of amylopectin (Ciésla *et al.,* 1992). These unique properties led to desired functional properties such as reduction of viscosity and high water solubility (Bhat and Karim, 2009; Lee *et al.,* 2003; Bao and Corke, 2002). *Ex situ* radiation modification is an environmentally friendly physical process, in contrast to chemical modification. It is economically viable, safe, and possesses several advantages over other conventional methods employed for modification and cross-linking of starch (Bhat and Karim, 2009).

2.7.2 Chemical modification

Chemical modification of starches involves three processes; cross linking, stabilization (substitution) and conversion. Cross linking improves process tolerance such as heating, stirring, pumping and packing. Cross linked starches are prepared by exposing an alkaline slurry (pH 7.5-12) of granular starch (30-45%) with a cross-linking reagent, e.g. phosphorus oxychloride or sodium trimetaphosphate, in the presence of a salt at temperatures between 25 and 50ºC.

Stabilization helps reduce retrogradation tendencies of starch. Stabilized starches are prepared by acetylation (esterification) and hydroxypropylation. Acetylated starch is prepared by slow addition of acetic anhydride or vinyl acetate to a starch slurry at pH 7.5-9.0, while hydroxypropylation involves the reaction between propylene oxides with starch in an aqueous alkaline slurry. After the reaction the pH of acetylated and hydroxypropylated starch slurries is adjusted to 4.5 and 5.5 respectively and starch is recovered by filtration and washing. Dihydroxypropylation of Brazilian cassava starch increased the swelling power, viscosity and freeze-thaw stability, and reduced retrogradation tendencies of the starches, thus improving their application in the food industry (Schmitz *et al.,* 2006).

Conversion is mainly employed to produce starches with reduced molecular weight, hence reduced viscosity of starch pastes. Three types of reactions are involved in this process; acid hydrolysis, oxidation and pyroconversion or dextrinization. Acid hydrolysis involves addition of an aqueous solution of an acid, usually sulphuric (H_2SO_4) or hydrochloric acid (HCl), to starch with a certain period of incubation. During this process α-1,-4 and α-1,-6 glycosidic linkages are hydrolyzed. Oxidation of starch is carried out by slowly adding an oxidizing agent, e.g. sodium hypochlorite, to the alkaline starch slurry. After completion of the reaction the starch slurry is neutralized and washed. Pyroconversion or dextrinization involves dry roasting of acidified starch (Thomas and Atwell 1999; Rutenberg and Solarek, 1984).

Chemical modification alters properties of native starches depending on the modification method employed. Starches from different sources may also respond differently to chemical modification. Acid hydrolysis of cassava starch from Nigeria increased gelatinization temperature, viscosity and produced more stable pastes (Ahmed *et al.,* 2005) while acid modification of starch from *Canavalia ensiformis,* a Mexican jack bean, resulted in reduction of viscosity, swelling power and retrogradation tendency and an increase in solubility but no effect on gelatinization temperatures was observed (Betancur and Chel, 1997). T_o and enthalpy of gelatinization of sweetpotato starches grown in India significantly decreased following acid hydrolysis but peak viscosity and cold paste viscosities of the starches remained unchanged (Singh *et al.,* 2005) while increases in T_o, T_p, and T_e but decrease in enthalpy were observed in acid-hydrolyzed rice starches (Thirathumthavorn and Charoenrein, 2005).

2.8. Mutation breeding

Induced mutations are highly effective in enhancing natural genetic resources and have been used in developing improved cultivars of cereals, fruits and other crops (Lee *et al.*, 2002). It is a simple, efficient, rapid and cheap option for obtaining desired genotypes from recalcitrant species. Mutagens differ in the “spectrum” of mutations produced (Lundqvist and Lundqvist, 1998) but this has little relevance for practical mutation breeding.

Although breeding of new varieties may take 12 to 15 years of intensive effort, identification of “stone mutants” among vegetatively propagated crops can significantly reduce the breeding period. Immediately after irradiation, mutation has taken place in the planting material. The use of inappropriate chemical screening tests for a large population of induced plants is chief among causes of unnecessary delay in noticing a mutant plant with desired traits (Micke, 1962). To increase the efficiency of mutation breeding, improvement of screening methods is more important than length of trials used for raising mutation probabilities (Makino, 2001). Selections from directly observable monogenic traits (seed, flower and stem colour and early flowering) are easier than selection from hidden quantitative traits such as starch, protein, oil, and inorganic components (quantitative traits) that require specific instruments for analysis (Makino, 2001).

The identification of threshold characters among quantitative traits could be encouraged to speed up the screening process of useful traits. Amylose exhibits a threshold character because it has a differential staining with iodine – thus a normal amylose content of starch (> 10%) stained bluish black and a novel starch stained reddish brown, suggesting a lower-than-normal level of amylose (Ceballos *et al.,* 2007). Waxy (*Wx*) maize starch shows properties similar to those of cassava starch (Watson, 1988). Apart from the field iodine test, the field glucose oxidase-peroxidase strip test shows a brown color for higher glucose content (Carvalho *et al.,* 2004). These threshold properties for quantitative traits have commercial advantages that have been extensively exploited to speed up the screening process in some quantitative traits.

The one plant one grain method, in which one grain is taken from each M_1 (M_1V_1) plant to sacrifice for the M_2 (M_1V_2) generation is the most effective method for the selection of easily discernible mutations in self-fertilizing crops (Yoshida, 1962). The spontaneous photon screening system which allows 600 samples to be measured in an hour to discriminate resistant mutant plants among a body of susceptible plants has been developed to speed up mutation detection in grains (Makino, 1998).

2.8.1 Basis of mutation breeding of plants

Mutation breeding induces variations in traits that appear to be recessive compared to the wild type plant (Muller, 1958; Bhattacharyya *et al.*, 1990). At the molecular level, mutations could be due to base pair changes, recombination or transposition which are independent on the mutagen used (Burr and Burr, 1989; Walbot, 1988). Cytologically, mutations can be unnoticeable gene alterations, insertions or deletions (INDEL) or various forms of translocation. The combined use of induced mutation techniques with *in vitro* culture methods has great potential in breeding programmes (Lee *et al.,* 2003).
Variation at DNA and chromosome levels is the basis of permanent mutation. Varieties differ in their mutability and the kinds of mutant alleles that may be obtained (Lundqvist and Lundqvist, 1988), because the gene(s) controlling the desired trait may not mutate, or the mutated gene may not be expressed, or transmitted or may have a lethal effect on the plant and therefore cannot be selected (Marie, 1967). All genes are induced at the same time but cannot mutate at the same rate because of differences in gene sizes (number of base pairs), gene location and chromosome structure. House-keeping genes occur in multiple copies and may mutate but the mutation cannot be recognized phenotypically. They may exist only as a cryptic genetic variation to some extent comparable with the situation of recessive mutations in polyploids. The number of such duplicated genes seems to be substantial.

Genes are no more regarded as beads on a string to be altered one by one or knocked out by irradiation (Mike, 1991). A gene may contain 100 - 10000 base pairs and codes for hundreds or thousands of amino acids making a complicated protein. This seems to open only two possibilities for mutagenesis: (a) an alteration that has a negligible effect on the protein - such a mutation will not be noticeable or (b) an alteration that changes the gene structure and the function of the gene product. Gene alteration may result in impaired or lost gene function with multiple consequences due to gene interaction, their dimension depending upon the role of the particular gene in the metabolism of the cell or the plant (Nathan *et al.*, 1991). Such an impaired gene function should not be confused with the phenomenon of “dominance and recessivity”. If such an impaired gene function requires homozygosity to be noticed, we deal with a typical recessive mutation but if the impaired function is already noticeable in the recessive condition, we deal with a dominant or at least semi- dominant mutation (Yamaguchi, 2001). Freshly arising mutations probably start in a heterozygous condition (with very rare exceptions). Those that express are immediately subject to selection. In most cases, the cell(s) carrying such a mutation will have a handicap and eventually be eliminated from the meristem by a kind of intrasomatic competition. On the other hand, detrimental mutations that require homozygosity to be expressed can be tolerated by the heterozygous cell and can be carried along without problems through mitosis, as long as DNA replication is not stopping the mutation site.

It is difficult to detect and evaluate the variations at DNA level accurately and this has been an obstacle to improve the efficiency of mutation breeding (Fukui, 2001). Various methods have been developed to detect variations at DNA and chromosome levels. Florescence *in situ* hybridization method (FISH, e.g., Ohmido *et al.*, 1998) is frequently used to detect chromosome aberrations. It allows detection as small as 1 kb even in a plant chromosome. A modified FISH method (M-FISH) allows chromosomes to be painted in different colours (Ohmido *et al.*, 1998). Genomic *in situ* hybridization (GISH) (Schwarzacher *et al.*, 1992; Jacobsen *et al.*, 1995) is also an effective method to detect genomic belongings of chromosomes in allopolyploid species. In contemporary times, many PCR based methods are now available. The identification of mutants using the targeting induced local lesions in genomes (TILLING) method (McCallum *et al.*, 2000) can deal with any population size due to its pooling strategy of samples. Mutation, biotechnology, genetic engineering and conventional breeding methods could employ molecular tools to speed up the screening process and confirmation of desired traits.

2.8.2 Gamma irradiation

Gamma rays are electromagnetic radiation emitted by radioactive decay and having energies in a range from ten thousand (10^4) to ten million (10^7) electron volts (EV). Gamma ray treatment of plants produces mainly chlorophyll variants i.e., albino and viridis were predominant. Khan *et al.* (1998) also reported more chlorophyll variants in sugarcane after gamma ray treatment. Although other variants such as plants with thin and curled leaves, vitrified and plants with comparatively smaller and narrow leaves were also observed. Lethal and mutagenic effects of ionizing radiation result principally from incompletely or incorrectly repaired DNA lesions (Ward, 1994). Among these lesions, strand breaks are considered to be the most important (Ali *et al.*, 2007) as they interrupt the continuity and integrity of the DNA double helix. An unrepaired single stranded break (SSB) in ssDNA (Fiers and Sinsheimer, 1962); an unrepaired double strand break (DSBs) in dsDNA (Frankenberg-Schwager *et al.*, 1981) and cross linking of DNA to itself or to proteins (Cress *et al.*, 1991) has been shown to be responsible for the lethal effects of ionising radiation. Ward (1995) reported that radiation induced mutagenesis is due to the involvement of multiple damaged sites (such as double-strand breaks).

Gamma irradiation (from a ^{60}Co source) accounts for 80% of mutant plant varieties released in China (Nagatomi, 1992) and 61% of more than 200 direct-use mutant varieties released in Japan (Hitoshi, 2008). In rice it was successfully attempted to extensively induce low amylose content for a better taste. Cooked grains from high-amylose rice types are usually firm, dry and not adhesive, while those of low amylose content are more adhesive, moist and soft (Mossman *et al.*, 1983; Sowbhagya *et al.*, 1987). The most preferred rice in Japan is 'Milky Queen' (Ise *et al.*, 2001) which is a mutant with a low amylose content (10%) and excellent taste relative to the parent 'Koshihikari' (with 17% amylose). The taste of cooked rice depends on the amylose content of the endosperm and lower amylose content results in a better taste (Ise *et al.*, 2001). Some of the gamma-ray mutagenesis include mutant rice with a semi dwarf allele *sd1* from the USA (Yamaguchi, 2001), mutant cassava 'Tek bankye', with high dry matter content (40%) and good poundability; mutant cacao 'Upper Amazon T85/799' with resistance to cocoa 'swollen shoot' virus from Ghana (Danso *et al.*, 2008); mutant rice 'Zhefu 802' with early maturity and high yielding potential even under poor management and infertile conditions, wide adaptability, high resistance to rice blast, and tolerance to cold from China; aromatic rice types 'RD6' and 'RD15', from Thailand (Ahloowalia *et al*., 2004); a shorter barley mutant 'Diamante' with an increased grain yield from Czechoslovakia (Bouma and Ohnoutka, 1991); and mutant flowers and horticulture cultivars from Europe (Lundqvist, 2008).

2.8.3 Radio Sensitivity Test

The success of mutation breeding greatly depends on the rate of mutation, the number of screened plants, and the mutation efficiency. The mutation rate is affected by the total dose of the mutagene employed, and can be modified by physical and biological factors. Higher doses inevitably bring about mortality, high pollen and seed sterility and deleterious mutations. To avoid excessive loss of actual experimental materials, radio-sensitivity tests must be conducted to determine LD_{50} (the safe dose at which half of the planting material survive) doses before massive irradiation of similar materials are accepted. The LD_{50} values vary with the plant parts. For example, cassava cuttings were given a series of irradiations after the appropriate doses (25 and 30 Gy) had been determined (Asare and Safo-Kantanka,1997). Radio-sensitivity tests on the regenerated cassava plantlets indicated LD_{50} of 40 Gy, and doses of 25, 30 and 35 Gy were identified as suitable for *in vivo* and *in vitro* grown plants (Ahiabu *et al.,* 1997). Reference doses could be obtained from the international Atomic Energy Agency (IAEA) for some seeds or sourced from literature. This is helpful for researchers who lack the facility to conduct their own tests before the actual mass irradiation. About 1400 botanical cassava seeds were irradiated with 200 Gy (^{60}Co) based on previous experience at the International Atomic Energy Agency (IAEA) with cassava as well as with other crops whose seeds have similar size as those of cassava (Ceballos *et al.,* 2008). A 50 Gy of γ-rays was determined to be the optimal dose for inducing mutations in a cyclic somatic embryogenic system in cassava PRC 60a under invitro propagation (Joseph *et al.,* 2004). A radio sensitivity test in six indica rice varieties to determine the optimum dose of ^{60}Co gamma irradiation showed that the optimum dose for planting materials is variety specific. Based on seedling height reduction, the dose rate of rice ranged from 200 - 300 Gy for IR29, 300- 400 Gy for IR51500, 300-400 Gy for IR63731, 400-500 Gy for IR58430, 300-400 Gy for Pokkali and above 500 Gy for Nona Bokra (FAO/IAEA, 1998). After irradiation of 1400 botanical seeds, Ceballos *et al.* (2008) found three mutants from the same mother. Three sister

M_2 genotypes were derived from the same M_0 mother (progeny which was not irradiated serving as a source of many mutants) and that was a strong indication that within the same species, some cultivars are more radio-sensitve than the others. Two radiosensitivity genes were discovered in soybean which could be used to screen cassava germplasm. The differences in radiosensitivity between the varieties were found to be controlled by a single recessive gene, $rs_{1.}$ The second recessive gene rs_2, acts only in seed irradiation (Takagi, 1969). The radio sensitive test or LD_{50} dose of the similar test material must be known before mass irradiation of the test sample follows. The greater the number of test planting material to be irradiated, the higher the chances of finding a useful mutant.

2.8.4 Mutation breeding of cassava

Mutation screening may be rapid when cassava haploids are used for mutagenesis, so that all mutants are immediately expressed (Joseph *et al*., 1999). Cassava is an out-crossing crop with heterozygous seeds produced under natural conditions. Irradiated cassava seeds should be well-suited to exploiting mutation breeding because vegetative propagation allows immediate fixing of useful traits found in any one of the segregated progeny. In vegetative propagation of mutagenized cassava stem cuttings, the obvious advantage is that a superior plant, identified at any stage of evaluation, can be cloned indefinitely, thus genotypic integrity is maintained through successive generations of cloning. Such superior useful variations are totally due to the irradiation effect if the putative mutant and the control are stakes (stem cuttings with average five adventitious buds) from the same original parent which was compared in the same environment or .

2.8.5 Mutation breeding of seed propagated plants

In cross pollinating seed propagated plants, dominant alleles provide vigour. One of the homozygous dominant alleles may become defective during irradiation and the dominant allele left will often show optimal functioning at the single dose level. Most alleles that have one detrimental allele are eliminated by selection. This is in line with observations that cross pollinating crops like maize tend to accumulate deleterious recessive alleles (thereby assuring a gene dose of one for dominant alleles) and derives a fitness advantage from this (Salerno *et al.*, 1981; Russel *et al.*, 1963). In self pollinating seed propagated plants, where homozygosity is the normal state, dominant and recessive alleles should have an equal chance to contribute to fitness as well as good performance in a crop plant (Simonguyan, 1989). Mutation screening methods for self-fertilized species are inappropriate for open-pollinated cross-fertilization species due to random mating which results in a very low frequency of homozygous mutants, which may segregate from generation to generation (Ukai, 1990).

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CHAPTER 3

Identification of novel morphological traits in induced cassava mutants

3.1 Abstract

The morphological characterization of induced cassava plants established from irradiated stakes with Co^{60} gamma rays led to the identification of solid mutants with linear leaf shape and various chimerical mutants at the M_1V_1 stage. The solid mutant was confirmed at the M_1V_2 stage. These observations indicated that the 35 Gy acute dose applied could also be effective in causing *in planta* modification of starch in roots. Four putative mutant plants were identified with an iodine test. They displayed exceptional starch quality characteristics at the M_1V_2 stage, which could possibly indicate free sugar plants.

3.2 Introduction

Induced mutation has created new genetic variations within crop varieties where natural genetic variation was limited and insufficient (Ahloowalia *et al.*, 2004; Maluszynski *et al.,* 2001). It also increases mutation frequency relative to natural spontaneous mutation. Induced mutations are more acceptable to the consumer than genetically modified organisms (GMO) (van Harten, 1998) because variation caused by induced mutations is not essentially different from that which originates from spontaneous mutations (Sigurbjörnsson, 1977; Heinze and Schmidt, 1995).

Gamma rays are energetic electromagnetic waves which do not leave charged particles like positrons or electrons in their targets. Excessive gamma irradiation is lethal. To prevent killing of all the planting material, a pre-test for survival of planting material is carried out at various doses (radiosensitivity test). The safe dose at which half of the planting material survives (LD_{50}) is noted as a reference dose (Brunner, 1995). A precise method for survival estimation was proposed by Neville *et al*. (1998). The common approach is to estimate the effective radiation dose which is accessed through the physiological response of the irradiated stakes (stem cuttings with an average of five adventitioud buds). Other researchers reported the dose rate that caused a

50% reduction of vegetative growth of the treated plant (LD_{50}) with respect to the control (Predieri, 2001). The LD_{50} for cassava stem cuttings was determined in previous research as 32 Gy (1 Gy = 1 J kg^{-1}= 100 rad) (Amenorpe *et al*., 2004). The radiosensitivity test result was used to estimate the most suitable single acute dose needed for mass irradiation of similar planting materials in ensuing experiments. Heinze and Schmidt (1995) suggested a single acute dose range of LD_{50} (±10%), or an acute dose resulting in 20% survival of treated material, could cause effective mutation estimation in the test materials.

Identification of cassava genotypes using morphological characteristics is reliable (Maduakor and Lal, 1989; Soyode and Oyetunji, 2009). Instead of describing plant morphology in abstract terms as in molecular biology, the real morphological features could help farmers, herbalists and botanists to recognize functional parts without much ambiguity. In a particular farming community, farmers know observable morphological characteristics very well, as these traits dominate their vocabulary during communication.

Selection of desired mutants is very effective if it is controlled by a single gene (Kearsey and Pooni. 1996). For example, in the local markets of Ghana, single gene traits such as pink or red ring colour is associated with mealiness, and storage roots with such colors are sold faster and at a higher price than the white ones (Amenorpe, 2002).

Plant height, yield, and starch contents are quantitative traits which are predominantly controlled by polygenes. Each gene contributes a small effect, which is called an addictive effect. Due to genotype x environment (GxE) interaction, the expression of quantitative traits fluctuates and is therefore not as reliable for morphological characterization as qualitative traits. Hence, phenotypic variance in cassava is higher than genotypic variance for traits of agronomic importance like tuber weight (Mathura *et al.*, 1986). Therefore it is difficult to make a precise description of all the morphological traits due to a large number of cassava genotypes cultivated commercially in a large diversity of ecosystems where constant GxE interaction occurs. Cultivars with more stable yield are therefore preferred. Among morphological descriptors commonly used in cassava are (i) apical leaf colour; (ii) apical leaf pubescence; (iii) central lobe shape; (iv) petiole colour; (v) stem cortex colour; (iv) stem external colour; (vii) phyllotaxix length; (viii) root peduncule presence; (ix) root external colour; (x) root cortex colour; (xi) root pulp colour; (xii) root epidermis texture; and flowering (Alves, 2002; Benesi, 2002; Nassar, 2005). The reliable morphological traits form the basis for phenotyping of populations. Morphological markers and molecular markers complement each other in diversity studies, since no marker can solely provide all the information needed in plant adaptation, breeding, evolution and conservation programmes without support from other techniques (Thormann *et al*., 1994; Karanja *et al*., 2009).

Cassava stems bear a large number of dormant adventitious buds which can give rise to adventitious shoots. Adventitious shoots are the ideal material for inducing desirable mutations in asexually propagated plants (Mikkelsen and Sink, 1978) because they ultimately originate and develop from a single cell which promotes solid mutants (Broertjes and Keen, 1980; Broertjes *et al.*, 1980) or homohistant mutants (Broertjes, 1977; Duron, 1992). Large numbers of solid, non-chimeric mutants obtained from adventitious bud techniques that have been commercialized, have been reported by Broertjes and van Harten (1978).

Cassava seeds contain multicellular embryos which are prone to chimera formation. The occurrence of a chimera can affect the stability of a mutant and is therefore undesirable. For example, a chimera was reported to be responsible for the occurrence of amylose synthesizing clones derived from an amylose-free potato mutant (van der Leij *et al.*, 1992). Most of the chimeric plants do not survive, but if they do, self pollination will allow segregation and desirable plants could be selected. Self pollination is meant to allow duplication of recessive mutant genes (GBSS responsible for waxy starch) for expression (Ceballos *et al*., 2008).

Cassava seeds are allogamous (cross-fertilized) and highly heterozygous (Jennings, 1963; Umanah and Hartmann, 1993). Moreover, low rates of flowering, difficulty in seed germination and the long breeding cycle further render seed based mutation of cassava arduous (Jennings and Iglesias, 2001). It is prone to inbreeding depression with disomic inheritance (Raji *et al.*, 2009). Due to seed characteristics, the variability generated by segregation in hybrid progeny is so great that there is little chance of the selection of improved types among seedlings and at the same time retaining the general traits of the adapted cultivars from generation to generation (Ceballos *et al.,* 2008). Variation as a result of irradiation of seeds may overlap and confounds the natural hybrid genetic segregation that makes each botanical seed unique. Since the variability generated by irradiation of seeds is often confused with variability generated by segregation of progeny from seeds, there is a need to test nodal stem cuttings with dormant adventitious buds to study the effect of gamma irradiation on cassava stem cuttings. In this chapter, morphological variation caused by gamma irradiation observed in the M_1V_1 stage and confirmed in the M_1V_2 stage, is reported.

3.3 Materials and methods

3.3.1 Sources of materials

The four controls used in mutagenesis were among the top ten dry matter, starch, sugar and cyanide safe landraces which were selected after evaluating 250 cultivated species collected across Ghana (Amenorpe, 2002). The controls were HO008, HO001, UCC090 and UCC026. The souce materials were grown vegetatively in the same environment and were of the same age before acute mass irradiation of stakes for planting was done.

3.3.2 Radiation treatment

Heinze and Schmidt (1995) suggested that a dose range of LD_{50} (±10%), or a dose resulting in 20% survival of the treated material, could cause effective mutation in the test materials. A radiosensitivity test was conducted for similar landraces (HO 008, Ankra and Medzontem) used in this study earlier and a 35 Gy LD_{50} dose was reported (Amenorpe *et al.,* 2004). A total of 1689 stakes were irradiated with 35 Gy gamma rays from a Co^{60} source and stakes planted with unirradiated control (HO008, HO001, UCC090 and UCC026) stakes. The 35 Gy chosen was based on extrapolation from the erradiation response curve obtained in 2004. The 35 Gy was expected to cause 28% survival following the method of Heinze and Schmidt (1995). The irradiation was done at the Gamma Irradiation Centre (GIC) of Biotechnology and Agricultural Research Institute (BNARI) in the Ghana Atomic Energy Commission (GAEC) on 13 June 2007 and planted the following day.

3.3.3.1 Field establishment of M_1V_1 and M_1V_2

The mutation breeding experiments were established at the GAEC's Green House Research farm on high clay content loam soil belonging to the Adenta series (Brammer, 1960) with irradiated stakes from four landraces planted one after another on the same field. The M_1V_1 and M_1V_2 fields were 1 and 3 acres respectively. The planting was linear and serpentine with 1 m x 1 m inter crop and inter row distances. There was no replica of the same stake for replication immediately after irradiation because mutagene was administered to change the adventitious buds before they germinate and developed into shoots on the same irradiated stake planted. That means adventitious buds on the stake (stem cutting with an average of five adventitious buds) would not maintain the same genetical constitution after irradiation. Induced plants derived from such nodes were therefore assumed to be different. The four controls were plants germinated from the four un-induced HO008, HO001, UCC090 and UCC026 stakes. Some alterations in morphology observed on the fields of M_1V_1 and M_1V_2 were characterized using standard cassava descriptors (Gulick *et al.,* 1983) in addition to revised descriptors (Fukuda *et al.,* 1998).

3.3.3.2 Coding based on surviving M_1V_1 and expected M_1V_2 plants

The generated codes for M_1V_1 and M_1V_2 fields are shown in Table 3.1 with the survived M_1V_1 and M_1V_2 plants. The coding for the M_1V_1 field was based on the number of irradiated stakes that survived. The codes from N1 to N349 were for induced HO008 plants and N350-N390 for HO001, N391-N432 for UCC090 and N433-N476 for UCC90 plants. The coding of the M_1V_2 field was based on the number of induced stakes planted from each M_1V_1 plant. Each M_1V_2 code had an "N" "prefix" and "P" "suffix". The "N" referred to the specific M_1V_1 plant that survived the irradiation and the "P" is the plant positions of sub-plants of "N" on the M_1V_2 field. Therefore P1, P2, P3… Pn showed the first to the last number of stakes planted on the M_1V_2 field obtained from the "N".

Table 3.1 The generation of codes for M_1V_1 and M_1V_2 cassava fields

Landraces	Survived	M_1V_1 Codes	M_1V_2 codes
HO008	349	N001 - N349	N001P1, N001P2,... N001Pn to N349P1, N349P2,...N349Pn.
HO001	41	N350 - N390	N350P1, N350P2,... N350Pn to N390P1, N390P2,...N390Pn.
UCC090	42	N391 - N432	N391P1, N391P2,... N391Pn to N432P1, N432P2,...N432Pn.
UCC126	44	N433 - N476	N433P1, N433P2,... N433Pn to N476P1, N476P2,...N476Pn.

3.3.3.3 Field and laboratory iodine test of starch in cassava storage roots

Potassium iodide 2% (2.0 g KI dissolved in 75% ethanol) was sprayed on a fresh slice from the central pith part of each storage root, both on the field and in the laboratory. Blue-black staining of cassava starch is typical for normal amylose-amylopectin content whereas reddish-brown staining is typical of amylose free starch.

3.3.4 Results

3.3.4.1 The survival rate of irradiated stakes

Generally, 28% of the total irradiated stakes survived the acute mass irradiation dose of 35 Gy, representing 476 plants established in the M_1V_1 field in 2007 (Table 3.2). The survival rate of induced plants across four landraces ranged from 73.3 to 8.6%. HO008 induced plants had the highest frequency of survival (73.3%), followed by UCC126 (9.2%), UCC090 (8.8%) and HO001 (8.6%). The survival rate for a particular landrace was termed “the specific landrace survival” whilst the survival rate across different landraces was termed “across landraces survival” or “between landraces survival” where the term “across” or “between” denotes inter-relatedness of different landraces. The “specific” landrace survival rates therefore ranged from 41.5% to 8.3%. Induced HO008 plants had the highest specific landrace survival (41.5%) followed by UCC126 (34.9%), HO001 (18.8%) and UCC090 (8.3%).

Table 3.2 The survival rate of irradiated cassava stakes

Landraces	Irradiated	Survived	Landraces survival (%)	
			Across	Specific
HO008	840	349	73.32	41.55
HO001	218	41	8.61	18.81
UCC090	505	42	8.82	8.32
UCC126	126	44	9.24	34.92
Average	422	119	25	28.18
Total	1689	476	100	
Correlation(A:B)	0.86			

3.3.4.2 Alteration of morphological characteristics of induced plants

Table 3.3 shows morphological differences between un-irradiated and irradiated plants. There were three morphologically different plants (in brackets) identified at M_1V_1 and confirmed at the M_1V_2 stage.

Table 3.3 Morphological differences between conrol and induced cassava plants

Control (Induced)	A	B	C	D	E	F	G	H	I	J	K
HO008 (N167)	9	1(2)	5	4	1	3	1	2	2	0	2
HO001 (N375)	3	1	3	1	3	4	3(1)	2	3	0	1
UCC090 (N422)	7(5)	1	7	4	3	3	1	2	2	6	2
UCC126	5	1	7	3	3	3	1	2	2	4	1

A. Colour of unexpanded apical leaves: 3=light green; 5=dark green; 7=greenish purple; 9=purple
B. Shape of central leaf lobe: 1=oblanceolate (Fig. 2a) ; 2=linear (Fig. 2b); 3=elliptic; 4=pandurate; 5=lanceolate
C. Petiole length: 0=absent; 3=short (5-10cm); 5=medium (15-20cm); 7=long (25-30cm)
D. Petiole colour: 1=light green; 2=dark green; 3=greenish purple; 4=purple
E. Stem colour: 1=silver green; 2=light brown or orange; 3=dark brown
F. Storage root surface colour: 1=white, 2= cream, 3= light brown, 4= dark-brown (Fig. 6a)
G. Colour of root cortex (Rind) at harvest: 1=white or cream, 2=yellow, 3=pink, 4=purple (Fig. 6a)
H. Colour of root pulp (parenchyma cells making the central pith) at harvest: 1=white, 2= cream, 3=yellow, 4=pink (Fig. 6b)
I. Number of levels of branching at harvest : 1=1st level; 2=2nd level; 3=3rd level
J. Average number of Tillers formed under the first reproductive branch at harvest: 0 = no tiller; 1 = one tiller...

K. Height to first reproductive branching (m) at harvest: 0= no reproductive branch; 1 = first reproductive branch at 1 m and below, 2 = first reproductive branch between 2m and 1 m

3.3.4.3 Changes of leaf shape

The change from an oblanceolate to a linear shape of the central leaf lobe is shown in Figure 3.1. Before irradiation, the shoot and root systems of the induced HO008 plant had a purple colour of unexpanded apical leaves, oblanceolate shape of the central leaf lobe, medium petiole length, purple petiole colour, silver green stem colour, light brown storage root surface colour, white coloured root cortex (rind of peel) and white coloured root pulp. It also had two levels of branching, no tillers and the first reproductive branch was between 1 to 2 m high (Table 3.3). After irradiation, one induced shoot of HO008 with only linear shape of central leaf lobes (solid mutant) was observed sharing the same stake planted (N167) with another shoot bearing oblanceolate central leaf lobes (normal) at the M_1V_1 stage (Fig.3.1a). The two un-identical shoots of N167 were separated at M_1V_2 stage. The solid mutant was observed to maintain the production of only the linear shape of central leaf lobes as confirmation of solid mutant status at the M_1V_2 stage (Fig. 3.1c).

Fig. 3.1. Cassava mutant plant N167 with linear leaf shape. Oblanceolate (normal) and linear (mutant) shape of the central leaf lobes shoots germinated from the same stake at M_1V_1 stage (a), and were separated into homohistant oblanceolate (b) and linear (c) leaf types at M_1V_2 .

The confirmation of mutant expression in the M_1V_2 showed that the mutant could be maintained through mitotic cell division and clonal propagation.

3.3.4.4 Changes of rind colour

Figure 3.2 shows changes from pink to cream and pinkish cream rind colours of N375. The control HO001 had a light green colour of unexpanded apical leaves, oblanceolate shape of the central leaf lobe, short petiole length, light green petiole colour, dark brown stem colour, dark brown storage root surface colour, purple coloured root cortex (rind), and cream colour of root pulp. It also had three levels of branching, no tillers and the first reproductive branch was between 0 to 1 m high (Table 3.3). After irradiation, changes from pink to cream and pinkish cream colours were observed under the same N375 plant.

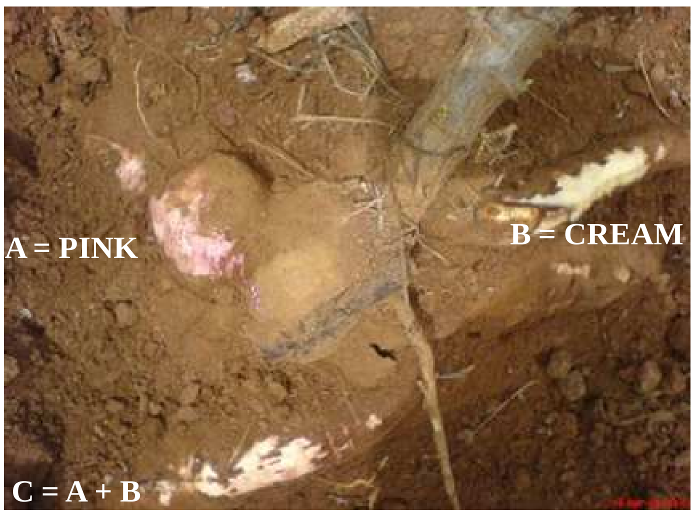


Figure 3.2. Cassava mutant plant N375 with alteration in rind colours. M_1V_1 clone N375 with pink (A) cream (B) and a pinkish cream (C) rind types.

3.3.4.5 Changes of unexpanded apical leaf colour

The UCC090 control had a greenish purple colour of unexpanded apical leaves, oblanceolate shape of the central leaf lobe, long petiole length, purple petiole colour, dark brown stem colour, light brown storage root surface colour, cream colour of root cortex (rind) and a cream coloured root

(a1)Normal part of N422 **(a2) Mutant part of N422**

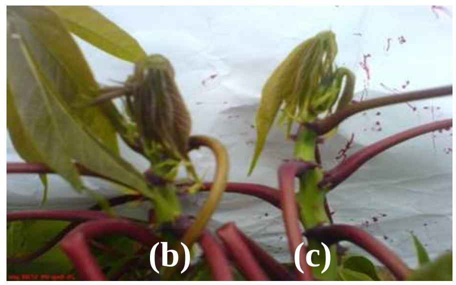


Fig. 3.3. Cassava mutant plant N422 with alteration in unexpanded apical leaves colours. M_1V_1 clone with both apical leaf types (a), and M_1V_2 clones with normal purplish green (b) and mutant dark-green(c) leaf types.

pulp. It also had two levels of branching, six tillers on average and the first reproductive branch about 1 to 2 m high (Table 3.3). After irradiation of UCC090 stakes, some tillers of N422 were observed to change from purplish green (normal) (b) to dark-green (mutant) (c) colour of unexpanded apical leaves (Fig. 3.3) at the M_1V_1 stage. During the M_1V_2 propagation stage, the normal and the mutant shoots of induced N422 plant were separated into respective plants.

3.3.4.6 Induced UCC126 plants

Control UCC126 had a dark green colour of the unexpanded apical leaves, oblanceolate shape of the central leaf lobe, long petiole length, greenish purple petiole colour, dark brown stem colour, dark brown storage root surface colour, cream coloured root cortex (rind) and the cream coloured root pulp at M_1V_1 stage. It also had two levels of branching, four tillers and the first reproductive branch height between 0 to 1 m (Table 3.3). Induced plants of UCC126 however, did not show any observable morphological differences from the control during the M_1V_1 stage.

3.3.4.7 Variations which were not expressed in M_1V_1 but in M_1V_2

The morphological changes that were observed only in M_1V_2 plants are presented in Figure 3.4. The variation in chlorophyll pigmentation (a), multiple shoots from the same node with different leaf forms and colours (b), stony head with retarded growth (c) and variation in plant storage root numbers as a result of increase in plant root vigor (d) were common across the four induced landraces.

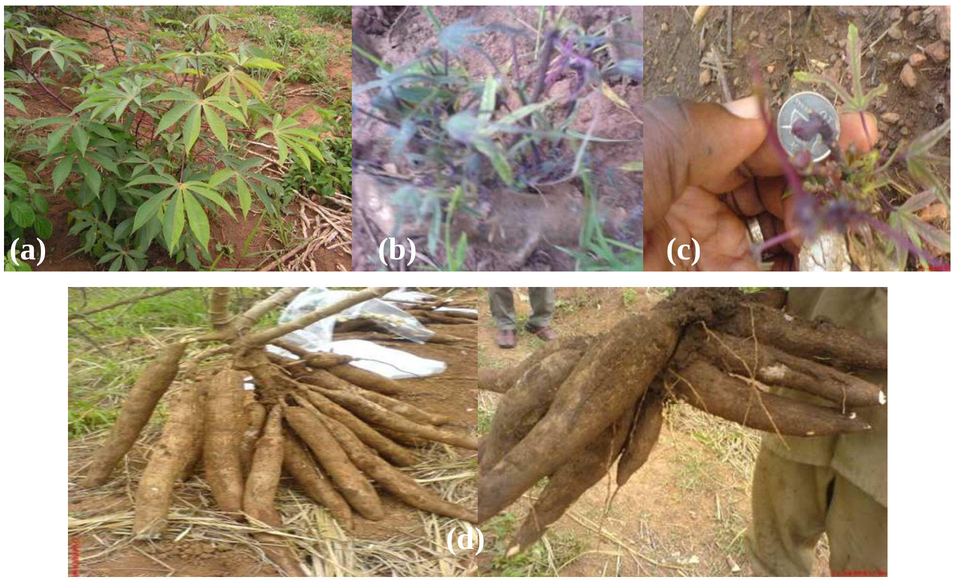


Fig. 3.4. Common changes in morphology and vigour of induced cassava at M_1V_2 stage. M_1V_2 clones with chlorophyll variation in leaves (a), multiple shoot with deformed leaves (b) stony head with retarded growth and vigor (c) and variation in storage root numbers (d).

When the cut roots were subjected to the iodine test, difference in reaction of iodine with starch in the central pith was observed (Figure 3.5). Starch in most of the induced plants and their controls reacted with iodine solution to turn blue black except induced plants N281P16, N357P5, N394P82 and N441P54 with shades of reddish brown or yellow or Khaki colours (Figure 3.5 (d) lab.).

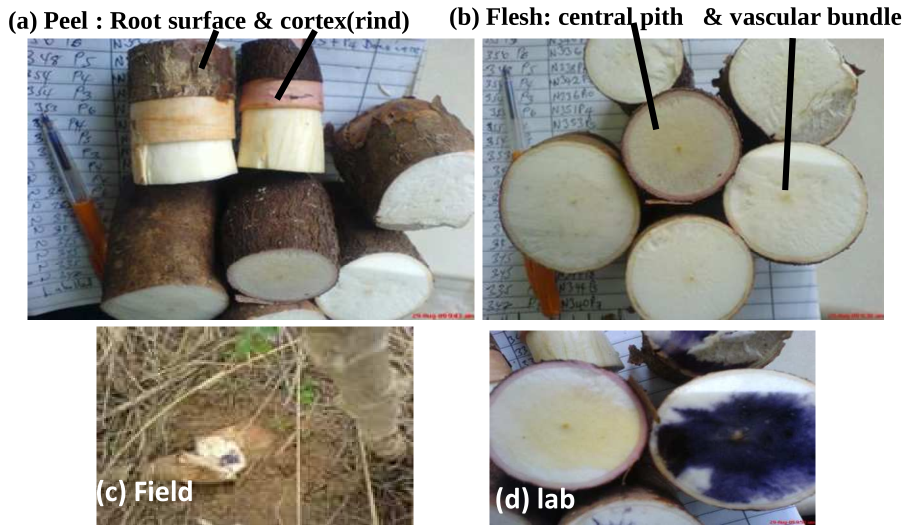


Fig. 3.5. The cassava root structure and preliminary field and laboratory iodine tests of M_1V_2 plants. M_1V_2 clones showing root structures of peel (a) and flesh (b), and changes of the central pith colour from blue-black (normal) (c) (d) to brown (d) in N281P16, N357P5, N394P82 and N441P54 clones only out of 4700 M_1V_2 clones tested in M_1V_2

3.5 Discussion

3.5.1 Survival rate of irradiated stakes

The germination of stakes in the field depends on water, air, genotype and the irradiation dose. Since planting of stakes from different landraces was done in the the same homogeneous field and location, then the variation in environmental factors must be assumed to be negligible. In addition, the effect of genotypic variation before irradiation was nullified since stakes for induced and control plants were originally from a common cell lineage of the same landrace. After irradiation of stakes, any significant difference experienced in the induced plants in terms of survival, morphology and reaction with iodine solution could be due to irradiation effect.

The survival rate varied across the four landraces. This agrees with Briggs and Konzak (1977) and D'Amato (1992) who reported that radio-sensitivity varies with the physiological condition of the plant. The survival rate of induced HO008 (73.3%) was the highest, followed by UCC126 (9.2%), UCC090 (8.8%) and HO001 (8.6%). The number of irradiated stakes had a strong positive correlation (r = 0.86) with the survival rate of induced plants. This suggests that the more stakes irradiated the higher the chance of obtaining survivors. Although the number of irradiated stakes for UCC126 was the least, it had the second highest surviving rate, which may indicate that a higher number of stakes alone might not be a sufficient factor controlling the survival of induced plants, therefore may also not be enough evidence for explaining the survival rate of all induced plants. This confirms that mutation is a random phenomenon and could not be totally controlled. Irradiated stakes with sufficient moisture might have higher chances of germination in moist soil than drier ones. The difference between the survival rate of HO008 and others was due to a larger number of fresh stakes available for irradiation. This suggests that HO008's stakes might have had a higher moisture content compared to the other landraces and therefore had a higher frequency of specific landrace survival than UCC126, followed by HO001 and UCC090. This was supported by a report that the most frequent primary target of ionizing radiation is the water molecule because correlations between the physiological status of plants and their radiosensitivity are often determined by the water content of the tissue (Britt, 1996). The irradiation of tissue is affected by hyperhydricity, which can increase the frequency of individual mutations (Cassells, 1998). The specific survival rate measures the physiological condition of the stake just before planting, varies within each landrace because moisture content of main stem and branches differ. Therefore the specific landrace survival rates could be an additional factor controlling the survival rate.

The numerous dormant adventitious buds (axillary buds) on stems were comparable to seed embryos awaiting favourable conditions to develop into shoots (Konan *et al.*, 2006; Ogbe *et al.*, 2001). Under good soil moisture, length of the stake planted, number of adventitious buds on the stake and the orientation of stakes to the soil surface, one to three adventitious buds could germinate into one to three main shoots (Fig. 3.4d). Depending on plant height, reproductive branches, age and tillering habbits of the three shoots, an explosion in the number of M_1V_2 plants could be experienced since the number of stakes obtained from each M_1V_1 plants ranged from 2-183 (data not shown). This emphasized that the number of initial cassava stakes required to start mutation breeding cannot be in tens of thousands like cassava seeds. Otherwise, more pressure was to be placed on land, labour and capital required to select the few useful mutants from ~ 7616 induced stakes planted on the M_1V_2 field.

3.5.2 Changes in leaf shape

The change in leaf shape and colour of N167 is an indication of two genes that mutated together and might belong to the same linkage group (located on the same chromosome) but are separated by vast stretches of introns. In cassava there are two distinct types of leaf types: broad and narrow. Jos and Hrishi (1976) concluded that the narrow leaf shape is dominant to the broad shape and that the trait is monogenic. The linear or narrow trait observed is a dominant monogenic trait but it cannot be determined whether a mutant plant is heterozygous or homozygous for the dominant allele by simple observation. This is one of the rare cases where a recessive trait mutated into a dominant trait.

If somatic or a genetic mutation was induced across adventitious buds of the same stake planted differs, then different morphological shoots could be observed on the same irradiated stake planted at the M_1V_1 stage (Fig.3.1a). The linear shape of the central leaf lobe obtained from N167 is a solid or stone mutant that originated from a single cell or very few cells in the adventitious bud which developed into a single shoot. Solid mutants like N167, were selected at the M_1V_1 stage because it could be spotted easily as plant having vigorous growth of uniform healthy linear leaves. This experiment therefore demonstrated the identification of solid mutants in the first generation, followed by cloning and separation of mutant shoots from the non-mutant shoots in the second generation. This agreed with Brunner (1995) who reported that the selection of mutants usually begins in the second or later generation for vegetatively propagated plants, because it will be much easier to recognize mutants, as they segregate. Through mitotic cell division and cloning, as in vegetative propagation, both altered traits were fixed permanently. That means that a new kind of plant has been developed and established in this experiment. Irradiation effects might be somatic or a genetic mutation. Somatic mutation occurs when the mutant cell continues to divide mitotically to support growth and development. This is an epigenetic mutation which causes the formation of distinct cells, surrounded by un-modified tissue (Brar and Jain, 1998). In vegetatively propagated crops such variation could be identified early if an appropriate screening strategy is adopted. It is established permanently through cloning, provided that it is identified with novel useful traits. In seed propagated crops most of the somatic mutations are not inherited and such variations will disappear in the next generation.

But when the trait is a genetic mutation, a permanent change in the DNA sequence that makes up a gene is involved. It might originate from the changes in sequence of bases which are located in exons or introns.

The two morphological types observed in the N167 may have been due to two adventitious buds close to each other which had experienced independent mutagenic effects. This underscores the importance of evaluating each shoot individually (as a suspected or putative mutant) and never a collection of the same plant from the same stock after irradiation. The focus is mainly on the selection of a few useful traits and rejecting thousands of induced plants which share similar characteristics with the control already in circulation among farmers. The extent of variation of putative mutants from the control is a useful indicator of successful mutation in vegetatively propagated crops.

3.5.3 Changes in leaf and rind colours

The most reliable prominent morphological traits used by farmers to identify their varieties in a particular location are colour variables (Elias *et al.,* 2001; Alves, 2002). Some morphological traits such as colour variables, especially colour of apical leaf, leaves, stem, petiole, root surface, rind (cortex) and pulp of root appear more salient than others, which is not surprising because colours of above ground parts are salient traits for identification, and colour of the pulp is under strong diversifying selection (Elias *et al.*, 2001). Colour variables are those that show the least intra-varietal variability. In the field, colour variables seem to play an important role in assessment of similarity, as they do for other Amazonian cassava cultivators (Boster, 1985). An induced plant may have the same morphological trait but differ in root cortex colour; therefore root system features could supplement the shoot system features in accurate identification of mutant. Some salient traits were, however, highly variable in a heterogeneous environment, and this might cause confusion between varieties. Colour variables are monogenic traits which are highly heritable compared to polygenic traits.

There is a difference between how the solid mutant plant N167 and chimeric mutant plant N422 were developed. In plant N167, two different shoots developed from the same stake of 15 cm with an average adventitioud bud of 5, while only one shoot was produced in plant N422, which later developed different tillers. At one side of the stem of plant N422, normal apical leaf colour tillers were produced, whilst the other side produced tillers with mutant apical leaf colours. This shows that in plant N167 only one cell was fully mutated whilst in N422 a layer (or chimera cells) was mutated which later developed into tiller mutants. A tiller mutant is described here for the first time as a vegetative shoot below the first reproductive branch with novel features caused by mutation. The chimeras could be depicted as periclinal, mericlinal and sectorial. Periclinal describes the stable, "hand-in-glove" arrangement of the tunica-corpus region; mericlinal, describes a type of periclinal where only part of a layer is mutant, and sectorial, describes a form where a solid sector through all apical layers is mutant (Satina and Blakeslee, 1941). Those adventitious buds located in the sectorial chimaeric region of the stem later developed into shoots with new morphology whilst the non-chimaeric region maintained the nature of the control. The observation of tiller mutants in N422 at both M_1V_1 and M_1V_2 confirmed that it could be sustained through mitotic cell division and clonal propagation.

The identification of three different cortex (rind) colours under the same plant may be an example of distortion of an enzyme responsible for maintaining the integrity of pink colour formation in plant N375. Generally, the production of solid mutants, without chimeric sectors which is an imperative for vegetatively propagated crops, usually take up to a minimum of four cycles of clonal propagation to generate homohistonts even under *in vitro* culture conditions dealing with less tissue. In Figs. 3.2, 3.3 for instance, dissociation of chimeras into solid mutants is still required at the M_1V_2 stage in order to separate the different types of leaves and rind colors, respectively, into individual plants that can breed true.

3.5.4 Other phenotypic changes observed in the M_1V_2 stage

The conventional method of describing the genotypes of the tunica and corpus regions is the use of the abbreviations L.I, L.II, and L.III which represent the outermost layer, the next tunica layer and the corpus, respectively (Satina and Blakeslee. 1941). Such designations are, in the case of chlorophyll chimeras, generally based on the appearance of leaves and other organs produced by derivatives of the apical meristem. This may not refer to the precise meristem cell layers, since chlorophyll is not synthesized or detectable in the tunica and corpus cells of the meristem itself (Dermen, 1960). The variation in chlorophyll pigmentation (Figs. 3.4 a;b;c) is one of the physiological damage symptoms with albinism being the most common.

The ability of the middle tunica layer cells (L.II) to divide into the outer layer (L.1) and inner layer (L.III) was reported in potato and was the reason why amylose-free chimera mutants suddenly started developing both amylose and waxy tubers (van der Leij *et al.*, 1992). In this study, normal appearing M_1V_1 plants suddenly developed chlorophyll variation in leaves at the M_1V_2 stage because they were harbouring chimeric L.II cells. These cells were waiting for apical dominance to be removed before they could be developed and expressed during the M_1V_1 stage. During vegetative production of stakes from M_1V_1 plants as planting material for the M_1V_2 field, the apical dominance was broken, allowing chimeric L.II cells to divide and produce new shoots with a variegated chlorophyll pattern and other chimaeric expressions (Fig. 3.4a-c). Thus, the vast majority of variegated-leaf chimeras have arisen by spontaneous nuclear or plastid mutation (Kirk and Tilney-Bassett, 1978). Gamma ray mutagenesis is responsible for the chlorophyll variants observed in shades of green (Fig 3.1, 3.3 and 3.4a). This agreed with Khan *et al.* (2009) who had reported several chlorophyll variants in sugarcane after gamma ray treatment. It is likely that adventitious shoots are derived from one or a few cells (Hovenkamp-Hermelink *et al.*, 1987; Broertjes and van Harten, 1985) and if they did for a chimeric L.II cell which segregates into two genotypes, a new vegetative shoot is observed with normal or variegated leaves on the same plant. If the adventitious bud is originated from a single cell or very few cells, the chances of obtaining chimaeric plants would be drastically reduced and there would be a higher probability for the mutated cells to develop into solid mutant plants.

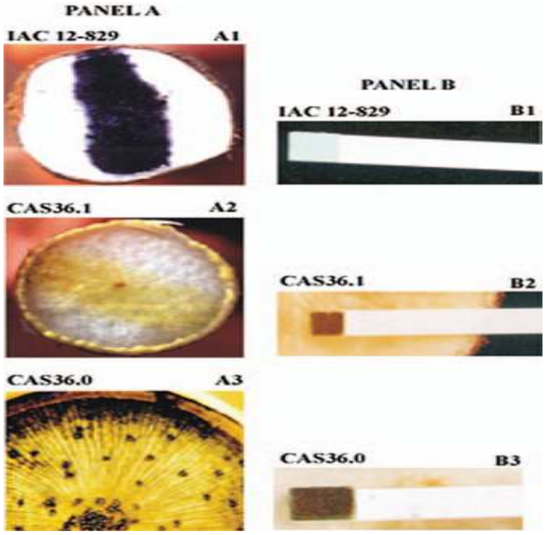
A node is expected to produce one adventitious bud which later develops into a single shoot but a node depicted in Fig. 3.4b rather produced multiple shoots with narrow, thin, smallish and physiologically damaged curled leaves. Leaf abnormalities could be due to chromosomal breakage, disrupted auxin synthesis and accumulation of free amino acids (Sparrow, 1961; Kanakamanay, 2008). This shows a disorganization of an adventitious bud by gamma irradiation which resulted in nuclear and plastid mutation with these symptoms. Cytokinins cause initiation of mitosis and rapid cell division and cells without cytokinins cannot progress to mitosis

(Valente *et al.*, 1998). After cell division, gibberellins stimulate cells and stems to elongate and grow (Berg and Berg, 2007). It implies that numerous shoots observed at a single node were due to over stimulation of cell division by cytokinins and cell elongation and differentiation into shoots by gibberellins (Fig. 3.4b and in Fig 3.4 c). Unfortunately the rapid shoot production was not supported by rapid root development, hence plantlets were not adapted well enough to absorb enough soil nutrients to survive in the field, resulting in physiologically retarded growth. According to Kovacs and Van'thof (1971) the reduced growth could be attributed to a delayed onset of first mitosis and an inhibition of DNA synthesis. Radiation induced mutagenesis is due to the involvement of multiple damaged sites (Ward, 1995), which are incompletely or incorrectly repaired DNA lesions. Strand breaks are considered to be the most important lesions (Ali *et al.,* 2007) as they suspend the connection and reliability of the double helix. An unrepaired single strand break in simple strand DNA (Fiers and Sinsheimer, 1962); an unrepaired double strand break in double strand DNA (Frankenberg-Schwager *et al.,* 1981) and cross-linking of DNA to itself or proteins were identified with the lethal effects of ionizing radiations (Cress *et al.*, 1991). This, and the fact that over-production of cells, which were not supported by cell differentiation at the apical meristem, were contributing factors to the stony head observed. Therefore the ratio of auxin to cytokinins in certain plant tissues determines initiation of specialized meristematic cells such as root versus shoot buds and their further differentiation into various plant organs. Gamma irradiation could therefore distort this ratio and alter the course of normal shoot and root developments into altered traits mostly observed.

3.5.5 Changes in iodine interaction with starch

The extraction of starch from the putative free-sugar mutant was difficult and as a result little starch was available for other physical and biochemical analyses. The detailed enzymatic basis of free-sugar cassava mutants was explained in Chapter 2.5.9-10. Some scientists explained free-sugar mutants in terms of reduction in amylose, leading to over accumulation in an excess of 100 times more free sugar (mainly glucose) than commercial varieties (Carvalho *et al*., 2004). Weak acids such as glucuronic acid or galacturonic acid and minor sugars such as mannose, also accumulate in free sugar plants (Reiter *et al.,* 1999). It is the amylose fraction of starch that reacts with iodine to give a blue-black colour in normal starch granules. It was reported that amylose-free starches, such as those from waxy cassava and cereal mutants, stained red with iodine solution. However, some other cereal endosperm mutants also stain abnormally with iodine solution. For example, the endosperm of sugary 1 mutants of rice contains a red-staining soluble glucan called phytoglycogen, as well as blue-staining starch granules (Nakamura *et al.*, 1996). Therefore a failure of starch to turn blue-black with iodine solution implies that waxy mutants and free-sugar mutants are suspected. Lack of amylose in addition to the observation of the blue-black colour implies that a waxy mutant is the most probabable candidate. The reduction in amylose content, in addition to the accumulation of reducing sugars, makes starch turn reddish brown with the iodine solution, therefore, makes the free-sugar mutant a prime suspect.

The importance of field (Fig. 3.5c) and laboratory (Fig. 3.5d) iodine tests is in its ability to identify amylose containing plants from amylose-free (amylopectin) mutants at an early stage in order to reduce the cost of mutation breeding. When the amylose level of starch falls far below 10%, the brown colour deepens, signifying the presence of more waxy starch. The brown colour clearly signifies that starch (amylose) biosynthesis was altered in plants N281P16, N357P5, N394P82 and N441P54 (Fig.3.5 c-d) affected by gamma irradiation. Comparing Fig. 3.5d to the reported free-sugar pictures of Carvalho *et al.* (2004) (Fig. 3.6, PANEL A) and reported free-amylose pictures of Ceballos *et al.* (2007) (Fig. 3.6, A and B), and personal communication Dr. Hernan Ceballos (author of 64) led to the conclusion that the putative mutant may be similar to the free sugar cassava mutant reported by Carvalho *et al.* *(*2004). Therefore plants N281P16, N357P5, N394P82 and N441P54 might be novel free-sugar mutants.



Source: Carvalho *et al.* (2004)

Source: Ceballos *et al. (*2007)

Fig. 3.6. The reaction of iodine solution with storage root cassava starch. Normal starch reacts with iodine to form blue-black but mutant starch form a reddish brown colour. Brown colour could mean free sugar is present as in panel A (CAS36.1 and CAS36.0) (Carvalho *et al.*, 2004), or amylopectin is present as in A and B (Ceballos *et al.,* 2007). To know which one is present, a glucose strip test is required. Free sugar forms a brown colour (B2 and B3) with a glucose strip (panel B, Carvalho *et al.*, 2004) but amylopectin does not react (B1)

Other scientists explained the free-sugar cassava plants in terms of a reduction in amylopectin production which leads to an accumulation of free sugars. Although starch is not soluble, it becomes water soluble free-sugar starch (phytoglycogen) only when enzymes responsible for amylopectin packing into an organized crystalline structure (debranching enzyme responsible for ∝-1-6 glucan hydrolysis) are anti-sensed to produce distorted crystalline structure (Taylor *et al.*, 2004). This is in agreement with Carvalho *et al.* (2004) who reported a free-sugar cassava with a glycogen-like starch. It possessed the gene coding for the ADPGpyrophosphorylase small sub unit needed for glucose residue production but lacked the gene coding for branching enzyme. The reduction in branching activity could mean over production and accumulation of linear α -1-4 amylose, increase in amorphous region, decrease in amylopectin and decrease in crystalline region of starch granules. The excess amylose production could lead to a shut down mechanism of amylose biosynthesis pathway but activation of free-sugar parthway. As over activity of gene coding for the ADPGpyrophosphorylase would cause an accumulation of glucose residue which accumulates as phytoglycogen. This is a diversion from normal water insoluble starch biosynthesis pathway to abnormal water soluble glucose-like starch pathway.

3.6 Conclusions and recommendations

The early characterization of M_1V_1 plants developed from cassava stakes irradiated with Co^{60} source of gamma radiation was helpful in the identification of a linear shape solid mutant and chimerical mutants. Most of the chimera mutants were not expressed in the M_1V_1 stage due to apical dominance in the shoot system but were fully expressed during the M_1V_2 stage when apical dominance was destroyed by removal of the apex of induced plants during the vegetative cutting of stems to be used as planting material in the M_1V_2 stage. Since apical shoot dominance had no effect on the root system, a chimera was clearly expressed in the root rind colour at the M_1V_1 stage. The good aspect of the chimera was that it indicates whether dose rate applied was efficient or not for predicting mutation rate in the population. The identification of chimera and solid mutants among plants indicated that the irradiation dose applied was effective and could lead to identification of *in planta* modified starch. Eventually, four putative mutants were identified with the iodine test to possess altered starch qualities, suspected to be a novel free-sugar cassava mutant at the M_1V_2 stage. It is recommended that blue-staining starch granule tests be conducted on putative free-sugar mutants N281P16, N357P5, N394P82 and N441P54 for confirmation of their free-sugar status in the subsequent mutation breeding stages, which was not covered here due to a lack of funding.

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CHAPTER 4

Variations in the anatomy of starch granules of induced cassava mutants

4.1 Abstract

Induced plants were evaluated individually based on morphological traits of starch granules using light and scanning electron microscopy. Many *in planta* mutations of starch granules occured, but not all of them are useful. A small number of useful ones were selected. Control landraces produced only up to trivalent compound starch granules, but induced putative free-sugar mutant N357P5 produced up to dodecavalent (12) compound starch granules. Linear central leaf lobe solid mutants (N167B, N169P4) and putative free-sugar mutants (N281P16, N357P5, N394P82 and N441P54) produced fissured granules but plants N398P3 and N481P26 showed highly fissured granules with higher water absorption properties and flake production properties than the rest. A monovalent granule producing plant (N110P2) simultaneously produced small uniform granules with smooth surfaces without dents/holes or compactness and fissures and were therefore suspected of having higher resistant starch properties compared to the control.

4.2 Introduction

A starch granule is solid material with a density of approximately 1.5 g/ml (Hoover, 2001). On average, a normal granule contains one-quarter amylose and three-quarter amylopectin. These macro molecules are insoluble in water at room temperature. They therefore exert a minimal osmotic effect on the plant cells in which they are found. Starch can be found in leaves, stems, pollen grains, roots (cassava and sweet potato), tubers (potato, yam), cereal grains (wheat, rice, corn, oat and barley) and fruits (banana, plantain). The sugar produced from photosynthesis is stored in leaf chloroplasts as small transitory starch granules during the day and in the night excess granules are hydrolyzed to sugar and translocated through the phloem to amyloplasts for storage. Transitory starch has a smaller granule size than the reserved starch. The sizes of the cassava starch granules range from 2 - 40 μm (Moorthy, 2002). The size and shape of the amyloplast is species dependent (Davis *et al*., 2003). Mutation induction could be used to modify the size and number of amyloplasts which would result in tailoring granule size distribution *in planta* (de Pater *et al*., 2006; Shapter *et al.*, 2008). Small sized amyloplasts could produce small sized starch granules and vice versa (de Pater *et al*., 2006). The modification of starch is relevant when considering the mechanism of starch degradation by enzymes, because enzymes are bond specific and can therefore hydrolyse only substrates complementary to their active site. As a result selective hydrolysis of amylose leaving amylopectin lamellae can be achieved when amylose specific enzymes are employed. It would not make much difference to acid hydrolysis of granules since acid provides broadbase hydrolysis of both amylose and amylopectin bonds at the same time. Many glucan metabolizing enzymes were inactive to native starch granules (Buléon *et al*., 1998). Smaller granule sizes present a superior surface area to volume ratio for a faster rate of enzymic hydrolysis (Shapter *et al.*, 2008). Varieties of maize with small starch granules were reported to have low amylose content (Sandhu *et al.*, 2004; Choi *et al.*, 2004). Large wheat granules were reported to have an increased enthalpy of gelatinization, lower gelatinization temperatures (Peng *et al*., 1999; Singh and Kaur, 2004) and result in softer textured flours than smaller granules (Gaines *et al*., 2000).

Larger than normal pores or holes in granules could also be induced by mutagens. The dents/holes, pores, pinholes or fissures on the surface of granules could aid hydrolysis of starch (Fannon *et al*., 2004; Benmoussa *et al*., 2006) because they funnel α-amylase enzymes directly to the centre of granules (Huber and BeMiller, 2001) and erode granules inside out (Fannon *et al*., 2004). The dents/holes, pores, pinholes or fissures would therefore increase cassava starch digestibility and expand its end uses.

Light microscopy (LM) produces coloured pictures which is ideal for near *in situ* characterization of starch granules. Scanning electron microscopy (SEM) produces three dimensional monochromatic images which could be used to study the ultra-surface structure of dried granules as a complementary tool for LM (O’Connell *et al.*, 2008). In the previous chapter solid mutants and chimerical mutants were identified at the M_1V_1 stage which was confirmed at the M_1V_2 stage during plant morphology and *in planta* starch modification studies. In this chapter LM and SEM were used to identify induced cassava plants with useful *in planta* modified starch granules.

4.3 Materials and methods

4.3.1 Irradiation, planting and harvesting of surviving M_1V_1 and M_1V_2 plamts

A total of 1689 stakes (stem cuttings with an average of five adventitioud buds) from four elite landraces of cassava were irradiated with 35 Gy at the Gamma Irradiation Centre (GIC) of the Ghana Atomic Energy Commission (GAEC) on 13 June 2007 for *in planta* modification of starch (see chapter 3 sections 3.3.1-3.3.4 for details). Irradiated stakes were planted on the 14^{th} of June, 2007 at the GAEC research field without fertilization, with un-irradiated four landraces serving as controls. The field was planted in line and in continuous serpentine turnings. All of the M_1V_1 plants were harvested one year after planting and analysed with LM and SEM. The M_1V_2 field was planted with 8442 induced stakes from M_1V_1 plants (HO008 (4966), HO001 (353), UCC090 (1614) and UCC026 (1509)) in 2008 which was harvested in 2009. Though all the plants' storage roots were tested with iodine, not all the M_1V_2 plants were harvested, due to the large population size and the need to evaluate plants individually. The following sampling strategy was used: Plants with clean to mild levels of African Cassava Mosaic Disease (ACMD) were identified and three plants with bigger diameter of the basal stem were randomly selected from plants arising from common cell lineage of M_1V_1 or related to the same M_1V_1. These three plants were harvested and fresh root weight determined separately with a scaled-pan. Percentage starch content was determined on a fresh root weight basis for each plant using the specific gravity method of Brautlecht (1953) as modified by the Danish EU-directorate (1996). Root samples from each plant were taken for characterization with LM and also for starch extraction for ultra-structure evaluation with SEM.

4.3.2. Characterization of granules by light microscopy

The transverse section of a freshly harvested storage root was pressed on a clean microscope slide to leave prints of starch granules near *in situ* (it is assumed that the storage root prints draw the picture of the transverse surface of the cassava root on the slide with starch granules that are not mechanically damaged and are representative of the same state as found in the living root. The root prints were used for establishing the identity of plants. The starch prints were stained with a drop of 2% potassium iodide (2.0 g KI dissolved in 75% ethanol) solution and covered with a cover slide. Care was taken not to dislodge or shift the components of compound granules. The samples were examined with an Exacta Optical Technology microscope (Mod. B3, S/n 220434, Germany). Ten pictures were randomly taken across each slide at 100x fixed magnification of the microscope and 4x fixed magnification of the digital camera attached to the microscope, thus making a total of 400x magnification for each sample examined. Three diameters were determined for three radomly selected granules from each picture, and a total of 30 diameters were measured across 10 pictures per genotype. The average image diameter size per genotype was determined and the object diameter size calculated using a formula:

$$\text{Object size} = \frac{image\ size}{magnification} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (1)\ .$$

The object sizes were used to classify the size of granules as small (1-10 μm), medium (11-25 μm) and large (>25 μm) (Lindeboom *et al*., 2004). Other morphological features of granules were determined based on the descriptors developed and presented in Figures 4.1: 1-11.

4.3.3 *Ex situ* characterization of granules by scanning electron microscopy (SEM)

4.3.3.1 Starch extraction and drying

Peeled cassava roots were washed with tap water and 500 g was chopped into 2- 4 cm^3 chips with stainless steel knives. It was blended in 500 ml tap water at 2000 rpm for 5 min in an industrial blender. The slurry was then mixed with 1 litre of tap water and sifted through a 500 μm mesh sieve (Endecotts Ltd., London) into a plastic bucket. Another litre of tap water added to the starch slurry, mixed and the starch left to settle for an hour. Well extracted cassava starch settles between 30 - 60 min and gives a good yield which is free from colour, proteins and fats (Moorthy, 1994). The supernatant was decanted and the residue dried at room temperature under ceiling fan until the moisture level dropped below 12%. Dried starch was bagged, labelled and sealed until further use.

4.3.3.2 SEM of starch granules

An aluminium pin stub was dipped into the dry starch with a moisture content between 8.5-11.9% determined with near infrared spectrometry (Type 8100 No. 334, PerCon Prüfgeräte GmbH, Germany) to pick up granules which were ready for sputter coating. For flake property determination of granules, two dry aluminium pin stubs were dipped simultaneously into the starch sample to pick up some granules by adhesion and the granules were crushed between the two stubs before sputter coating. Mounted starch granules were coated with gold (20-30 nm thick) for 135 s at 20 mA in a Biorad sputter coater (Microscience division, England) and examined using a Jeol 6400 SEM (Tokyo, Japan). Images were captured by Orion version 6 digital capture cards (E.L.I. *sprl*, Belgium).

4.3.3.3 Water absorption properties

Moisture in the dry starch sample (50 g) was initially determined by near infrared spectrometry (Inframatic 8100, No. 334, PerCon Prüfgeräte GmbH, Germany). Starch was dried under vacuum for 24 h and after being re-exposed to atmospheric moisture, the moisture was determined daily for a 12 day period. This was carried out to compare the moisture content, moisture loss and moisture re-absorption of cassava starch from control landraces to two flaky starches obtained from induced N398P3 and N426P26 plants.

4.4 Results

4.4.1 The surviving stakes of the M_1V_1 and M_1V_2

Table 4.1 represents the number of surviving plants from the M_1V_1 and M_1V_2 stages. Control landraces were HO008, HO001, UCC090 and UCC026 and surviving induced plants (enclosed) are presented in column 1. A total of 476 M_1V_1 and 8177 M_1V_2 plants survived. Also the average numbers of surviving M_1V_1 and M_1V_2 plants were 28% and 97% respectively (a 69% difference). The number of M_1V_2 plants was 1617.9% more than M_1V_1 plants. The number of cuttings of M_1V_2 plants had 100% specific landrace survival except HO008 which had 95% survival.

Table 4.1 Plant survival rate at the M_1V_2 generation

Plants	**Number of stakes**			**Number of stakes from M_1V_1 plants**		
Landrace (Induced)	Irradiated (M_1)	Survived (M_1V_1)	Survived within a landrace (%)	Planted	Survived	Survived within a landrace (%)
HO008 (N1-N349)	840	349	41.55	4966	4701	95
HOO1 (N350-N390)	218	41	18.81	353	353	100
UCC90(N391-N432)	505	42	8.32	1614	1614	100
UCC26(N432-N476)	126	44	34.92	1509	1509	100
Average	422	119	28.18	2111	2044	97
Totals	1689	476		8442	8177	

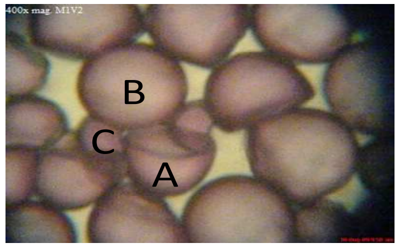
4.4.2 Morphological descriptors for characterization of granules

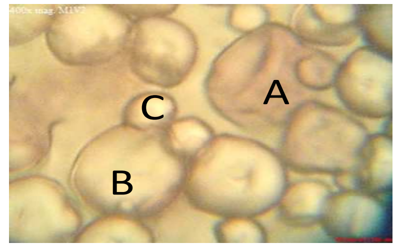
The morphological descriptors developed for near *in situ* and *ex situ* characterization of cassava starch granules are shown in Figures 4.1:1-11. These descriptors covered the size and shape of granules, the presence or absence of compound granules, multiple-head granules, dents or holes in granules, compact granules, smooth or rough surface of granules, deep fissures and surface protrusions.

Figure 4.1.(1-11). Descriptors for near in situ characterization of cassava starch granules using coloured LM (400x enlargement) photographs and ex situ characterization using monochromatic SEM photographs.

1. Types of monovalent starch granules from root pulp (Parenchyma) (400x).

An “A” granule is half bowl shaped (A). The “B” granule is round or oval (B). The “C” granule is irregular in shape and smaller than “A” and “B” (C).





2. Size of starch granules from root cortex (rind) (400x) *Care should be taken to avoid contamination of root pulp granules with cortex granules on slide).

1 small (1-10µm)
2 medium (11-25µm)
3 large (> 25µm)

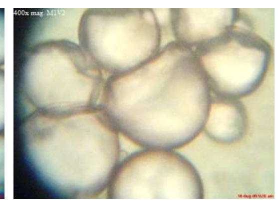
small (1-10µm)

3. Size* of starch granules from root pulp (Parenchyma)(400x)

1 small (1-10µm)
2 medium (11-25µm)
3 large (> 25µm)


small (1-10µm)

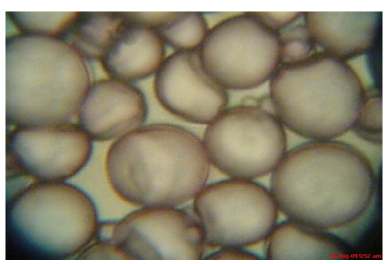

(11-25µm)

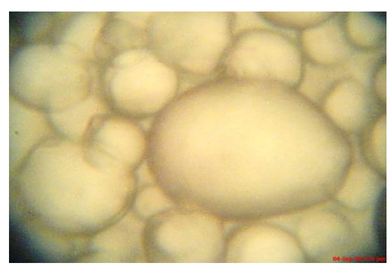

C (>25µm)

*Cassava granules should be classified as small (5-10 μm), medium (10-25 μm) and large (>25 μm) (Lindeboom, *et al.*, 2004).

4. Form of starch granules from root pulp (Parenchyma) (400x)

1 uniform *
2 non-uniform


uniform

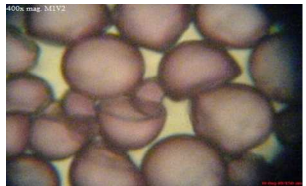

non-uniform

* A granule is uniform when 2- 10 photographs taken across a slide produce similar granule sizes, and if there is not much variation in sizes of granules in a particular photo.

5a. Type of compound* granules of starch from root pulp (Parenchyma) (400x)

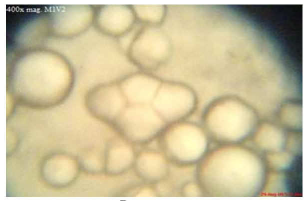
(*compound granules composed of two or more large granules stacked together)

1 monovalent (monov)
2 divalent (div)
3 trivalent (triv)
4 tetravalent (tetrav)
5 pentavalent (pentav)
6 hexavalent (hexav)
7 heptavalent (heptav)
8 octavalent (octav)
9 nonavalent (nanov)
10 decavalent (decav)
11 undecavalent
12 dodecavalent


monovalent


divalent


trivalent

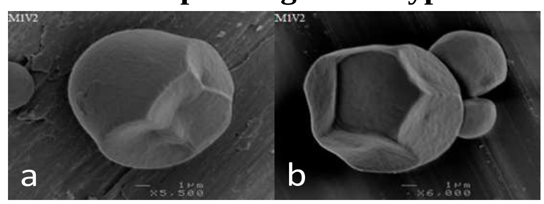

tetravalent


dodecavalent


undecavalent

5b SEM pictures confirming the presence of different compound granule types with large marks all over the surface.

Tetravalent (a) and hexavalent (b) types of compound granules with three and five large marks on the surface of disengaged granules.



6a. Multi-head* of a starch granule from root pulp (Parenchyma)(400x)

(* Multi-head granules composed of many small granules attached at one side)

1 present
2 absent


present

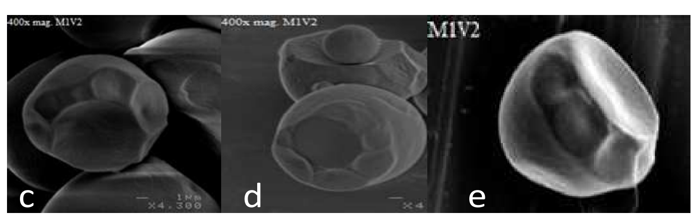

present


absent

6b. SEM pictures confirming the presence of multi-headed* granules

*Confirmed with SEM by many small attachment points at one side of disengaged granules

The disengaged granular marks (c-e) left on the head of granules were small and more than those normally observed on the surface of compound granules.

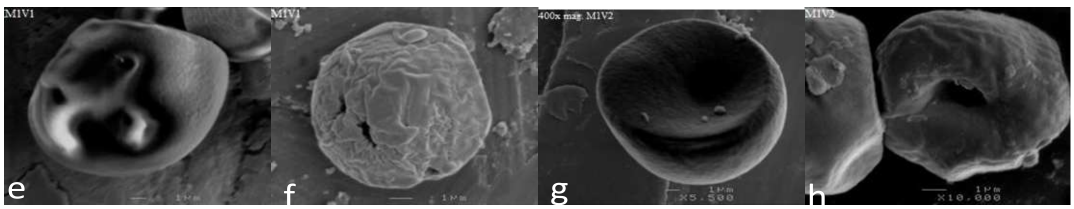


7a. Dents/holes on the surface of starch granules from root pulp (parenchyma)(400x

1 present
2 absent

present present absent

7b. SEM pictures (e-h) confirming presence of dent/hole on the surface of starch granules .



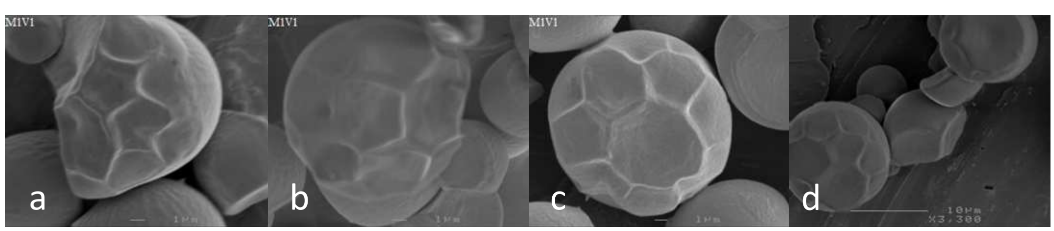
8a. Compact* starch granule from root pulp (parenchyma) (400x)

* occurred in high yielding clones when individual granules are in physical contact, these contact marks are irregular.

1 compact
2 Non-compact

compact compact non-compact

8b. SEM pictures (a-d) confirming the presence of compact granules



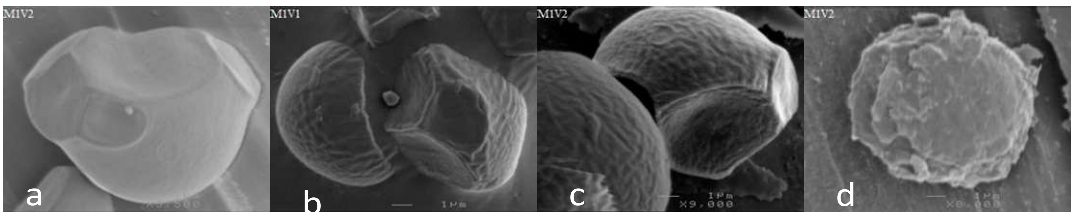
9a. Smooth or rough surface of starch granule from root pulp (parenchyma) (400x)

1 smooth surface
2 rough surface

smooth surface

rough surface

9b. SEM pictures confirming the presence of smooth (a) and rough (b-d) surface of granules



10a. Deep fissures on the surface of starch granules from root pulp (parenchyma) (400x)

1 present
2 absent

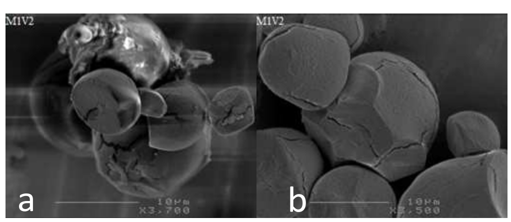
present

present

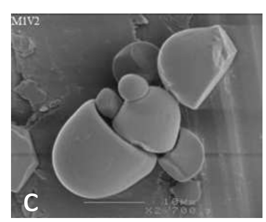
present

10b. SEM pictures confirming the presence and absence of deep fissured starch granules

Presence of exceptional fissured on granules of clones N398P3 (a) and N426P26 (b) but absent (c) in the native clones UCC90 and UCC26.



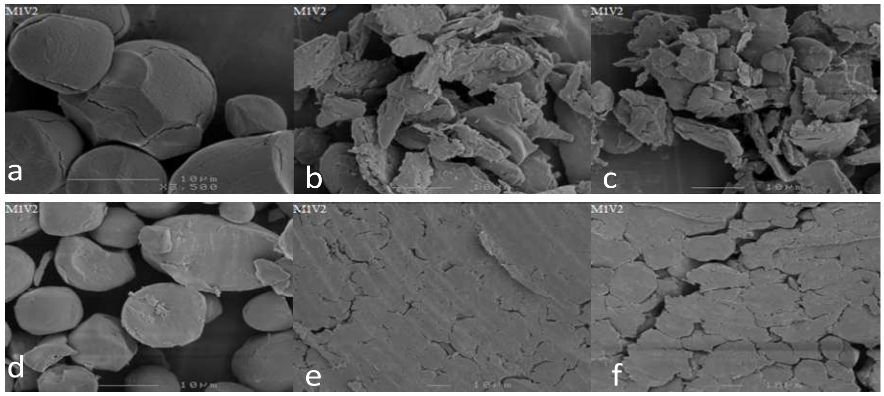
present present



absent

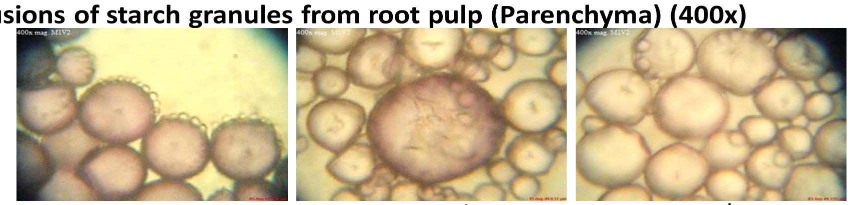
10c. SEM pictures showing the flaking potential of fissured starch granules

The fissured granules from irradiated clones (a) produced flakes (b-c)(N398P3, N426P26) but non-fissured granules of native clones (d) produced a paste (e-f) instead of flakes



11. Surface protrusions of starch granules from root pulp (Parenchyma) (400x)

1 present
2 absent



Present (on multi-head granules) present present

4.4.3 Characterization of cassava starch granules with light microscopy

Figure 4.2 shows the histogram of starch granules based on the near *in situ* characterization of 1302 storage roots sampled from M_1V_2 cassava plants according to the classification system described in Figure 4.1. There were 108 plants producing small (1-10 μm) size granules, representing 8% of 1302 plants analyzed. This was followed by 628 medium size (48%) and 566 large size (43%) granules. The total number of plants producing uniform granules was the same as non uniform granules (50%). One-quarter (325) of plants had compound starch granules and three-quarter (977) had single starch granules. Only 108 plants (8%) produced granules with multiple heads and the rest (1194; 92%) with single heads. The number of plants

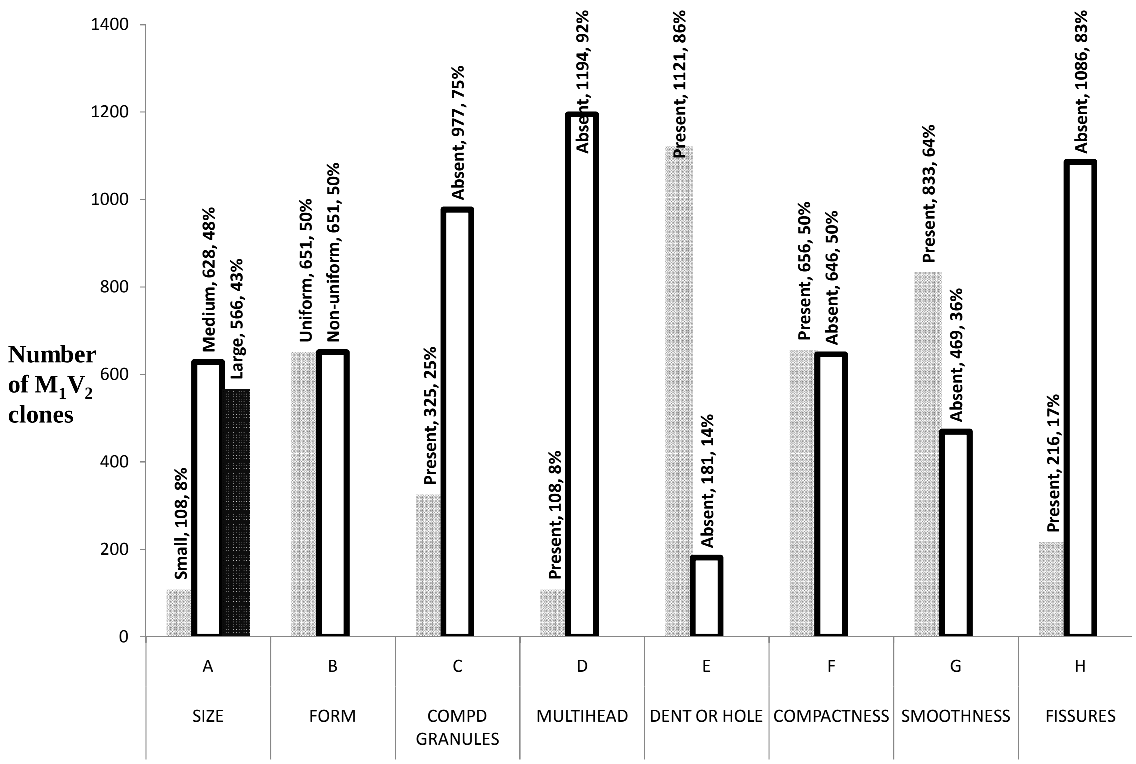


Figure 4.2: Light microscopy (LM) characterization of starch from 1302 M_1V_2 cassava plants into size, shape (form), compoundness, multi-head, dent/hole, compactness, smoothness and fissures on the surface of granules. A-H are keys for various surface features of granules as explained under Table 4.2.

with multi-heads (108; 8%) was very small as were those with small granule sizes. Granules with dents/holes were found to be common (1121; 86%) compared to granules without them (181; 14%). The percentage of compact granules (656; 50%) was the same as non-compact granules (646; 50%). The smooth surface granules (833; 64%) were more than rough surface granules (469; 36%). Granules with fissures were smaller in number (216; 17%) than granules without them (1086; 83%). Based on 1302 rinds analyzed it was also observed that all cassava rinds (root cortex) produced smaller size compound starch granules than storage root flesh. Care should be taken that these are not transferred during cutting for the preparation of the microscope slides.

Figure 4.3 shows detailed LM characterization of starch from 1302 M_1V_2 cassava plants into compound granules. One-quarter (325; 25%) of plants analyzed had compound starch granules and three-quarter (977; 75%) had single (monovalent) starch granules. The monovalent granules could be classified further into A, B or C type granules based on shape (Figure 4.1.1) whilst further classification of compound starch granules was based on the number of granules adhering together instead of shape (Figure 4.3). The following had produced only one type of compound granule in the pool of monovalent granules: A total of 50 plants (N113 P5, N125P5, N298 P3, N46P12, N56P5, N58P4, N94P3, N137P4, N148P2, N266P3, N280P6, N283P16, N308P5, N316P3, N332P4, N367P2, N51P8, N183P13, N207P4, N234P9, N275P2, N278P2, N281P15, N292P2, N302P18, N304P11, N314P3, N329P11, N451P9, N481P25, N106P5, N144P20, N193P19, N219P5, 237P4, N291P3, N306P5, N446P4, N42P2, N81P4, N119P10, N133P3, N155P3, N171P14, N351P4, N390P6, N468P8, N188P6, N400P2 and N279P6) (4%) produced divalent compound granules, 168 plants (13%) produced trivalent compound granules, four plants (N56P2, N105P3, N311P8, N398P8) (0.31%) produced tetravalent compound granules, one plant (N225P20) (0.08%) produced pentavalent compound granules and one plant (N59P2) (0.08%) had hexavalent granules in the midst of monovalent granules. The rest produced combinations of compound granules in the midst of monovalent granules. For example, there was one plant (N415P5) with a mixture of divalent and tetravalent compound granules found in the same root, representing 0.08% of 1302 plants analyzed. In a similar manner of abbreviation of valences of granules as in Figure 4.3, 60 plants (N162P10, N162P10, N71P7, N113P2, N132P4, N160P12, N241P2, N187P2, N114P4, N167P13new, N192P14, N254P5, N267P4, N314P6, N104P5, N108P14, N125P8, N138P5, N162P15, N162P15, N173P7, N187P2, N247P6, N155P2, N345P4, N354P4, N313P6, UCC090 (control), N336P14, HO001 (control), N305P5, N432P5, HO008 (control); N413P2, UCC026 (control), N464P8, N111P5, N116P9, N74P3, N55P12, N85P4, N125P3, N128P2, N202P12, N132P2, N158P2, N75P3, N95P2, N168P7old, N405P12, N277P4, N341P6, N266P6, N272P3, N304P3, N106P9, N442P113, N478P3, N300P7 and N405P11) produced a mixture of divalent and trivalent compound granules *in planta* (5%). Also, 12 plants (N107P4, N108P19, N122P7, N113P3, N118P23, N129P6, N107P17, N154P8, N299P4, N343P2, N167P11 and N355P3) produced divalent, trivalent and tetravalent granules as a mixture in the root, representing 1%, two plants (N124P7, N341P11) had a mixture of divalent, trivalent, tetravalent, and octavalent compound granules in the root, representing 0.15%, three plants (N107P17, N200P2 and N343P2) with a mixture of divalent, trivalent, tetravalent and pentavalent granules in the storage root (0.23%), one plant (N145P5) had divalent, trivalent, tetravalent and hexavalent compound granules (0.08%). One plant (N200P2) had divalent, trivalent, tetravalent, pentavalent and octavalent compound granules (0.08%), while two plants (N96P3, N104P2) had divalent and octavalent compound granules producing storage roots with a mixture of compound granules (0.08%). One plant (N122P8) had divalent, trivalent and pentavalent compound granules (0.08%). There were nine plants (N104P2, N339P3, N224P3, N267P5, N357P5, N63P7, N129P4, N214P6 and N412P7) with trivalent and tetravalent granules in the same root (0.69%), while one plant (N274P2) had a mixture of trivalent and pentavalent granules in the storage root (0.08%), three plants (N225P9, N384P8 and N238P13) had combined trivalent and hexavalent granules in the same root (0.31%), two plants (N81P2 and N262P11) had trivalent and octavalent compound granules produced in the same root (0.15%), two plants (N187P8, N154P4) with trivalent, tetravalent and heptavalent compound granules produced in the same plant (0.15%). Finally the putative free-sugar mutant (N357P5) had the most diverse compound granules because it had trivalent, tetravalent, hexavalent, undecavalent and dodecavalent granule combinations in the same storage root, and representing 0.08% of 1302 plants analyzed (Figure 4.3).

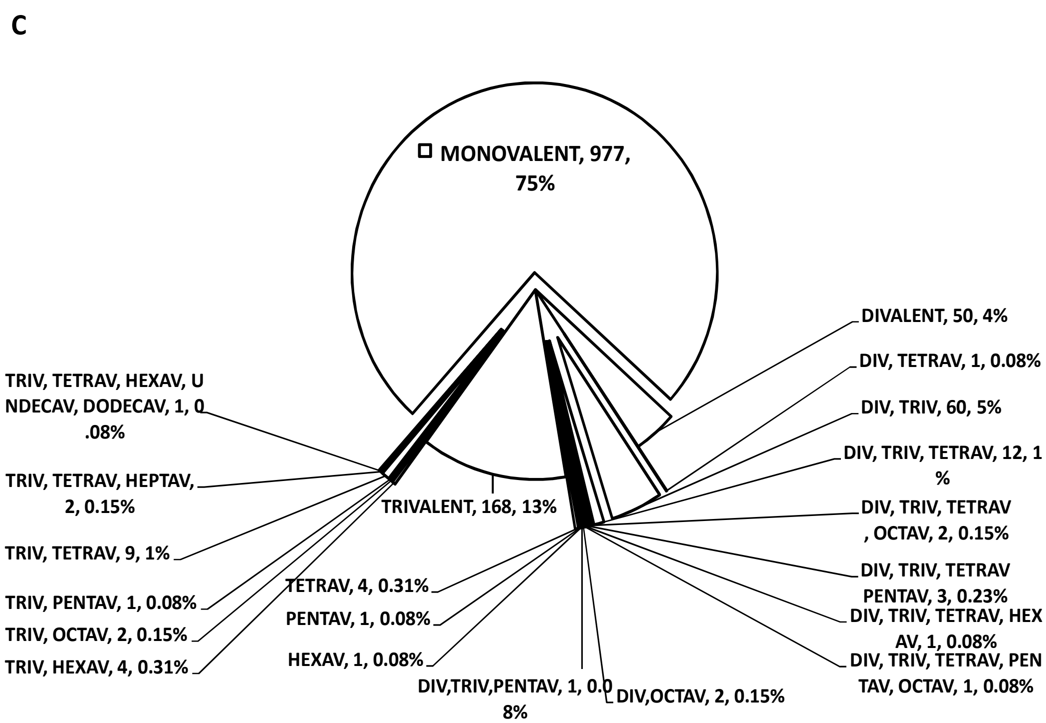


Figure 4.3: Detailed light microscopy (LM) characterization of starch from 1302 M_1V_2 cassava clones into compound granules (C)

Table 4.2 shows a few examples of how starch granules may differ within the different clusters in the dendrogram. All control starch granules had a large size and non-uniform form of compound granules up to trivalent, with presence of dents/holes. They however, lacked multi-headed and deep fissured granules. Some of the control starch granules had compact and smooth granular surfaces (Table 4.2). After irradiation of the HO008 landrace, differences in starch granules were observed from induced plants. Normal plants with oblanceolate central leaf lobes (N167A) and lanceolated (linear) central leaf lobe mutants (N167B) produced granules which were distinct from each other, although they germinated from different adventitious buds located on the same 15 cm piece of stake. Some traits were observed in putative mutants but not in the control. The oblanceolate plants produced only monovalent starch granules. In addition, a putative free-sugar mutant (N281P16) also differed from the control in terms of medium sized starch granules with deep fissured granules. A putative mutant, N110P2, produced small sized and uniform starch granules, monovalent and single headed granules, lacking dents/holes and compactness but having a smooth surface without fissures. See the dendrogram of induced HO008 plants [Figure 4.4(C3) S; Appendix 4.1. S.; Table 4.2].

The N357P5 plant, derived from induced HO001, produced the most diverse compound granules such as trivalent, tetravalent, hexavalent, undecavalent and dodecavalent poly-granules whilst the control produced up to trivalent granules. The putative free-sugar mutant N357P5 also had multi-heads, rough surfaces and deep fissured starch granules, properties which were absent in the control.

The N398P3 plant, derived from induced UCC090, produced granules which differed from the control starch granules by possessing medium sized, monovalent, compact and rough surface granules. N398P3 also had exceptionally deep fissures and should possess better flaking and water absorption properties than the controls (Figure 4.1:10c; Figure 4.4.8 and Table 4.2). The N422 plant at the M_1V_1 stage had two different kinds of branches which were separated into different plants at the M_1V_2 stage. Granules from the normal purplish green apical leaf colour plant (N422P44) still had large sized granules like the control UCC090.

Table 4.2 Comparison of control to putative mutant granule structures

Induce landraces	SIZE (A)	SHAPE (B)	COMPOUNDNESSS (C)	MULTIHEAD (D)	DENT/HOLE (E)	COMPACTNESS (F)	SMOOTHNESS (G)	FISSURES (H)
HO008 (control)	3	2	DIV, TRIV	2	1	1	2	2
N167 B Linear leaf	2	1	DIV, TRIV, TETRAV	2	1	2	2	1
N167 A Oblanceolate leaf	2	1	2	2	1	2	2	2
N169 P4 Linear Leaf	2	2	TRI	2	1	1	2	1
N281 P16 (free-sugar)	2	2	TRIV	2	1	1	2	1
N110 P2	1	1	2	2	2	2	1	2
HOO1 (Control)	3	2	DIV, TRIV	2	1	2	1	2
N357 P5 (free-sugar)	3	2	TRIV, TETRAV, HEXAV, UNDECAV, DODECAV	1	1	2	2	1
UCC90(control)	3	2	DIV, TRIV	2	1	2	1	2
N394P82 (free-sugar)	2	1	TRIV, TETRAV	2	1	1	2	1
N398 P4	3	1	TETRAV	2	1	1	2	1
N398 P3 (flaky)	2	2	2	2	1	1	2	1
N398 P2	3	1	2	2	1	2	1	2
N422 P44 p.g.a.l	3	1	2	2	1	2	1	2
N422 P114 d.g a. leaf (mut)	2	2	2	2	1	2	1	2
UCC26 (control)	3	2	DIV, TRIV	2	1	2	1	2
N441P54 (free-sugar)	3	2	2	2	1	1	2	1
N481P25 (crack)	3	1	DIV	2	2	1	1	1
N481P26 (flaky)	2	2	2	2	1	2	1	1
N481 P27	3	2	2	2	1	2	1	2

A. SIZE: 1= Small (1-10µm); 2= Medium (11-25µm); 3= Large (More than 25µm).
B. SHAPE: 1= Uniform (all 5-10 pictures have small or medium or large sizes); 2= Non-uniform.
C. COMPOUND GRANULES: 1= Presence of compound granules (this can be expanded as i=monovalent (monov), ii=divalent (div), iii=trivalent (triv), iv= tetravalent (tetrav); v=pentavalent (pentv), vi=hexavalent(hexav), vii=heptavalent(heptav), viii= octavalent (octav), ix = nonavalent (nonav), decavalent (dicav), xi = undecavalent (undca), xii = dodecavalent (dodecav); or 2 = absence of compound granules.
D. MULTIHEAD: 1=presence; 2=absence.
E. ENT/HOLE:1= Presence of granules with holes; 2= Absence of granules with holes
F. COMPACTNESS:1= Presence of compact surface of granules; 2= Absence of compact granules
G. SMOOTHNESS:1= Smooth Surface; 2= Rough surface
H. FISSURES:1= Presence of deep fissures;2= Absence of deep fissures.

Plant N422P44 had uniform granules whilst non uniform starch granules were observed from the dark green putative mutant (N422P114). The starch granules of N422P114 also differed from the control with medium sized monovalent granules. The majority of N422 granules did not differ from the control UCC090 landrace, suggesting that apical leaf colour might not cause significant differences between granular structures of putative mutants and the control.

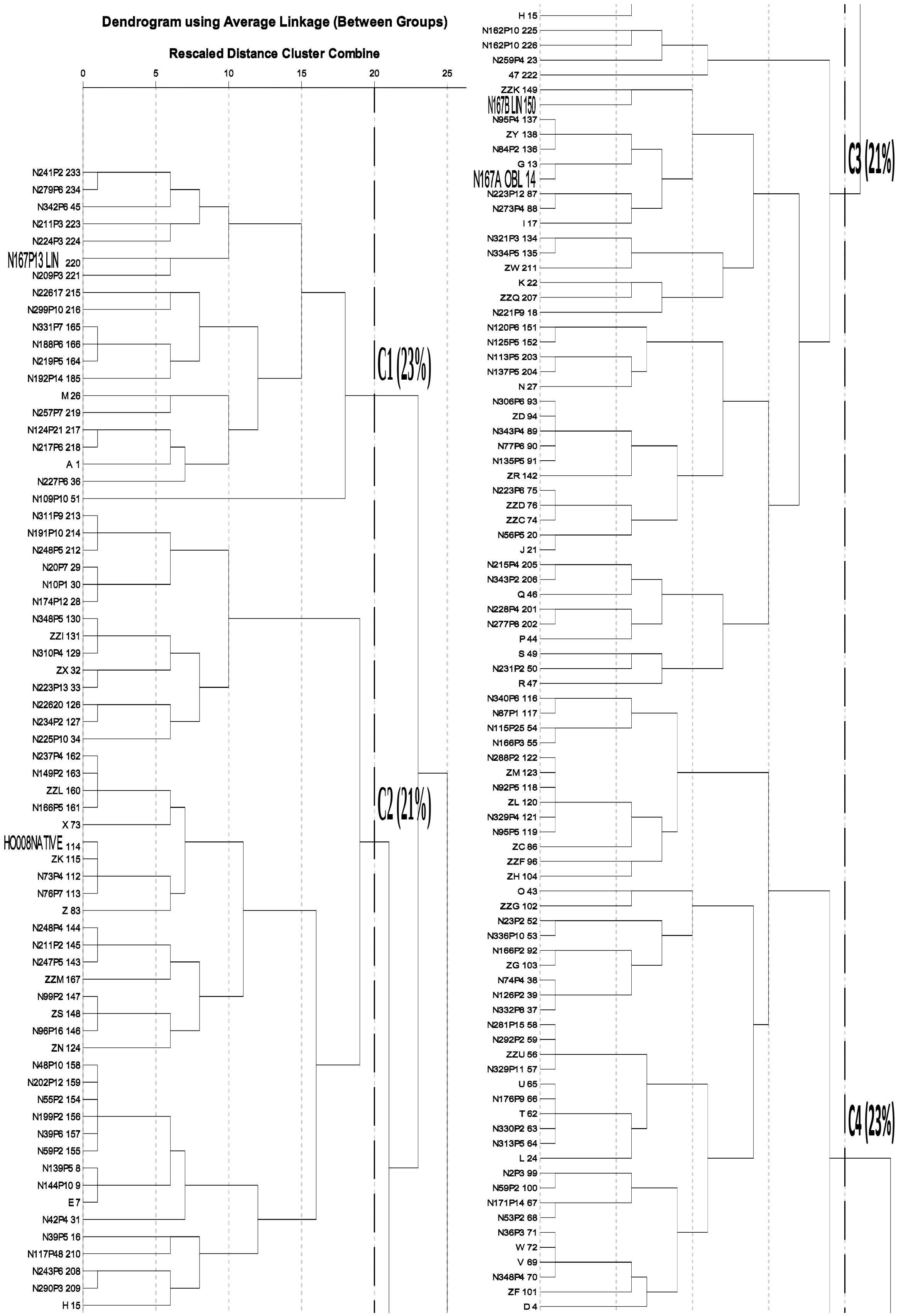
The fourth induced plant UCC026 produced a useful putative free-sugar mutant N441P54, with monovalent, compact, rough surface and deep fissured starch granules. The starch granules of N481P26 differed from the control UCC026 landrace by possessing medium sized granules which were monovalent rough surface granules with exceptionally deep fissures in addition to having different flaking and water absorption properties than the control (Figure 4.1:10c; Figure 4.8 and Table 4.2).

4.4.4. Cluster analysis of cassava starch granule traits

The Predictive Analytics Software (PASW®- applied statistical software version18) was used for the between-groups linkage method of hierarchical cluster analysis of starch granule data. The measured interval was based on squared Euclidean distance and a dendrogram was plotted with names of plants using all clusters of size, shape, compound, multi-head, dents/holes, compact, smooth and deep fissures of granules combined as variables and rescaled distance cluster combine (Figure 4.4 to 4.7). At zero rescaled distance (0% dissimilarity), all plants within the same cluster were similar because they have common starch granule shapes. Plants between 0 to 25 rescaled distances were 0 to 25% different from each other. They belonged to different clusters which may share very few starch granule properties and are therefore considered very dissimilar. Similarity of plants therefore increases toward x=0 whilst dissimilarity increases towards x = 25 rescaled distances. As distance between induced plant and control landrace increases in the dendrogram, the chances of finding useful mutant among non-useful mutants would increases.

Figure 4.4 represents an expanded dendrogram containing clusters of induced HO008 plants. If all the genotypes are represented individually in the dendogram, many pages of dendogram would be required. An expanded dendrogram provided an opportunity for representing a group of “less important” genotypes with letters A – ZZU, whose meaning were explained in Appendix 4.1. The cluster in which the control HO008 occurs (C2) could be separated from the rest by a reference line drawn at an x value of 20, which divided the dendrogram into seven clusters (C1-C7) at 20% dissimilarity. One notable member of C1 is lanceolated (linear) leaf mutant (N167P13 lin) producing starch granules that differed from the control (HO008 landrace) in C2. The C1 is different from C2 at 23% and C2 from C3 at 21%. Both lanceolated leaf mutant (N167B) and oblanceolated leaf (normal) plants (N167A) were located in C3. The C3 was different from both C4 and C5 at 21%. C4 was also different from C5 at 23%. The C5 contained putative free-sugar mutant (N281 P16). The C5 differed from C6 at 23.5% whilst C6 could be differentiated from C7 at 22%.

The dendrogram of HO001 is shown in Figure 4.5. The cluster of landrace HO001 (C1) could be separated from most indced plants by a reference line drawn at an x value of 18, which divided the dendogram into three clusters (C1-C3) at 20% dissimilarity. The control HO001 was more related to some induced plants in the C1 than the putative free-sugar mutant (N357P5) in the C2. The C1 was different from C2 at 19%. C1 and C2 differed from C3 at 25%. The C3 contained only the N374P5 and differed from the rest at 25%.



Dendrogram using Average Linkage (Between Groups)
Rescaled Distance Cluster Combine
0
5
10
15
20
25
C1 (23%)
C2 (21%)
C3 (21%)
C4 (23%)

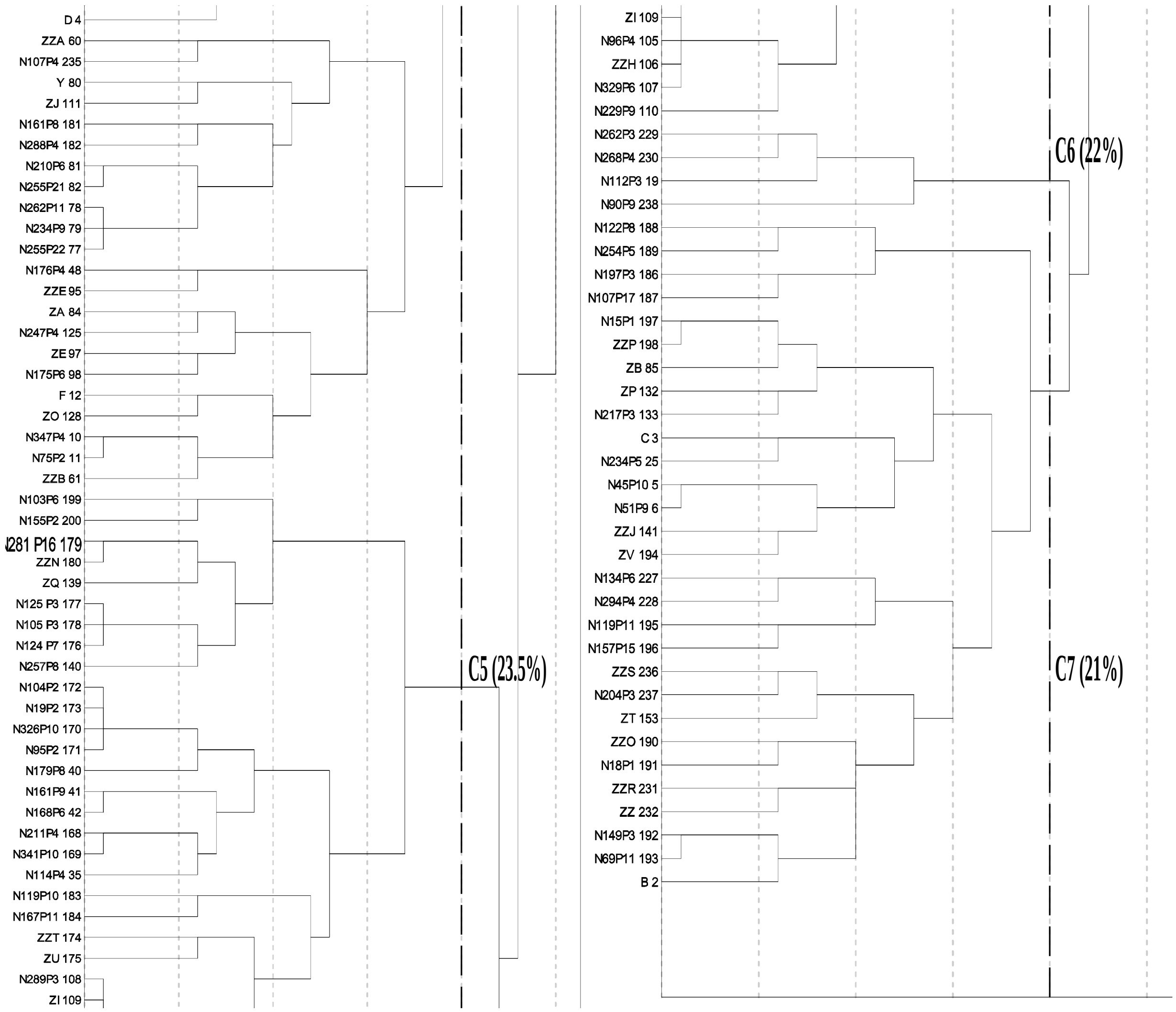


Fig.4.4. Average linkages between group cluster analysis showing an expanded dendrogram of 977 induced and a control HO008 plants based on eight morphological traits of cassava starch granules (not all individuals shown)

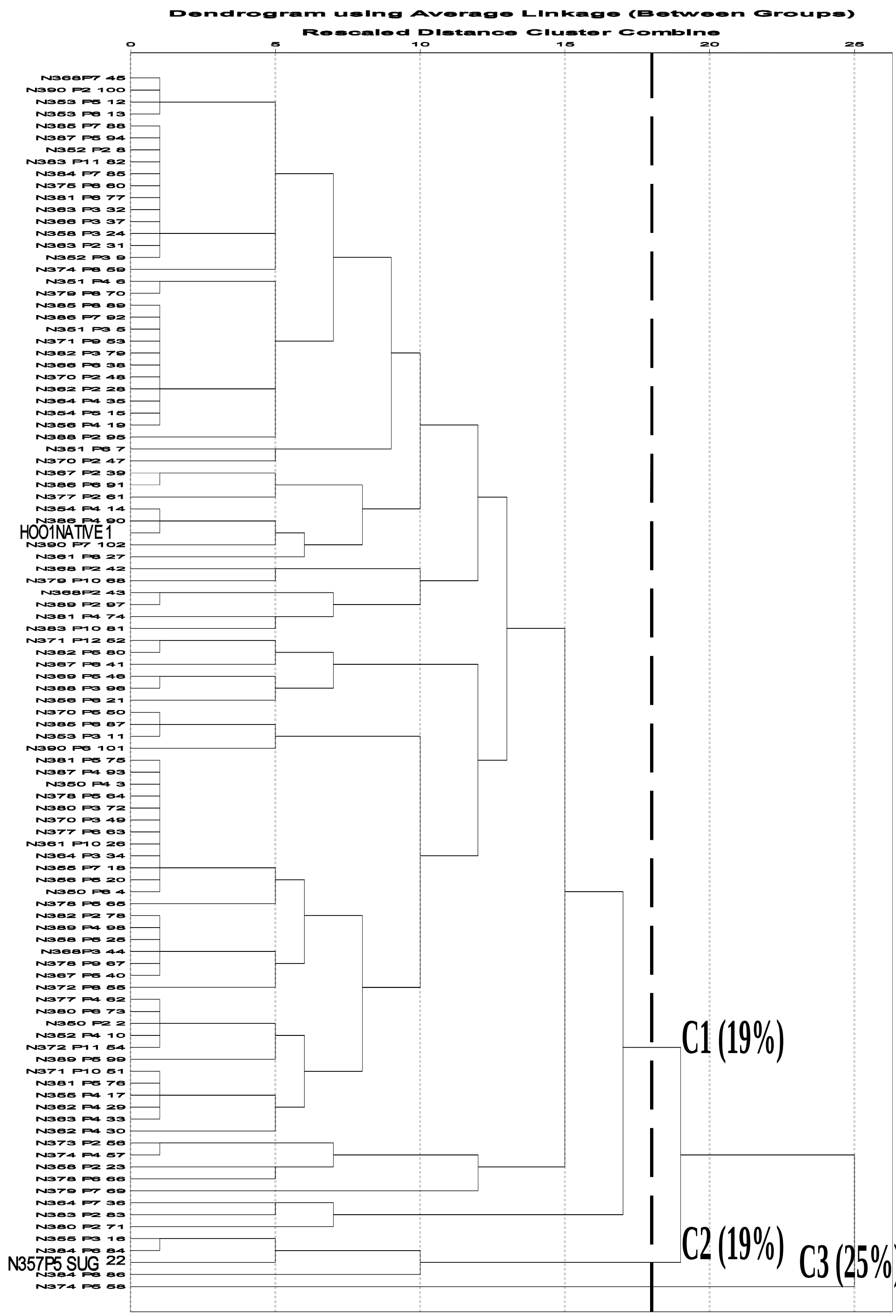


Fig.4.5. Average linkages between group cluster analysis showing a dendrogram of 101 induced and a control HO001 based on eight morphological traits of cassava starch granules

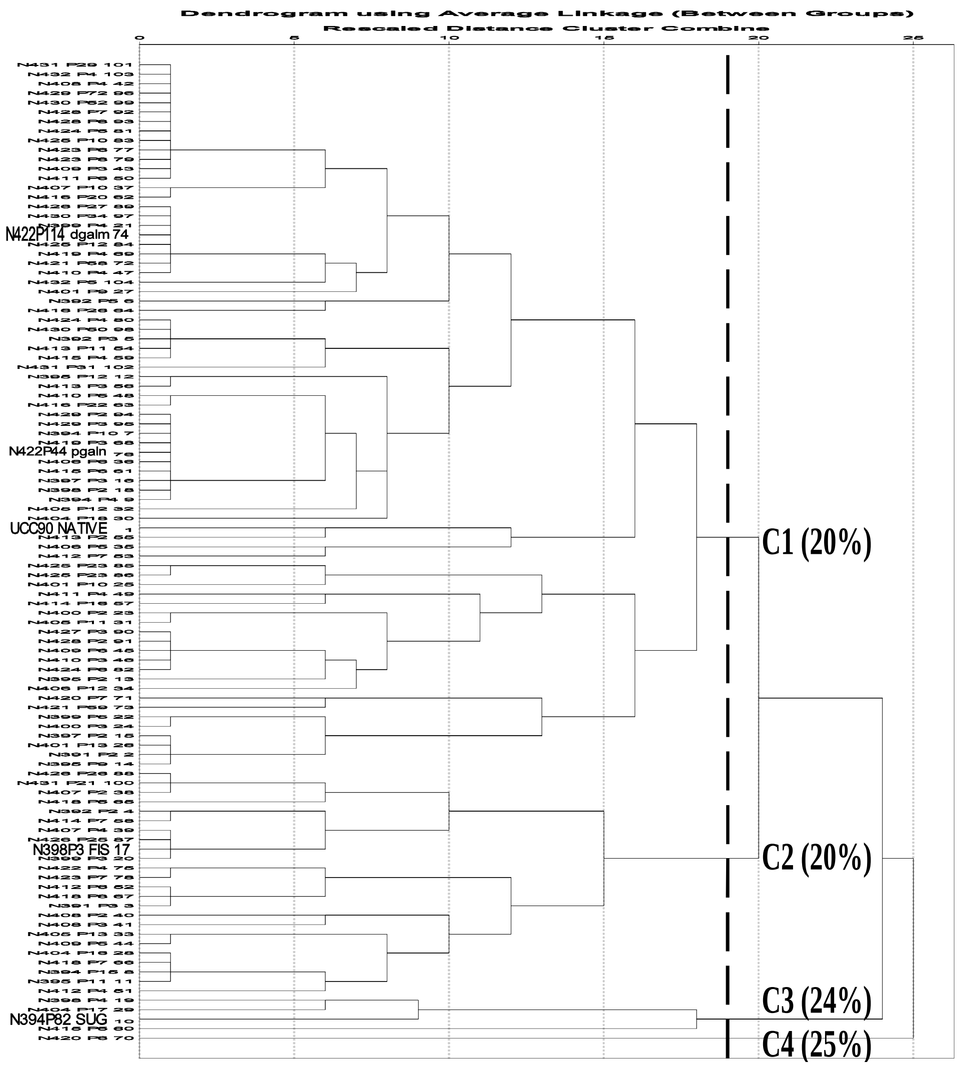


Fig.4.6. Average linkages between group cluster analysis showing a dendrogram of 106 induced plants and a control UCC090 based on eight morphological traits of cassava starch granules

Fig.4.6 shows the dendrogram of UCC090 clusters. The cluster of the control UCC090 (C1) could be separated from the rest by a reference line drawn at an x value of 19, which divided the dendogram into four clusters (C1-C4) at 19% dissimilarity. The C1 was a major cluster containing the putative dark green apical leaf mutant (N422P1 dgalm), purple green apical leaf normal (N422P44 pgan) and the control (UCC090). The dgalm and pgan plants of N422 were dissimilar at 12% in terms of starch granule proprtties and they also differed from the control at 16%. The C1 differed from C2 at 20%. The C2 contained exceptionally fissured granules in N393P3 FIS. The C1 and C2 differed from C3 at 24%. The C3 had a putative free-sugar mutant

(N394P82 SUG) and C4 had only N420P6 which was 25% different from the rest in terms of starch granules.

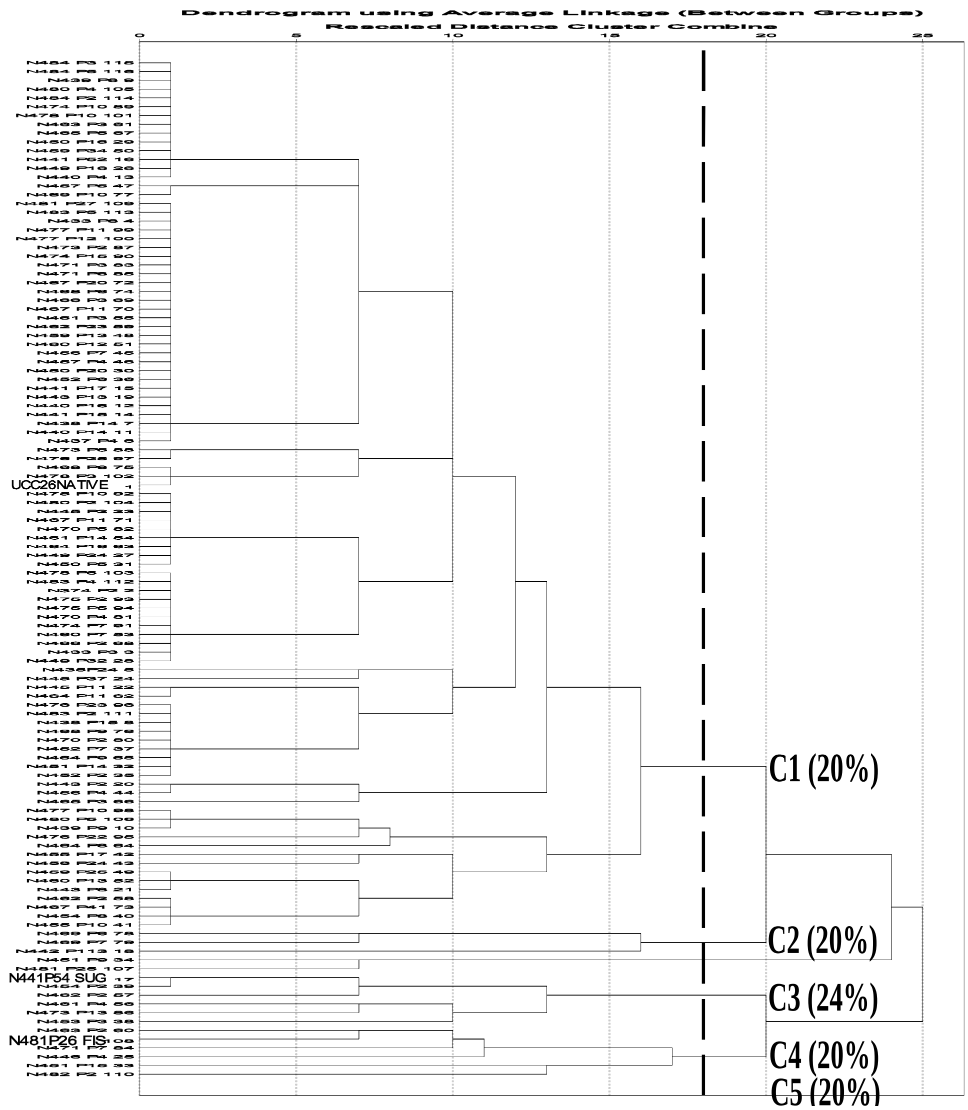


Fig.4.7 Average linkages between group cluster analysis showing a dendrogram of 115 induced plants and a control UCC026 based on eight morphological traits of cassava starch granules

The clustering of induced plants around the control UCC026 is depicted by the dendrogram in Fig.4.7. The dendrogram was divided into five clusters (C1-C5) by a reference line drawn at an x value of 18 with 18% dissimilarity. The C1 contained the control UCC026 among the majority of induced plants. The C1 differed from C2 at 20%. The C1 and C2 differed from C3 at 24%. The C4 had a putative free-sugar mutant (N441P54 SUG) whilst the C5 had a putative mutant (N481P26) with exceptionally fissured granules. The C3 differed from C4 and C5 at 25%.

4.4.5. Water re-absorption properties of flaky cassava starch granules

The profile (Figure 4.8) showed that fissured starch granules (N398P3 and N481P26) tended to have a higher moisture content (>90%) than the controls (UCC90 and UCC26). Under vacuum the control starches tend to loose more water (< 6.5%). Upon exposure to the environment the starch granules of the two induced plants tended to re-absorb water at a faster rate than the controls up to two days, after which their lead in water absorption prevails.

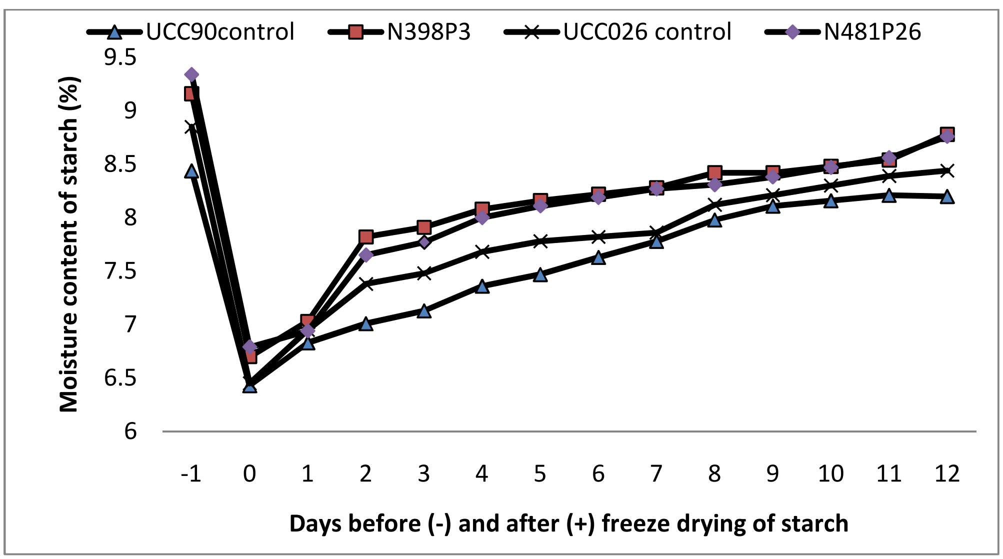


Figure 4.8. Water re-absorption of starch granules after drying under vacuum

4.4 Discussion

4.5.1 The survival rate of M_1V_1 and M_1V_2 cassava plants

The M_1V_2 field had a 1617.9% increase of surviving plants over the M_1V_1 field. This presented a picture of how explosive the next generation would be if no selection is made at the M_1V_2 stage, or how much more resources should be committed to the research. Mutation is a single cell event that requires individual plant evaluation until one mutation is found to be useful (Ceballos *et al.,* 2008). However, cassava is a perishable crop and all plants cannot be uprooted and processed at the same time, so that sampling is inevitable. Sampling is also controlled by the processing logistics available. The period of sampling must also be reasonably short to prevent significant variation between the first and the last samples. In view of this, the number of stakes used for initial irradiation was not as many as in seed propagated crops. To increase the efficiency of mutation breeding, improvement of screening methods is more important than the number of cuttings used, and the length of time used for raising possible mutated plants (Makino, 2001).

The survival rate of M_1V_1 plants is much lower than that of M_1V_2 plants because M_1V_1 plants were directly obtained from stakes exposed to acute irradiation which killed some stakes instantly, while leaving some to survive the irradiation effects. It was only the induced and surviving plants of M_1V_1 that gave rise to induced M_1V_2 plants. The general increase in specific landrace survival is an indication of the good physiological status of cuttings used for planting in the M_1V_2 stage. But the death of a few plants of HO008 origin relative to others at the M_1V_2 could be due to the lingering effect of irradiation in some landraces. Stakes from three landraces had a 100% specific landrace survival, whilst HO008 induced plants had a 95% survival. The visible changes in HO008 were nevertheless a terminal manifestation of micro-events. Changes at the sub-molecular level of plants occur when a cassava stake is exposed to gamma irradiation initially. If this change is not reversed within a relatively short time, it leads to a change in physico-chemical properties which follow directly or through modification in genetic structure. This gives rise to physiological changes that are expressed in a range of minor forms to the ultimate death of some plants later on (Gordon, 1957).

4.5.2 Size, form and shape of cassava starch granules

The size of cassava granules is classified as small (5-10 μm), medium (10-25 μm) and large (>25 μm) (Lindeboom *et al.*, 2004). All the controls contained large and non-uniform starch granule sizes. It was expected that the majority of induced plants should have large or medium sizes like the controls. Indeed, 92% of the induced plants had these properties. Only 8% (108) of 1302 plants analyzed had small sized starch granules. This indicated that reduction in starch granule size could be due to gamma irradiation. Figure 4.2 indicated that the chances of finding modified starch granules are higher in small granule plants than in large and medium sized ones. Ceballos *et al.* (2008) were the first to report a cassava mutant with reduced size of starch granules (5.80 µm) which were about a third the average normal size (10-25 µm). A putative mutant, N110P2, produced small sized and uniform starch granules, monovalent and single headed granules, lacking dents/holes, was not compacted and had a smooth surface without fissures. [Figure 4.4(C3) S; Appendix 4.1 S; Table 4.2].

Gamma irradiation could tailor new morphology of starch granules by changing the number of amyloplasts formed as well as their size. A positive correlation between the size of amyloplasts and the size of starch granules was reported (de Pater *et al*., 2006). Thus, small transition starch granules are produced from small chloroplasts whilst larger storage starch granules are produced from large amyloplasts. There are differences in functional properties of small and large starch granules which suggest that the granule size distribution is an important parameter in the food industry. Small granules have a higher affinity for water at room temperature. It seems that there is a less ordered arrangement of the polysaccharide chains in the smaller granules compared to the larger ones (Chiotelli and Meste, 2002). Uniform small granules are a prerequisite for application in soup preparations as thickeners. It also imparts a creamy texture to food preparations and could be used as fat replacer in foods. It is also valuable in applications such as cosmetics, binders in pharmaceuticals, paper, photographic films and plastics (Lindeboom *et al*., 2004).

Figure 4.1.3 showed that uniform sized granules (50%) were as common as non-uniform (50%) sized granules, and both uniform and non-uniform sized granules were far more in abundance than small sized granules (8%). Small sized granules could therefore be a more determining factor for finding a mutant compared to uniform sized granules because small size is a scarce trait. Mutants discovered based on small size would be more trusted or reliable than mutants based on common form of granules sizes which may be confused with that of the control. Small sizes of starch granules were found to have advantages in a great variety of applications. Starches with small granules also display a lower swelling power but higher solubility than starches with large sized granules (Kaur *et al.,* 2002). Small starch granules gelatinize at a higher temperature than larger ones (Baruch, 1985; Geddes *et al.,* 1965) and are therefore more suitable for coating food for deep frying. The reduced granule size and the obvious irregularities in their surface would lead to facilitated hydrolysis (Lehman and Robin, 2007) or would require reduced quantities of enzymes for hydrolysis. A significant portion of the small B - type granules from barley is not completely gelatinized in the mash and the undegraded residue causes mechanical or filtration problems during subsequent processing (Tillett and Bryce, 1993). Additional processing costs required for the isolation of large or small granules is incurred when granules are not uniform. Thus, *in planta* production of starches with predominantly large or small and uniform granules would be very desirable. The characterization of size (Figure 4.2) showed a bimodal distribution of starch granules which represents large and medium sized granules. The small granule size is relatively very small and could not be considered for trimodal classification. This is in agreement with the results of Garcia *et al.* (1996) who reported bi-modal distribution of granules in wheat, barley and rye. There is a strong correlation between mealiness, the size of the starch granules and dry matter contents. Plants with mealy roots have large starch granules, while non mealy types have small granules (Safo-Kantanka and Owusu-Nipah, 1992). The size of starch granules is influenced by storage root size. Starch granules were significantly smaller in small storage roots than in larger storage roots.

4.5.2.1 The relationship between A, B and C monovalent starch granules

Cassava starch granules are generally described as round or oval with a flat surface on one side containing a conical pit which extends into a well (Moorthy, 1994; Thomas and Atwell, 1999) while Nuwamanya *et al.* (2010) described cassava starch granule as truncated or kettledrum, or perfectly round. It was observed that 75% of 1302 plants studied produced monovalent (single) starch granules which could be classified into A, B and C types based on shape (Figure 4.1.1).

An A-type granule was more varied than a B-type granule and was truncated or kettledrum or half bowl or calabash shaped. This observation matches the description by Thomas and Atwell (1999) whilst the B type granules were round or oval which agrees with Moorthy (1994). All A type granules had one or more flat surfaces depending on origin. The third type of starch granules was C, with amorphous shape, and because of its smaller size than A and B granules, it always occupies the spaces between them.

Care should be taken not to confound amorphous C type with polygonal A type of granules under higher magnification of SEM. The C types are much smaller than A and B types of granules under SEM. Figures 4.1.5b (b) and 4.1.6b(c-d) show the C granules attached to larger ones. Intact compound granules and multi-head granules were not observed under SEM but attachment points or contact marks left on granules were convincing evidence. Compound granules disintegrate during preparation activities such as abrasive blending of storage root flesh to purify starch granules from cells, starch sieving, drying and grinding of caked starch into powder.

Most A type granules that have been detached from compound granules may have multiple flat surfaces making them more polygonal than those that originated from the A amyloplasts as A granules with a single flat surface. Compound granules are developed from the pressure of many surrounding granules developed together within a single amyloplast. Such granules are small at maturity and polyhedral in shape. These polyhedral granules are compressed together to form compound granules which, at low magnification, have the appearance of a single granule (Shapter *et al.*, 2008). When compound granules and multi-headed granules are separated, it would surely increase the population of C and A types of granules, but not B type of granules which are round or oval. Multihead granules may shed their numerous heads to increase the population of C granules (Figure 4.1.5a-6b). In contrast, the large, ‘simple’ granules were developed from larger individual amyloplasts. The B type of starch granules might have developed from A amyloplasts or oval B amyloplasts or from outgrowth on some part of normal

A or B type granules. Figure 4.1.11 could depict further development of B granules from existing B types. This observation agreed with the report that the B - type granules develop within evaginations of the A types of granules or amyloplasts and remain independent, without the final appearance of a compound granule while the A - type granules develop in the body of the amyloplasts (Langeveld *et al*., 2000). Parker (1985) described the relationship between A- and B - type starch granules in the inner endosperm of some developing tetraploid and hexaploid wheat examined by electron microscopy. At about 14 days after anthesis, B - type granules were initiated in the stroma of some A - type amyloplasts, often in association with the peripheral groove and tubuli. The B - type granules were also found within narrow protrusions extending from the surface of A - type amyloplasts, but it was not certain whether they were initiated in the protrusions or passed into them from the stroma. From 25–35 days after anthesis, the A - type amyloplasts contained one A - type starch granule and numerous B - type granules, which were often attached to the parent amyloplast by a narrow constriction.

Finally, it should be noted that the size of protrusions observed under 400 x magnification with LM were far larger than the protrusions reported previously. For example very small protrusions of ca 10-50 nm were observed at the surfaces of native wheat starch granules whereas larger spherical protrusions (20 and 500 nm) were evident on the surface of native potato starch granules (Thompson *et al.*, 1994; Baldwin 1995). Taking into account the sizes of these structures, it becomes evident that 'protrusions' observed in Figures 4.1.11 were not of the same size as protrusions reported previously by Thompson *et al.* (1994) and Baldwin (1995). This is the first *in planta* protrusions in cassava starch granules to be reported.

4.5.2.2 Compound and multihead cassava starch granules

It was observed in Figure 4.3 that one-quarter (325) of plants had compound starch granules and three-quarters (977) had single starch granules. Compound granules ranged from divalent granules to dodecavalent (12 sub-units) granules. All controls had up to trivalent granules. The dodecavalent compound granules found in the putative free-sugar mutant N357P5 was an exceptional association of granules above the original HO001 control. N357P5 produced the most diverse compound granules (Table 4.2). The two other plants in the same cluster with

N357P5 at > 20% difference could be identified for further studies. Only 108 plants (8%) produced granules with multiple heads and the rest (1194; 92%) single heads. Multi-head (108, 8%) producing plants were of the same number as small size producing plants. All controls lacked multi-head granules. Further study of compound granules showed that all plants produced various kinds of minute compound granules in the rind (cortex). This indicates that few plants produced compound granules in both the cortex and the storage root flesh but the majority of plants limited the production to the cortex alone. Compound granule development of root flesh might therefore be interrupted by gamma irradiation in some plants whilst in others both compound granules and multi-head granule sites were excited and developed more. Most of the multi-headed starch granule plants also produced compound starch granules but not all compound granule producing plants were producing multi-head granules.

Cassava stem cuttings (stakes) produce adventitious fibrous roots from a basal-cut surface and buds under the soil. The soil is penetrated by thin roots, and their enlargement begins only after that penetration has occurred. The number of thin adventitious roots that would change eventually into storage roots is predetermined before bulking starts. Few of these predetermined roots start to bulk under favourable conditions by accumulation of starch between the cortex parenchyma cells and the stele root located at the centre of the pith (Amenorpe *et al.*, 2007). McAllister *et al.* (1994) reported that examination of the internal tissues (i.e. cortex) of the cassava peels revealed discrete starch granules contained within parenchymatous cells. The storage roots have relatively reduced ability to absorb water and nutrients compared to the remaining adventitious fibrous roots (Alves, 2002). Since only the flesh of storage roots' and cortex were studied, one cannot conclude that the compound granules and the mutiheaded granules are only confined to the storage root of starch. Therefore, the search for these granules should be expanded in future to cover the cortex of adventitious fibrous roots, although their main function is to absorb water and minerals. If they do not produce them, then their presence has something to do only with storage and the mechanism of bulking. None of the multi-head granules was found in the rind (cortex) of the storage roots, which shows that the population of compound granules in these parts should also be monitored.

4.5.2.3 Dents or holes in cassava starch granules

It was found that induced plants frequently produce starch granules with dents/holes (1121; 86% vs. 181, 14% without dents/holes) 86% vs. 181, 14% without dents/holes) (Figure 4.2). In line with this, 14% (181) of 1302 plants studied produced granules without holes. A typical smooth granule producing plant is N110P2 (Figure 4.4 (C3) S; Appendix 4.1. S.; Table 4.2). The dents or holes were defined as a single characteristic because it was not possible to differentiate between small dents and holes at 400 x magnification under the LM. Using dents/holes as criteria for identifying a mutant would also confound the natural occurrence of dents/holes in starch from the controls. However, the exceptionally large dents/holes observed in induced plants under LM (Figure 4.1.7a) were far beyond the size observed in control starch granules. Pores were reported to have been found along the equatorial groove of large granules of wheat, rye, and barley starches but not on other starches (rice, oat, potato, tapioca, arrowroot and canna) (Fannon *et al.,* 1992). Moreover, it was reported that cassava starch granules seem to be smoother than those of potato amidst regions of relatively flat surfaces without visible structures, pores and protrusions. However, using very high definition Atomic Force Microscopy (non-contact method) Juszczak *et al.* (2003) managed to find pores or protrusions in potato and tapioca starches. Such protrusions were minute or infinitesimal with dimensions in nanometers (Chapter 4 Section 4.5.2.1) compared to the current study. Therefore, the presence of large dents/holes may be an indication of mutation.

Dents or holes were found to be of advantage in technological processes. If absent, they can be created. Industries depend on α-amylase hydrolysis or partial hydrolysis with dilute acids to produce holes in granules for commercial ventures. Researchers also use these methods to open up pores on granules. Such pores are so small that advanced procedures like SEM, confocal laser scanning microscopy (CLSM), environmental scanning electron microscopy (ESEM), atomic force microscopy (AFM) and transmission electron microscopy (TEM) have to be used for observation. A few cases include an *in vitro* enzymatic degradation of maize, potato and wheat starch granules by the three different α-amylase fractions. This begins by eroding the inside out, leaving the external parts of starch granules with small holes and crater-like areas on the surface as observed by SEM (Meireles *et al.*, 2009). Another example is the production of porous starch granules with empty interiors to be used as encapsulant by digestion of freeze-dried potato starch with α-amylase from *Bacillus sp* which was observed with SEM and CLSM (Apinan *et al.,* 2007). It is obvious that industries such as perfume, deodorant, talcum powder and "teargas" security based industries, need holes in starch granules (because holes increases surface to size ratio) as a "bag" for carrying active ingredients. In the food industry, the encapsulation of functional foods in porous starch granules could make them more stable. A single granule may take up to 50% of its weight of peppermint oil, for example. The granule can then be spray coated to protect the contents. By selecting a coating material it is possible to provide a controlled release over a longer period of time (Apinan *et al.,* 2007). The plants producing dents/holes of granules *in planta* (Figures 4.1.7a; b) could serve as a cheaper option for encapsulation properties. More applications of dents/holes in granules are covered in Chapter 2 under Section 2.4.3 (granules with holes).

The surface pores are assumed to be the open ends of interior channels in the granules. Consequently, the radial amorphous channels in starch granules and the number of pores affect the pattern of attack by amylases and some chemicals (Fannon *et al*., 1992; 1993; Gallant *et al.,* 1997). If a resistant starch (starch with amylose > 50%) has more pores and channels, it would reduce its resistance to hydrolysis.

4.5.2.4 Compact and smooth cassava starch granules

It is obvious from Figure 4.2 that there was no remarkable difference in the percentages of starch granules being compact (656; 50%) and non-compact (646; 50%) or smooth surfaced (833; 64%) and rough surfaced (469; 36%) to be attributed to mutation. These features may therefore not be attributed to mutation. These showed that they were normal granules developed from a crowded amyloplast of granules physically pressing against each other resulting in compaction points and rough surfaces observed. It is a typical symptom of high starch yielding clones (Shapter *et al.,* 2008) that when many granules developed together within a single amyloplast, they were compressed together. This implies that single smooth surfaced starch granules have developed from less populated amyloplasts or they were alone in the amyloplast.

The observation of these features under near *in situ* condition and confirmation under *ex situ* conditions showed that they were not caused by drying or processing of the starch.

4.5.2.5 Fissures on the surface of cassava starch granules

It was observed that 17% (216) of the 1302 induced plants studied had fissured starch granules produced *in planta*, whilst all of the control ganules were not fissured. All the putative free-sugar mutant and the linear leaf mutants (Table 4.2) also had fissured starch granules. There were differences between clusters of these putative plants and their respective control landraces [Fig.4.4. (1-4)]. Plant N398P3 produced extremely deep fissured granules. When crushed, the fissured granules broke up into flakes, while starch granules from a control cultivar formed a compacted clump (Figure 4.1.10c). Flaking starch granules might possibly find application in food and bio-ethanol industries, since the improved access to acid or amylase may accelerate hydrolysis into glucose for fermentation into ethanol or production of glucose syrup. The fissured granules (N398P3 and N481P26) were observed to contain more water, and also re-absorb more water after having been dried than their respective controls (UCC090 and UCC026) (Figure 4.8). The higher hydroscopic nature of fissured granules may be due to the larger surface area to volume ratio that is exposed to the moisture in the air.

Starch granules with a suppressed starch branching enzyme (SBE) were shown to have severe fissures and rough surfaces (Blennow *et al.*, 2003). The fact that all waxy mutants have a granule structure containing amylopectin with wild - type crystalline organization (Buléon *et al.*, 1997) confirms the fact that amylose is not required for the biogenesis of normal granule structures. Without amylose, amylopectin chain structure could result in sub optimal chain packing into the clusters. The smallest cluster was found to be made of amylopectin side chains, and its amorphous lamellae region was calculated to be ~7 X 10 nm in size (Yamaguchi, *et al.*, 1979). A minimum level of amylose is supposed to narrow amorphous lamellae regions between the amylopectin clusters. Too much amylose at the amorphous lamellae and too little amylopectin could distort the arrangement and packing of amylopectin side chain double helixes. This could result in incoherently held and packed crystalline regions with the amylopectin double helices and therefore result in fissuring of granules. Starch granules that are composed of pure amylopectin do not possess fissures. It is therefore possible that the irradiation has affected the SBE or the genes that code for it.

4.6 Conclusions and recommendations

Many and varied *in planta* modified starch granules were found amongst the mutants, through evaluation of individual plants. Some of these variations may be industrially useful. All plants with an altered morphological shoot system and storage roots that failed to be stained blue-black with iodine (reported in Chapter 3) were found to produce altered starch granules from the controls. Dendrograms of plants based on morphological starch properties did not help much because it is not possible for all traits to be mutated at the same time and contribution to the mean from larger numbers of non mutant traits could mask and prevent discovering of useful mutant plants. However, the dendrograms showed that induced plants with the greatest difference in starch morphology fell in the cluster that was distant from the controls. The way forward is to locate a useful mutant among the relatively scarce mutant traits which is found in the induced plants but absent in the controls. The ranking from relatively scarce to the most common traits of starch granules is as follows: small size (108; 8 %) = presence of multi-heads (108; 8%), absence of dents/holes (181; 14%), presence of fissures (216; 17%), presence of compound granules (325; 25%) and absence of smooth surface (469; 36%). If a less scarce trait is to be considered as a deciding factor for a mutant, there must be an exceptional difference between it and the controls. For instance, none of the controls produced up to tetravalent compound granules. The production of up to dodecavalent (12) compound granules by the putative free-sugar mutant (N357P5) is an exceptional *in planta* modification compared to control starch. In addition, linear central leaf lobe mutants (N167B, N169P4), and putative free-sugar mutants (N281P16, N357P5, N394P82 and N441P54) also produced fissured granules, but plants N398P3 and N481P26 showed extremely deep fissures. A monovalent plant (N110 P2) with small uniform and smooth granules without dents/holes, compactness and fissures is suspected to be having more resistant starch properties. It is recommended that more research should be carried out to find out why compound granules are found in all cassava root cortex (rind) but not in the majority of the storage root flesh.

In this study it was discovered that some gamma induced plants had *in planta* modified cassava starch granules.

4.7 References

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CHAPTER 5

Variations in the composition of starch of induced cassava mutants

5.1 Abstract

Four controls HO008, HO001, UCC090 and UCC026 with 19.7%, 22.0%, 22.6 % and 20.9% amylose contents were compared with their respective induced plants for variation in the amylose content. The four highest amylose producing plants N441P54 (32.7%), N223P12 (31.1%), N373P2 (27.2%), N329P11 (26.8%) contained enough amylose to be classified as high amylose cassava. The four lowest amylose producing plants N96P16 (14.0%), N353P6 (13.9%), N166P2 (13.2%), N476 P22 (11.7%) also contained low enough amylose to be classified as a low amylose cassava plants with high potential for processing into waxy (very low amylose) cassava starches. The wide range of amylose content observed amongst mutagenized plants caused an increase in genetic diversity of amylose. None of the induced plants of UCC90 were selected among the overall highest and lowest amylose producing plants across landraces. HO008 dominated the selection by producing half of the eight plants selected from the 8442 stakes of M_1V_2 stakes planted.

5.2 Introduction

Starch granules are semicrystalline and are composed of two types of molecules: the linear and helical amylose and the branched amylopectin. Although in absolute mass only about one quarter of the starch granules in plants consist of amylose, there are about 150 times more amylose molecules than amylopectin molecules. Amylose is a polymer that is 99% linear and amylopectin is branched approximately 15-45% by α-(1; 6) - bonds occurring at every 12 to 30 D-glucose residues on the linear α - (1; 4) - D-glucopyranose main chains (Ral *et al.*, 2008). Amylose is a much smaller molecule than amylopectin but has a longer chain length than amylopectin (Albert *et al.*, 2005). The smaller size enables amylose leaching at a faster rate than amylopectin and the longer chain length allows an increase in amylose viscosity during pasting and the highest viscosity is attained when longer chains of amylose hang out of granules than shorter amylopectin chains. There is no interaction of iodine molecules with each other except under strictly anhydrous conditions. Neutral iodine molecules may give polyiodides in aqueous solution. Where each turn of the amylose helix holds about two iodine atoms, a blue colour is produced due to donor acceptor interaction between water and the electron deficient polyiodides (Orlando, 2003). Single helix amylose behaves similarly to the cyclodextrins by possessing a relatively hydrophobic inner surface that holds a spiral of water molecules, which are relatively easily lost, to be replaced by hydrophobic lipid or aroma molecules. It is also responsible for the characteristic binding of amylose to chains of charged iodine molecules (chains of I_3^- and I_5^-) forming the polyiodides such as I_9^{3-} and $I_{15}^{3-}$for development of blue black colour (Orlando, 2003).

The fact that different adaptations of the iodine method, as well as newer methods, produce inconsistent results of amylose content for the same starch sample suggest inconclusion amongst researchers (Gérard *et al.,* 2001; Fitzgerald *et al.,* 2009; Zhu *et al.,* 2008). The situation is worse for the determination of amylose from mutant samples because biosynthesis of starch without some of the enzymes may produce an intermediated macro molecule between amylose and amylopectin that could also bind with iodine and produce results of higher amylose content (Baba and Arai, 1984; Colonna, 1984; Kasemsuwan *et al.,* 1995). The presence of “intermediate” material with long branches is troublesome since it cannot be clearly identified as amylose or amylopectin and may be assigned to either category, depending on the characterization method employed. Intermediate fractions are more prevalent in starches of atypical genotypes (Klucine and Thompson, 2002). Gérard *et al.,* (2001) and Zhu *et al.* (2008) observed different levels of amylose in the same test sample using size exclusion chromatography (SEC), concanavalin A (ConA), differential scanning calorimetry (DSC) and iodine binding capacity (IBC) methods. They concluded that SEC (with a 2.6×200 cm column) on HW75 S gel columns is the only method that can provide accurate amylose values, whereas CL 2B gel columns were not suitable for mutant starches. Measurement of IBC is still a standard method for comparing samples, though it can give incorrect amylose values for mutant starches. DSC, which for unknown reasons overestimated amylose values for maize and wheat starches, should be used with care. Response to Con A gave accurate values for normal starch, but erroneous values for mutant starches (Lansky *et al.,* 1949).

The colorimetric iodine method was chosen for analysis of induced plants due to several reasons. The recommended 2 m HW75Sgel SEC column was no longer available therefore a TSK gel was used instead. Three TSK gel columns (TSK gel G5000PW, TSK gel G3000PW and TSK gel G2500PW (TSK gel, TOSOH Bioscience GmBH, Stuttgart, Germany) were connected in series to give a total length of 90 cm. They were protected with a 15 cm guard column (TSK-PWH) resulting in a total length of 105 cm. A trial experiment was run with these columns using a high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) in-line with a Low Temperature Evaporative Light Scattering (ELSD) detector (ELSD-LT II, Shimadzu Corporation). The results were reproducible but the length of columns was too short to separate amylose from amylopectin. Moreover, the glueing effect of the cassava starch tends to clog the guard column, which must be replaced after every 30-40 sample injections. The cost for replacement therefore excluded HPLC from the methods used in this study.

A test sample was also analysed with the amylose/amylopectin assay kit (Megazyme International Ireland Ltd., Bray, Ireland) (Gibson *et al.,* 1997) but the results were unusually high for the induced plants. Moreover reproducibility of results for induced cassava plants was very low. The colorimetric iodine method was therefore chosen for the analysis because the test run was more reproduceable than the Mega-enzyme method with most of the results agreeing with literature.

Ceballos *et al,* (2007) reported that amylose yield of plants is under genetic influence and the age of the plant or the environmental factors seem not to play a major role. Cloning also maintains the genotype of cassava until induced to change. Therefore any significant change in amylose after mutagenesis of plants, which are planted in the same environment as compared to the control, could be attributed to the effect of irradiation. The objective of this study therefore was to induce and select the overall four highest and overall four lowest amylose producing induced plants at the M_1V_2 stage.

5.3 Materials and methods

5.3.1 Source of cassava planting materials

The four cassava landraces with high starch yielding, early bulking and mealy plants from previous research during 1996 to 2002 (Amenorpe *et al.*, 2002) were selected for mutagenesis and evaluation at the Ghana Atomic Energy Commission (GAEC) in 2007. A total of 1689 stakes (stem cuttings with an average of five adventitioud buds) were irradiated with 35 Gy gamma rays from a Co^{60} source and stakes planted with unirradiated control (HO008, HO001, UCC090, UCC026) landraces. The 476 (N1-N476) surviving plants (M_1V_1) were used to generate 8442 stakes planted on the M_1V_2 field in 2008 (See Chapter 3 Sectios 3.3.1-3.3.4 and Chapter 4 section 4.3.1 for details). The starch samples were obtained from the M_1V_2 stage in 2009 and analyzed at the University of the Free State in 2010.

5.3.2 Yield and total starch estimation on the field

Due to large population size, lack of logistics and the need to evaluate all plants individually, only three disease free plants arising from a common cell lineage in M_1V_1, with bigger diameter of the basal stem were randomly selected for harvest at the M_1V_2 stage. The fresh storage root weight and starch were determined individually with a scaled-pan and the specific gravity method of Brautlecht (1953) as modified by the Danish EU-directorate (1996) respectively.

5.3.3 Viscosity determination

The setback viscosity of the cooked starch was determined according to the ISI method 17-1e (International Starch Institute, 2002) with modifications. Distilled water was added to 10 g starch (db) sample in a 600 mL beaker until the total mass reached 500g. The suspension was heated in a water bath at 95°C for 30 min with constant stirring. It was cooled in a running water-bath to 50°C and the viscosity was determined in centipoises (cp) with spindle no. 2 at 100 RPM, using a Brookfield Digital Viscometer model RTDV II (Brookfield Engineering Laboratories Inc, Stoughton MA 02072, USA).

5.3.4 Determination of apparent amylose content

A modified colorimetric method of De la Cruz and Kush (2000) was used for amylose determination in starch granules. In this method, defattening was not necessary since storage root starches e.g. cassava, as well as tubers, legumes and waxy cereal starches do not contain lipids while cereal starches are characterised by a lipid content of about 1% (Buléon *et al.*, 1998). A stable alkali condition was first created for iodine to preferentially complex with the amylose helical structure. An excess of standard iodine solution was added and the resulting stable blue complex was measured spectrophotometrically. Amylose content was estimated with reference to potato amylose standards (Sigma-Aldrich, Germany). To 100 mg starch samples in 100 m1 volumetric flasks, 1 mL of 95% ethanol was added, followed by 9 mL 1M NaOH solution. The mixture was heated in a boiling water bath for 30 min to gelatinize the starch completely. After cooling for an hour to room temperature, distilled water was added to the mark. An aliquot of 0.5 mL was pipetted into a 10 mL test tube and 0.1 mL 1M acetic acid was added followed by 0.2 mL iodine solution. It was topped up with 9.2 mL distilled water to make a total volume of 10 mL. Absorbance was read at 620 nm in a spectrophotometer (Spectronic Unicam, Heλios, and Cambridge, United Kingdom). Triplicate measurements were made for each sample and average values reported.

A standard curve was prepared with potato amylose (De la Cruz and Kush (2000), of which 40 mg in a 100 mL volumetric flask was moistened with 1 mL of 95% ethanol, after which 9 mL 1M NaOH was added. The suspension was heated in a boiling water bath for 5-10 min. After 20 min of cooling to room temperature, it was topped up to the original volume with distilled water. Aliquots of 1, 2, 3, 4, and 5 mL of the amylose solution were placed in a 100 mL volumetric flask and 1M acetic acid of 0.2, 0.4, 0.6, 0.8 and 1.0 mL respectively were added. The blue-black colour was allowed to develop. The Absorbance (y) at 620 nm was plotted against concentration (x) of anhydrous amylose (mg/100ml) and the formulae for calculating the amylose concentration in starch sample $x=(y-0.0002)/0.178$ was deduced from the standard equation $y = 0.178x + 0.0002$ (Figure 5.1). The dilution factor of 20 for the samples is included in the conversion formulae, and the amylose concentration calculated in percentages (w/w) by the equation (1).

$$\text{Amylose \%} = \frac{\text{Amylose concentration} \left(\frac{\text{mg}}{100\text{mL}}\right) \text{obtained from starch x 100}}{\text{Initial concentration of starch} \left(\frac{\text{mg}}{100\text{mL}}\right)} \quad (1)$$

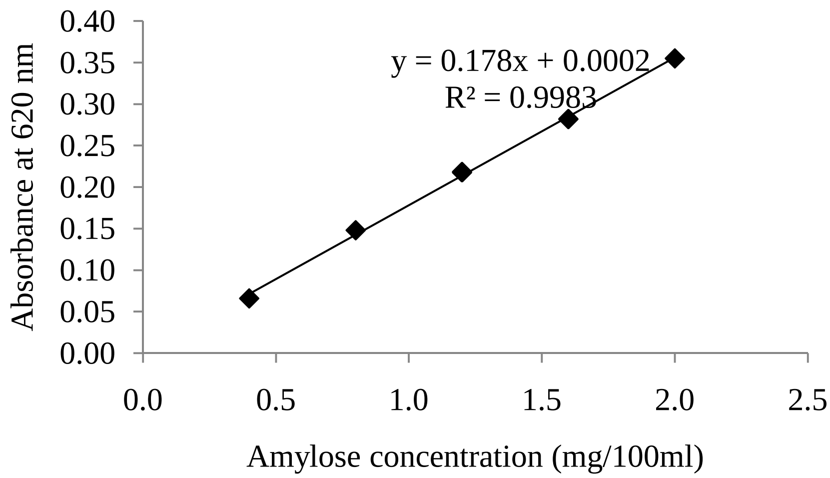

Fig. 5.1 Standard curve of absorbance versus anhydrous amylose concentration

5.4 Statistical analysis

Totals, means, standard deviations and correlations of plants arising from a common cell lineage were computed with Excel at the end of each table for general statements. For specific statements on genotypes, due to very large population size and the fact that each induced plant is genetically and morphologically different (refer to Chapter 3) it would be difficult reporting individual genotypes using statistical analysis of means and standard deviations obtained from different genotypes. Therefore evaluation must be based on individual plants (Ceballos *et al.*, 2008). Although triplicate measurements were done for the same field sample in the laboratory to ensure accuracy of the average results, standard deviation of mean was not reported for the triplicate values. If done, it would only explain the repeatability of the method and the degree of accuracy of various instruments used in obtaining the individual results in reference to the mean rather than focusing on the significant differences among genotypes. Breeder has no other choice where there is not enough relevant planting material to replicate than to report average laboratory results for each plants.

5.5 Results

5.5.1 Storage root yield, starch and amylose contents, and viscosity of HO008 M_1V_2 plants

The storage root yield, percentage starch estimate and the viscosity of starch from induced HO008 plants ranged from 2.5 to 7.6 kg, 20.4 to 30.4% and 303 to 515 cP whilst the respective values for the control HO008 were 2.5 kg, 25.4% and 390 cP respectively (Table 5.1).

Table 5.1 Storage root yield, starch and amylose contents, and viscosity of HO008 plants

Individual	Root yield (kg)	Starch content (%)	Starch viscosity (cP)	Amylose (%)	Average* amylose (%)	Four highest/ lowest selections
N330 P2	2.5	22.0	393	25.6	25.6	
N223 P12	2.5	21.4	325	31.1		1
N223 P13	6.5	21.5	303	23.6		
N223 P6	2.0	21.6	360	21.7	25.5	
N329 P11	7.0	30.3	383	26.8		2
N329 P4	5.0	29.9	515	26.3		4
N329 P6	4.5	26.6	413	22.8	25.3	
N348 P4	6.0	33.0	375	26.8		3
N348 P5	5.5	29.5	363	22.3	24.7	
HO 008	2.5	25.4	390	19.7	19.7	Control
N96 P16	3.5	27.7	333	14.0		2
N96 P4	3.5	27.5	359	21.6	17.8	
N95 P4	5.0	30.4	310	18.4		
N95 P5	7.5	27.9	333	17.4		
N95 P2	7.6	29.8	283	14.4	16.8	3
N39 P6	2.4	20.4	335	17.7		
N39 P5	2.6	24.0	330	15.4	16.5	
N166 P5	2.5	22.0	378	20.3		
N166 P3	3.0	21.7	433	14.7		4
N166 P2	2.5	21.8	395	13.2	16.1	1
Totals	84.1	514.4	6290.0	413.9	187.8	
Means	4.2	25.7	370.0	20.7	20.9	
STDEV	1.9	4.0	55.2	5.1	4.3	

The average* referred to the mean value for M_1V_2 plants harvested which were of the same M_1V_1 plant descent

The amylose content of starch from individual induced plants ranged from 13.2 to 31.1% and the control landrace had 19.7% amylose. The average amylose content of M_1V_2 plants from the same M_1V_1 plant ranged from 16.08 to 25.6%. The selected four highest amylose producing plants were N223 P12, N329 P11, N348P4 and N329 P4 and the four lowest amylose producing plants were N166 P2, N96P16, N95 P2 and N166P3 compared to the control HO008.

The correlations between root yield, starch, viscosity and amylose of HO008 plants at the M_1V_2 stage were 0.69, -0.21 and 0.11 respectively (Table 5.2). The correlations between starch and viscosity and amylose were 0.05 and 0.13 respectively. Correlation between viscosity and amylose was 0.24.

Table 5.2 Correlation between HO008 traits

	Starch (%)	Viscosity(cP)	Amylose (%)
Root yield	0.69**	-0.21	0.11
Starch		0.05	0.13
Viscosity			0.24

* $P \leq 0.05$, ** $P \leq 0.01$

The storage root yield, percentage starch and starch viscosity from induced HO001 plants ranged from 1.0 to 9.5 kg, 21.0 to 35.0 kg and 330 to 455 cP respectively, whilst the control HO001 had values of 4.7 kg, 23.4 kg and 513 cP (Table 5.3). The amylose content of starch from individual induced plants ranged from 13.9 to 27.2% and the control HO008 had 22.0% amylose. The average amylose content of M_1V_2 plants arising from a common cell lineage in M_1V_1 ranged from 19.2 to 26.3%. The selected four highest amylose producing plants were N373P2, N383P10, N387P4 and N389P5 and the four lowest amylose producing plants were N353P6, N354P4, N357P5 and N358P2 compared to the control HO001landrace.

Table 5.3 Storage root yield, starch and amylose contents, and viscosity of HO001 plants

Individual	Root yield (kg)	Starch Content (%)	Starch Viscosity (cP)	Amylose (%)	Average* amylose (%)	Four highest/ lowest selections
N387 P4	6.0	23.0	388.0	26.5		3
N387 P5	4.0	23.9	383.0	26.4		
N387 P3	3.0	21.0	408.0	26.1	26.3	
N383 P10	4.0	22.0	410.0	26.8		2
N383 P2	4.0	24.4	388.0	26.1		
N383 P11	2.5	23.0	340.0	23.3	25.4	
N373 P2	5.0	32.5	348.0	27.2		1
N373 P8	9.5	31.8	338.0	22.9	25.0	
N389 P5	3.5	23.0	338.0	24.5		4
N389 P2	4.5	24.5	383.0	23.3		
N389 P4	1.0	23.5	395.0	21.8	23.2	
HO001	4.7	23.4	513.0	22.0	22.0	control
N358 P5	4.9	34.7	413.0	21.2		
N358 P3	2.5	35.0	360.0	21.1		
N358 P2	2.5	34.5	338.0	20.1	20.8	4
N354 P3	5.0	34.7	338.0	21.0		
N354 P5	4.8	33.7	360.0	20.6		
N354 P4	5.0	34.8	455.0	19.0	20.2	3
N357 P2	3.5	34.6	355.0	22.6		
N357 P4	2.5	34.6	360.0	21.1		
N357 P5	2.0	34.0	330.0	15.4	19.7	2
N353 P3	2.5	35.0	375.0	21.0		
N353 P5	5.9	34.5	408.0	22.7		
N353 P6	7.0	34.0	368.0	13.9	19.2	1
Totals	99.9	710.1	9092.0	536.6	202.1	
Means	4.2	29.6	378.8	22.4	22.5	
STDEV	1.8	5.6	42.4	3.4	2.7	

The average* referred to the mean value for M_1V_2 plants harvested which were of the same M_1V_1 plant descent

The correlations between root yield and starch, viscosity and amylose were 0.33, -0.44 and -0.08 respectively (Table 5.4). The correlations between starch and viscosity and amylose were -0.13 and -0.65 respectively. Correlation between viscosity and amylose was -0.35.

Table 5.4 Correlation between HO001 traits

	Starch (%)	Viscosity(cP)	Amylose (%)
Root yield	0.33	-0.44*	-0.08
Starch		-0.13	-0.65**
Viscosity			-0.35*

* $P \leq 0.05$, ** $P \leq 0.01$

The storage root yield, percentage starch and starch viscosity from induced UCC090 plants ranged from 1.0 to 7.5kg, 21.0 to 33.5%, 283 to 468cP while that of the control UCC090 had values of 3.3 kg, 25.4% and 490 cP respectively (Table 5.5).

Table 5.5 Storage root yield, starch and amylose contents, and viscosity of UCC090 plants

Individual	Root yield (kg)	Starch content (%)	Starch viscosity (cP)	Amylose (%)	Average* amylose (%)	Four highest/ lowest selections
N419 P4	1.0	29.0	400.0	25.2	25.2	3
N412 P4	2.0	24.0	283.0	26.8		1
N412 P6	1.5	23.0	345.0	22.0	24.4	
N413 P2	2.0	23.0	355.0	25.4		2
N413 P3	4.5	24.0	370.0	21.3	23.3	
N394 P82	7.0	25.1	343.0	23.1	23.1	4
UCC090	2.3	25.4	490.0	22.6	22.6	control
N398 P3	4.0	31.0	468.0	19.8		
N398 P2	5.0	30.0	368.0	19.4		
N398 P4	7.5	33.5	450.0	18.9	20.5	3
N405 P13	2.0	21.5	358.0	21.8		
N405 P11	7.5	22.5	420.0	20.9		
N405 P12	2.5	23.0	413.0	17.7	20.1	2
N408 P2	4.0	21.0	393.0	20.6		
N408 P3	6.0	21.5	400.0	20.6		
N408 P4	5.0	23.0	380.0	19.6	20.3	4
N429 P2	2.5	28.6	440.0	22.1		
N429 P3	6.0	28.9	410.0	15.8	19.0	1
Totals	72.3	458.0	6646.0	383.5	198.5	
Means	4.0	25.4	390.9	21.3	22.1	
STDEV	2.2	3.8	50.4	2.7	2.2	

The average* referred to the mean value for M_1V_2 plants harvested arising from a common cell lineage in M_1V_1

The amylose content of starch from individual induced plants ranged from 15.8 to 26.8% and the control had 22.6% amylose. The average amylose content of M_1V_2 plants from the same M_1V_1 plant descent ranged from 25.2 to 19.0%. The selected four highest amylose producing plants were N412P4, N413P2, N419P4, N394 P82 and the four lowest amylose producing plants were N429P3, N405P12, N398P4 and N408P4 compared to the UCC090 control.

The correlations between root yield and starch, viscosity and amylose were 0.20, 0.19 and -0.52 respectively (Table 5.6). The correlations between starch and viscosity and amylose were 0.44 and -0.23 respectively. Correlation between viscosity and amylose was -0.46.

Table 5.6 Correlation between UCC090 traits

	Starch (%)	Viscosity(cP)	Amylose (%)
Root yield	0.20	0.19	-0.52**
Starch		0.44*	-0.23
Viscosity			-0.46*

* $P \leq 0.05$, ** $P \leq 0.01$

The storage root yield, percentage starch and starch viscosity from induced UCC026 plants ranged from 1.5 to 9.5kg, 22.5 to 31.8%, 365 to 473cP and the control UCC026 had values of 2.0 kg. 25.6% and 500 cP respectively (Table 5.7). The amylose content of starch from individual induced plants ranged from 11.7 to 32.7% and the control had 20.9% amylose. The average amylose content of M_1V_2 plants of the same M_1V_1 plant descent ranged from 16.2 to 32.7%. The selected four highest amylose producing plants were N441 P54, N470 P3, N468P8 and N439 P9 and the four lowest amylose producing plants were N476P22, N476P23, N474P5 and N474P2 compared to the control UCC026.

Table 5.7 Storage root yield, starch and amylose contents, and viscosity of UCC026 plants

Individual	Root yield (kg)	Starch content (%)	Starch viscosity (cP)	Amylose (%)	Average* amylose (%)	Four highest/ lowest selections
N441 P54	3.0	24.2	410.0	32.7	32.7	1
N439 P9	3.0	22.5	425.0	25.8	25.8	4
N468 P8	5.0	27.1	393.0	26.5		3
N468 P9	3.0	27.5	365.0	25.8		
N468 P6	1.5	27.0	370.0	22.9	25.1	
N470 P3	9.5	25.2	380.0	26.5		2
N470 P7	7.0	31.2	388.0	23.1		
N470 P8	5.5	26.8	390.0	23.0	24.2	
UCC 026	2.0	25.6	500.0	20.9	20.9	control
N473 P7	7.5	30.5	405.0	19.0		
N473 P15	4.5	31.8	390.0	18.7	18.8	
N472 P13	3.5	27.1	395.0	18.4	18.4	
N474 P10	5.5	29.5	415.0	19.1		
N474 P2	5.0	32.2	430.0	18.2	18.3	4
N474 P5	5.5	29.5	400.0	17.7		3
N476 P25	4.5	28.8	473.0	19.5		
N476 P22	4.5	29.0	465.0	11.7		1
N476 P23	5.0	31.0	390.0	17.3	16.2	2
Totals	85.0	506.5	7384.0	386.6	200.4	
Means	4.7	28.1	410.2	21.5	22.3	
STDEV	2.0	2.7	36.5	4.8	5.2	

Correlations between storage root yield, percentage starch, starch viscosity and amylose contents of UCC026 plants at the M_1V_2 stage, were 0.33, -0.24 and -0.08 respectively (Table 5.8). The correlations between starch and viscosity and amylose were -0.08 and -0.65 respectively. Correlation between viscosity and amylose was -0.35.

Table 5.8 Correlation between UCC026 traits

	Starch (%)	Viscosity(cP)	Amylose (%)
Root yield	0.33	-0.24	-0.08
Starch		-0.08	-0.65**
Viscosity			-0.35*

* $P \leq 0.05$, ** $P \leq 0.01$

5.6 Discussion

5.6.1 Screening strategy

The strategy for selection of the highest/ lowest amylose producing plants started from the field.

A. Each M_1V_1 generated at least five stakes planted in the M_1V_2 field. Three M_1V_2 plants of the same M_1V_1 stake descent were harvested and storage root and percentage starch yields data were obtained and ranked on a landrace basis. High starch yielding plants were selected using a selective index of 15%. Low starch yielding plants were also selected with a 15% index to broaden the genetic base for starch producing plants.
B. The percentage amylose was determined and separately ranked for each one of the four landraces. The four highest and lowest amylose producing plants were selected based on individual amylose values compared with their control. Once a plant was selected, two other related plants from the same M_1V_1 stake descent were also added for comparison based on amylose content. This was necessary to broaden the genetic base for amylose producing plants.
C. Three other putative mutants identified during field iodine screening of the entire population were added for further evaluation (refer to Chapters 3-4).
D. The overall four highest and lowest amylose producing plants were selected simultaneously with other traits determined.

5.6.2 Comparison of four control landraces based on amylose content

The amylose content of 22.6% observed for the UCC090 control was similar to 22.0% for the HO001control and 20.9% for the UCC026 control but higher than 19.7% for the HO008 control. The difference in amylose content observed may be attributed to genetic variations among the control landraces. The amylose contents of the four landraces were within the reported range of 15 to 26% (Sánchez et al, 2009); 17.9 to 23.6% (Defloor *et al.*, 1998); 17 to 25% (Fernandez *et al.*, 1996); 18 to 25% (Moorthy, 2004); or 13.6 to 23.8% (Rickard *et al.*, 1991) signifying that the method used for normal plants produced acceptable results which agreed with literature. The amylose results observed were, however, higher than the average amylose content of 16.6% ± 2.32 obtained from 2000 different genotypes of CIAT as reviewed in Ceballos *et al.,* (2007), similar to 20.7% average amylose content reported for 4000 different cassava genotypes (Sánchez *et al.* 2009) and lower than 25.4 to 28.8% values reported (Wickramasinghe *et al.,* 2009). These differences in amylose content of the four controls and literature were due to different genotypes used in Ghana for this studies and CIAT. It could also be due to different method used in this studies and CIAT because the amylose level may vary depending on the methods used for analysis (Gérard *et al.* 2001).

5.6.3 Evaluation of storage root yield, starch and amylose contents, and viscosity of induced cassava plants

The selection of the four highest and four lowest amylose producing plants simultaneously led to selection of inherent storage root yield, percentage starch and viscosity traits accompanying it. This agreed with the report that when a useful mutant is finally found, it is worth the search because it would be accompanied by many changes in biochemical, morphological and functional properties (Ceballos *et al.,* 2008). It was observed that the control values were within the range obtained for induced plants for the other measured traits. The four highest amylose plants N223P12, N329P11, N348P4, N329P4 also possessed storage root yield, starch and viscosity values of 2.5 kg, 21.4%, 325 cP; 7.0kg, 30.3%, 383 cP; 6.0 kg, 33.0%, 375 cP and 5.0 kg, 29.9%, 515 cP compared to 2.5 kg, 25.4%, 390 cP values of the control HO008. The four lowest amylose producing plants N166P2, N39, N95P2, N166P3 also produced 2.5 kg, 21.8%, 395 cP; 2.6 kg, 24.0%, 330 cP; 7.6 kg, 29.8%, 283 cP and 3.0 kg, 21.7%, 433 cP values of storage root, starch and viscosity respectively. The storage root yield, percentage starch and starch viscosity of induced plants, respectively, ranged from 2.5 to 7.6 kg, 20.4 to 30.4% and 303 to 515 cP whilst the control HO008 had 2.5 kg, 25.4% and 390 cP respectively. The degrees of variation between the control and four highest/lowest induced plants in terms of storage root yield, percentage starch and starch viscosity were probably due to irradiation effects.

In induced plants of HO008, the amylose content ranged from 13.2% (N166P2) to 31.1% (N223P12) compared with the control with 19.7% amylose, and reported values in Section

5.6.2. This implies gamma irradiation might have caused a considerable variation in the amylose extender genes, as some of the induced HO008 plants produced significantly higher/lower amounts of amylose than the control HO008 and reported values. The amylose content of starch was reported to be affected by the expression of the amylose extender genes (Noda *et al.,* 1992). Since irradiated stakes were of single genotype descent, planted in the same environment, the percentage amylose and standard deviation (5.1) from the mean (20.7%) observed were probably due to the irradiation effects. The amylose content plays a key role in the digestion of starches, as starches with low amylose contents were found to be more digestible than starches with high amylose content (Riley *et al.,* 2004; Noda *et al.,* 1992). The four highest amylose producing plants N223P12 (31.1%), N329P11 (26.8%), N348P4 (26.8%), N329P4 (26.3%) were therefore expected to be digested and absorbed at a slower rate than the four lowest amylose producing plants N166P2 (13.2%), N39 (15.4%), N95P2 (14.4%), N166P3 (14.7). The low digestibility of high amylose starch prevents a sharp rise in blood glucose level and prevents insulin and other endocrine responses when ingested (Wolever *et al.,* 1992; Jenkins *et al.,* 1982). High amylose starch would therefore be of benefit to diabetics and other health conscious individuals. Plants with very high amylose contents will not be suitable for products in which adhesion will be required but would rather be suitable for industrial production of alcohol, glucose and high fructose syrups (Hough, 1985).

The selection of the four highest and lowest amylose producing plants does not necessarily correspond with the selection of the highest or lowest storage root yield and starchy plants (Tables 5.1; 5.3; 5.5 and 5.7). In Table 5.2, storage root yield was observed to have a very strong positive correlation with root starch content (0.69), weak positive correlation with amylose content (0.11) and weak negative correlation with starch viscosity (-0.21). Starch content had weak positive correlation with amylose content (0.05) and starch viscosity (0.13). Viscosity also had weak positive correlation with amylose content (0.24). Viscosity was reported to have a strong positive correlation with the fraction of long chain amylopectin molecules and a negative correlation with short chain amylopectin molecules (Lu *et al*., 2008), therefore, induced HO008 plants are generally suspected to have more long chain amylopectin than short chain amylopectin. Pérez (2000) reported that amylose content had a low positive correlation with cassava starch content and an insignificant correlation with flour. It is also in agreement with Batey *et al.* (1997) who observed that when the amylose content is over 22%, the flour quality for viscous products decreased. It is also in agreement with Ral *et al.* (2008) who reported negative correlations between long chain amylopectin with viscosity but failed to establish convincing correlations between amylose and viscosity.

The amylose yield of plants is stable and genetically determined and neither the age of the plant nor environment seems to play a major role in its determination (Ceballos *et al.*, 2007). The relative stability of amylose content across environments in cassava explained why correlation between amylose content and storage root yield or starch content would not be very reliable. These values fluctuate depending on the wet or dry season, or whether the cassava is hydrolyzing or accumulating starch. During the early part of the rainy season, more starch is hydrolyzed to sugar to support new leaves. Under rainy conditions, more water is absorbed to support an increase in starch production and consequently root expansion (Amenorpe *et al.*, 2007).

It was expected that the more the leaching out of amylose from the starch granules, the higher the viscosity. However, it was observed that the highest amylose producing plant N223P12 (31.1%) was among the plants that displayed the lowest viscosity (325 cP) (Table 5.1). This is in contradiction with the results of Galliard and Bowler (1987) who ascribed high viscosity of starch to leaching out of the amylose fraction of the starchy system during pasting. These observations of plant N223P12 was similar to a cassava mutant reported by Ceballos *et al.* (2008) which also contained higher-than-normal amylose content (30.1% in stead of values between 20.7-22.2%) but low peak viscosity of 22 cP in stead of values between 976 to 1080 cP. This implies that a high amylose content of granules does not necessarily imply that there would be a corresponding increase in both amylose leaching and starch viscosity. This indicates that having high amylose is just one of the numerous factors controlling viscosity. There are some factors controlling the releasing mechanism of the amylose into the solution and others control the networking of molecules in the solution during pasting and gelation which affect viscosity. Starch viscosity is influenced by several factors, mainly temperature, water content, stirring, granule shape, swelling power, amount and type of amylose/amylopectin which has leached from the granules and amylopectin-amylose entanglement. Therefore, any change in the viscoelastic behavior of a starch-based medium may be interpreted as the result of changes in at least one factor of this list (Eliasson, 1986; Doublier, 1987; Ellis *et al.,* 1989).

Since the viscosities were determined for cooked starch undergoing cooling and re-crystallization of amylose at 50°C, with all other factors assumed to be constant, the long chains of amylopectin and amylose in starchy plant N223P12 probably have a much lower tendency than others to re-associate into a more organized gel, which decreased the viscosity (Tester and Karkalas, 2004; Atwell *et al.,* 1988). Whilst, plant N166P2 had a low amylose content, it might have had a greater tendency than starch from other plants to re-crystallize long amylopectin B and C type chains into a more ordered semi-crystalline state, which eventually increased the viscosity during cooling. The higher the proportion of amylopectin long-chains, the more the crystalline region is distorted and the easier the hydrogen bonds are broken in disordered double helixes when heated in excess water. As a result, longer amylopectin single helix chains may leach out and cause an increase in pasting viscosity during heating and also an increase in gelation viscosity during cooling (Gudmundsson and Eliasson, 1990; Lu *et al.,* 2008; Charles *et al.,* 2005; Shibanuma *et al.,* 1996).

In general it was also observed that the correlation between viscosity and amylose for induced HO008 (0.24) plants was positive whilst negative correlations were observed for induced HO001 (-0.35), UCC090 (-0.46) and UCC026 (-0.35) plants (Tables 5.2, 5.4, 5.6 and 5.8). This indicated that HO008 had more of the fraction of long chain amylopectin molecules while the rest of the plants had more of the short chain amylopectin molecule fractions. The differences in the physicochemical and structural properties of the starches from induced plants are therefore influenced by individual differences caused by the mutagenesis. Thus, each genotype might have a different response to irradiation. It is therefore not reliable to extrapolate correlations from one induced genotype to another. These variations are expected, because this is not a natural population. It is a tailored population of induced plants based on results of high and low amylose content.

The average amylose content of two to three similar induced plants from the same M_1V_1 stake was provided in Tables 5.1, 5.3, 5.5 and 5.7 to buttress the point that selections based on mean values were not necessary for finding a specific useful mutant. Selection based on mean values could easily select average performing plants but would prevent the highest or lowest extreme mutant plants to be identified. That means it favours the majority contributors to the mean which are mostly the non-mutants. Individual clonal evaluation focuses on very rare plants with significant useful traits differring from the control landraces. That is why cassava mutants reported in literature were based on individual analysis instead of means (Ceballos *et al.,* 2008; Carvalho *et al.,* 2004). The range of amylose content (13.2% to 31.1%) observed in induced plants of HO008 was observed to be broader than in HO001 plants.

The landrace HO001 (22.0% amylose) was induced to broaden the amylose content from 13.9% (N353P6) to 27.2% (N373P2). The four highest amylose plants of HO001 ranked from highest categories of plants N373P2, N383P10, N387P4 to N389P5 and the lowest four amylose producing plants ranked from lowest plant N353P6, N357P5, N354P4 to N358P2. The selected individual four highest/lowest amylose producing plants were significantly higher/lower than the control HO001. The plants with the highest amylose content may be more suitable for specific resistant food applications while the lowest amylose containing plants (mutants) could support glucose syrup and bioethanol production (Szczodrak and Pomeranz, 1991; Englyst *et al.,* 1992). The genetic base of amylose production was therefore expanded through mutagenesis.

The ranking of the storage root yield, starch content and viscosity did, as expected, not follow the decreasing order of the four highest/lowest amylose producing plants. This was due to unstable negative correlations observed between these traits as explained earlier for the HO008 plants. The putative free-sugar mutant N357P5 had a relatively lower storage root yield (2 kg), significantly higher starch content (34%) but lower viscosity (330 cP) than the control HO001 with 4.8 kg, 23.4% and 513 cP respectively. Carvalho *et al*. (2004) described the first natural free-sugar mutant (CAS36) as having glycogen-like starch, lacking amylose and accumulating over 100 times more free sugar (mainly glucose) than commercial varieties. In the field iodine test, the lack of blue-black colour indicated that starch biosynthesis might have been affected and that a low amylose containing free-sugar plant was suspected (Chapter 3). However, the laboratory analysis with the modified De la Cruz and Kush (2000) method did not agree with these predictions. A relatively high level of amylose was rather observed. Such contrasting results with the colorimetric method were also reported by Gérard *et al.* (2001), even with normal starches. These authors finally concluded that it was due to the presence of intermediate poly glucans and it is only the SEC (with a 2.6×200 cm column) on HW75 S gels that could give reliable amylose content analysis, a method which would be useful for mutant starches. Since such columns are no more available, one has to rely on alternative amylose determination methodologies until a suitable method is developed for intermediate poly glucans.

The focus of this chapter was to induce and identify the highest and/or lowest amylose producing cassava plants. Any of these plants may also have a novel trait such as putative free-sugar mutant and fissured granules. This confirms that mutation breeding is an uncontrollable process which may lead to any unexpected useful mutant which is not anticipated. This is in agreement with Ceballos et al. (2008), that even the useful mutant might be accompanied by many changes in biochemical, morphological and functional properties. The apparent analytical methods to determine amylose content may not be able to distinguish between intermediate macro-molecules which are capable of forming complexes with iodine like amylose, although they might neither be amylose nor amylopectin (Gérard et al., 2001) and this account for higher values of amylose observed for the free-sugar plant. This implies that appropriate methodologies for analysing such intermediate polyglucans are also lacking.

The ranges of amylose content between 13.2% and 27% for induced HO001 and 13.2% and 31.1% for HO008 were observed to be broader than that of induced UCC090 plants. A range of 15.8% (N429P3) to 26.7% (N412P4) amylose content was observed in induced UCC090 plants. The amylose contents of the four highest amylose producing UCC090 plants N412P4 (26.8%), N413P2 (25.4%), N419P4 (25.2%) and N394 P82 (23.1%) were higher than that of the control UCC090 (22.6%) except the free-sugar putative mutant N394P82 (23.1%). The amylose content of plants N412P4, N413P2 and N419P4 were high enough to render the starch number one material to be processed into lenthel (very high resistant) starches which could be included in slowly digestible diets for improvement of colon health (Englyst *et al.*, 1992). Induced plants N429P3 (15.8%), N405P12 (17.7%), N398P4 (18.9%) and N408P4 (19.6%) were lower in amylose content than the control UCC90. The standard deviation (2.73) from the mean (21.3%)

observed was probably due to irradiation effects. The storage root yield, starch content and starch viscosity values for UCC090 induced plants did not follow the ranking order of the four highest/lowest amylose producing plants due to the unstable nature of the relationship between storage root yield, percentage starch and amylose content, similar as has been explained earlier for HO008. There was a strong negative correlation (-0.52) between storage root yield and amylose content and a weak negative correlation between percentage starch and amylose content (-0.23). The storage root yield of the putative free-sugar mutant N34P82 was significantly higher than the UCC090 control. Plant N34P82 had similar amylose content as the control but with a significantly lower viscosity than the control UCC090. The amount of variation between the four control landraces and the four highest/lowest induced plants were probably due to irradiation effect. UCC090 induced plants had a lower range of amylose values (15.8% to 26.7%) whilst UCC026 induced plants had the broadest amylose content range.

In UCC026 induced plants the amylose ranged from 11.7% (N476P22) to 32.7% (N441P54) with the control UCC026 at 20.9% amylose. The standard deviation (4.8) from the mean (21.5%) observed was probably due to irradiation effects. The selected individual four highest amylose producing plants were N441P54 (32.7%), N470P3 (26.5%), N468P8 (26.5%) and N439P9 (25.8%) and were significantly higher than the control UCC026. The four lowest amylose producing plants were N476P22 (11.7%), N476 P23 (17.3%), N474P5 (17.7%) and N474P2 (18.2%). Plant N476P22 was significantly lower than the control. These variations induced by irradiation had broadened the genetic base of amylose producing plants and may bring out changes in biochemical, morphological and functional properties (Ceballos *et al.*, 2008). The four highest/lowest amylose producing plants, in addition, possessed considerable variation in values of storage root yield, percentage starch and starch viscosity compared to the control UCC026 control. The storage root yield, percentage starch and starch viscosity from induced UCC026 plants ranged from 1.5(N468 P6) to 9.5 kg (N470 P3), 22.5(N439 P9) to 31.8 % (N473 P15), 365(N468 P9) to 473 cP (N476 P25) and the control UCC026 had 2.0 kg, 25.6% and 500 cP respectively. Since amylose level is relatively constant across environments and as months after planting do not seem to have any effect on it (Ceballos *et al.*, 2008), the significant variations of the observed amylose content between the four highest/ lowest induced UCC026 plants were probably due to irradiation effects.

5.6.4 The overall four highest/lowest amylose producing plants

The fact that 11 out of 14 sensory parameters were reported to have strong correlations with amylose content of rice (Champagne *et al.*, 2004) indicates the importance of amylose variation with food taste qualities. The relative proportion of amylose and amylopectin greatly influences the physicochemical, technological and nutritional properties of starch (Karim *et al.*, 2007; Jane *et al.*, 1999; Fredriksson *et al.*, 1998). The overall four highest/lowest plants selected across the induced four landraces (HO008, HO001, UCC090 and UCC026) are presented in Table 9.

Table 5.9 The overall four highest/lowest amylose plants selected across landraces

Control source of individual plants	Individual plants	Root yield(kg)	Starch content (%)	Starch viscosity(cP)	Amylose content (%)	Ranking of selections
UCC26	N441 P54	3.00	24.20	410.00	32.74	1
HO008	N223 P12	2.50	21.40	325.00	31.13	2
HO001	N373 P2	5.00	32.50	348.00	27.15	3
HO008	N329 P11	7.00	30.30	383.00	26.83	4
	UCC090	2.33	25.40	490.00	22.55	
	HO001	4.75	23.36	513.00	22.02	
	UCC 026	2.00	25.60	500.00	20.92	
	HO 008	2.50	25.40	390.00	19.73	
HO008	N96 P16	3.50	27.70	333.00	13.99	4
HO001	N353 P6	7.00	34.00	368.00	13.91	3
HO008	N166 P2	2.50	21.80	395.00	13.20	2
UCC026	N476 P22	4.50	29.00	465.00	11.65	1
	Totals	46.58	320.66	4920.00	255.82	
	Means	3.88	26.72	410.00	21.32	
	STDEV	1.77	4.05	66.23	7.16	

The overall four highest amylose producing plants in decreasing order of percentage amylose were N441P54 (32.7%), N223P12 (31.1%), N373P2 (27.2%), N329P11 (26.8%) and the four lowest amylose plants were N96P16 (14.0%), N353P6 (13.9%), N166P2 (13.2%), N476 P22 (11.7%). There were significant differences between these plants and their respective controls UCC026 (20.9%), HO008 (19.7%), HO001 (22.0%) and HO008. Significant differences were also observed between these plants and the average amylose contents of cassava reported by other researchers, i.e. 17.9 to 23.6% (Defloor *et al.*, 1998), 17 to 25% (Fernandez *et al.*, 1996), 18 to 25% (Moorthy, 2004) or 13.6 to 23.8% (Rickard et al., 1991).

Since the traits are already expressing, it would therefore not be necessary to undergo self pollination to increases the chances of pairing and expression of the recessive desirable waxy genes (GBSS1) (Ceballos et al., 2008). The overall four lowest amylose plants could be classified among rapidly digestible starches which could therefore be considered for baby foods, industrial alcohol, and glucose and high fructose syrups (Englyst *et al.*, 1992) and products in which adhesion will be required (Hough, 1985). Low (waxy) amylose content was reported to give soft and sticky textures to food products whilst higher amylose provides hard texture to food products (Juliano and Hicks, 1996).

Starch was previously thought to be completely digested. However, it is now recognized that a portion is resistant to digestion by human enzymes and will pass into the colon, where it may or may not be fermented by gut bacteria (Bravo *et al.*, 1998; Yang *et al.*, 2006). The amylose level observed in comparison to the reported values in literature is high enough to render the starch from plants N441P54, N223P12, N373P2 and N329P1 number one material to be processed into lenthel (very high resistant) starches. Starch is classified as rapidly digestible starch, slowly digestible starch or resistant starch (Englyst *et al.*, 1992). Resistant starch has been defined as "the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals" (Asp, 1994). The enzyme resistance of starch is affected by amylose content (Chanvrier *et al.*, 2007), moisture (Kim *et al.*, 2006), heating time, temperature (Eerlingen *et al*, 1993; Szczodrak and Pomeranz, 1991), the ratio of amylose to amylopectin and granular properties (Eggum *et al.*, 1993). Foods with significant resistance levels, incorporated into the dietary-prevention strategies, are reported to have beneficial effects on colon health (Topping and Clifton, 2001) and may also have a reduced glycemic load with possible protective effects against non-insulin-dependent type II diabetes, obesity and heart diseases (Behall *et al.*, 2006; Kendall *et al.*, 2004; Kim *et al.*, 2003). High amylose starches also increases total and meal fat oxidation in humans and rats (Kabir *et al.*, 1998). The preferential digestion of the meal fat portion of the food, leaving the resistant starch portion, creates false satisfaction, consumption of less food and natural slimming down.

It was observed that none of the UCC090 induced plants were selected among the four highest and four lowest amylose producing plants across the four controls although they had some mutant plants. This underscores the importance of individual clonal evaluation as compared to their respective control landraces as a reliable method of discovering mutants rather than comparison of mutants across different landraces. If selections were done across different landraces, most useful mutants identified in UCC090 induced plants in the previous chapters would have been overlooked. HO008 dominated the selection by producing half of the eight induced plants selected from the 8442 stakes planted on the M_1V_2 field. The average amylose values of controls (HO008, HO001, UCC090 and UCC026) respectively ranged from 16.08 to 25.6%, 19.2 to 26.3%, 19.0 to 25.2% and 16.2 to 32.7 for M_1V_2 induced plants. Irradiation has broadened the amylose range and increased amylose genetic diversity. The expansion of the amylose genetic base observed might be accompanied by a change in starch biosynthesis and therefore resulted in the display of different functional properties. The application of starch in industries is also primarily controlled by the amylose to amylopectin ratio, gelatinization and viscosity tendencies (Wickramasinghe *et al.,* 2009; Nwokocha *et al.,* 2009; Yuan *et al.,* 2007). The reported cases of gamma irradiation responsible for increasing genetic diversity are based on the fact that mutagenic effect of ionizing radiation results principally from the production of multiple damage sites in single and double DNA strands and when the DNA lesions were not correctly repaired or completed (Ward, 1995). Among these lesions, strand breaks are considered to be the most important as they interrupt the continuity and integrity of the double helix (Ali *et al.,* 2007). As a result, gamma irradiation accounts for 80% of new varieties released in China (Nagatomi, 1992) and 61% of more than 200 direct-use mutant varieties released in Japan (Nakagawa, 2008). Ionic radiation was used successfully as a tool to extensively reduce amylose content of rice which improved taste (Kobayashi and Nishimura, 2007). Gamma irradiation was used to produce 'Tek bankye', a cassava mutant with high dry matter content (40%) in Ghana (Danso *et al.,* 2008). Although commercialisation of cassava and adoption of improved varieties encourages genetic erosion (Moyo *et al.,* 1998; Brush, 2000; Benesi *et al.,* 2004), the broadening of the genetic base of amylose by gamma irradiation of cassava stakes had increased the genetic diversity which would help alleviate genetic erosion.

5.7 Conclusions and recommendations

The overall four highest amylose producing plants N441P54 (32.7%), N223P12 (31.1%), N373P2 (27.2%), N329P11 (26.8%) were high enough in amylose content to render the starch number one material to be processed into lenthel (very high resistant) starches. Simultaneously, the four lowest amylose producing plants selected N96P16 (14.0%), N353P6 (13.9%), N166P2 (13.2%) and N476 P22 (11.7%) had low enough amylose content to be classified as high amylopectin cassava plants with high potential for processing into waxy (very low amylose) cassava starches. The differences in amylose values across landraces were due to both irradiation effects and genotypic variations. The amylose content range of the controls from 19.7% (HO008) to 22.6% (UCC090) was significantly narrower than the amylose range from 11.7% (N476 P22) to 32.7% (N441P54) observed in induced plants. This indicates that the mutagenesis of the four control landraces had resulted in the broadening of the genetic base of amylose, which was also observed to be associated with the variation in the viscosities of starch.

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CHAPTER 6

Variations in physico-chemical properties of starch granules of induced cassava mutants

6.1 Abstract

X- ray diffraction spectroscopy and DSC characterization was carried out on control and induced cassava plants. Gamma irradiation caused a change from A-type X-ray diffraction pattern in control landraces to C-types in seven of the induced plants. This was accompanied by a significant variation in crystallinity indexes and gelatinization properties. On the basis of T_o of induced plants, the overall four lowest plants were N167BP12 (40.9°C), N398P3fissur (47.0°C), N413P2 (54.5°C) of control HO008 (68.5) descent and N357P4 (56.0°C) of control HO001 (63°C) descent whilst the overall four highest plants were N389P5 (72.0°C) of control HO001 (63.0°C) descent, N473P2 (72.7°C) of control UCC026 (62.0°C) descent and N95P5 (73.3°C) and N39P5 (83.7°C) of control HO008 (68.5) descent. Out of these eight plants, five (N167BP12, N398P3fissur, N389P5, N95P5 and N39P5) had merged gelatinization and melting transitions. Therefore, all induced plants could be classified into three gelatinization categories. The first group consisted of three induced plants which lacked gelatinization transition. The second group included 99% of the induced plants with normal gelatinization transition properties similar to controls. The third group consisted of 11 plants which displayed merged gelatinization and melting transitions, occurring within gelatinization zone. The first and third categories included most of the putative mutants described in the research chapters and they were suitable for shortening the starch processing time required in most commercial starch applications in industry.

6.2 Introduction

The starch granule is a semi-crystalline structure composed of crystalline and amorphous regions. The crystalline region is formed primarily by two starch chains coiling together to form double helices which is networked into ordered clusters of crystalline lamellae (Hizukuri, 1985; Hizukuri *et al.,* 1983; Waigh, 1999; Matveev *et al.,* 2001). The space between clusters form amorphous regions is mainly occupied by amylose with relatively longer chains than amylopectin. The interaction of short amylopectin chains with amylose results in the formation of heterogeneous double helixes that co-crystallize within the crystalline lamellae (Jenkins and Donald, 1995). The amount of double helices increase with increasing amylopectin content (Jenkins and Donald, 1995) but decrease with increasing amylose content (Charles *et al.*, 2005), suggesting that the amylopectin is the main factor responsible for granule crystallinity (Cheetham and Tao, 1997; Gernat *et al.*,1993, Paris *et al.*, 1999). Higher crystallinity results in higher gelatinization temperature (Defloor *et al.*, 1998a).

The crystalline nature of starch granules is characterized into X-ray patterns (A, B, C and V) which can be identitied with predefined A, B, C and V peaks by X-ray diffraction spectroscopy (Shujun *et al.*, 2005). The A type is associated mainly with cereals such as maize and wheat. The X-ray spectra of these starches display stronger diffraction peaks at 15º and 23º and an unresolved doublet at 17°, 18° 2θ. The B type is from tuber starches such as potato and canna. The strongest diffraction peak of the B type appeared at 17º and 5° 2θ with a d spacing of 5.4 Å, in addition to a few small peaks at 20º, 22º and 24º 2θ (Shujun *et al.*, 2005; Cheetham and Tao, 1998; Chandrasekaran, 1998). The A type adopts a close-packed arrangement with eight water molecules between each double helical structure, while the B-type has an open structure with 32 water molecules, surrounded by six double helixes (Szymoska *et al.*, 2003; Imberty *et al.*, 1988). The C type pattern is a mixture of A and B types. The hard (outer) part of pea starch granules contains an abundance of short chain amylopectin, identified as A-type crystallinity, while the softer (mostly interior) part of pea starch granules is identified with long amylopectin chains identified as B - type crystallinity. Therefore the presence of both long and short amylopectin chains in a particular granule diffracts an intermediate pattern called the C - type crystallinity pattern. The C - type has been observed in arrowroot and pea starch (Cheetham and Tao, 1998; Chandrasekaran, 1998; Zobel, 1988; Hizukuri, 1985; French, 1984). During retrogradation of starch, some of the leached amylose coils to form a single helix which complexes with lipids to form an amylose-lipid-complex. This complex can crystallize into a gel that diffracts X-rays in a V - type pattern (Lopez-Rubio *et al.*, 2008) or is caused by amylose being complexed with substances such as aliphatic fatty acids, emulsifiers, butanol and iodine (Cheetham and Tao, 1998).

The crystallinity of native cereal starches is A - type but this could be changed to an intermediate C - type through chemical modification (Cheetham and Tao, 1998) or through plant conditioning in the field. Cheetham and Tao (1998) have reported a transition of crystalline type in maize from A through C to B, accompanying a decrease in the degree of crystallinity from 41.8% to 17.2% across a range of apparent amylose content from 0% to 84% by growth conditions in the field. The chemical reagent used for modifying the crystalline portion of amylopectin, however, will be banned in the health-conscious period (Chaisawang and Suphantharika, 2006). Gamma irradiation is energetic electromagnetic wave which does not leave any residue such as protons, neutrons, electrons, alpha (α) or beta (β) particles in their target to cause harm. Nive cassava plants have been reported to produce A-type crystallinity just as some cereals (Nuwamanya *et al.,* 2010; Defloor *et al.,* 1998a; b), although some varieties may exhibit a C-type pattern (Lorenz and Kulp, 1992; Eberstein *et al.,* 1980).

The application of starch in industries depend on gelatinization, viscosity, crystallinity, amylose and amylopectin ratios and the nature of granules (Wickramasinghe *et al.,* 2009; Nwokocha *et al.,* 2009; Yuan *et al.,* 2007). During gelatinization, starch absorbs heat in excess water to fully break hydrogen bonds between double helices. Amylose leaches from the swollen granules to form a paste which increases the viscosity. After the starch granules are totally destroyed, the viscosity decreses. Cooling of the cooked paste involves reconstruction of the network of double helixes toward gelation and/or solubilisation (Tester and Karkalas, 1996). The temperature at which starch begins to undergo these changes is known as the gelatinization temperature. Most of the cassava starch granules gelatinize between 60-85°C (Paes *et al.,* 2008), 65-70°C in 45% water (Lacerda *et al.,* 2009). The gelatenization transitions temperatures are characterized into onset (T_o), peak (T_p) and conclusion (T_c) or endset (T_e) temperatures. On an industrial scale, the energy input for gelatinization is a significant part of processing costs (Ellis *et al.,* 1998). The objectives of the work reported in this chapter were to identify induced plants with modified x-ray diffraction pattern, crystallinity and gelatinization properties. This could be used to replace the chemically modified starches in industrial applications.

6.3 Materials and methods

6.3.1 Source of material

The four highest yielding, early bulking and mealy landraces from previous research (Amenorpe *et al.*, 2002) were mutagenized with 35 Gy gamma irradiation from a Co^{60} source and evaluated at the Ghana Atomic Energy Commission (GAEC) from 2007 to 2009. Out of 1689 stem cuttings planted, 476 (N1-N476) M_1V_1 plants survived. The M_1V_1 vegetatively propagated plants were used to produce 8442 stakes (stem cuttings with an average adventitioud bud of 5) planted in 2008 (See chapter 3 sections 3.3.1-3.3.4 and chapter 4 section 4.3.1 for details). The starch samples were obtained from 12 months old M_1V_2 plants in 2009 for laboratory analysis at the University of the Free State, South Africa in 2010.

6.3.2 Determination of amylose content

The modified colorimetric method of De la Cruz and Kush (2000) used for amylose determination was explained in Chapter 5 paragraph 5.3.4. Data of amylose content has also been taken from Chapter 5.

6.3.3 X- ray diffraction spectroscopy of cassava starch granules

The cassava starch granules were tightly packed in 50 mm x 50 mm, 3/5 mm thick rectangular plastic cell holders, slotted into a SIEMENS D500 X- ray diffractometer (an automated SIEMENS D500 X- ray powder diffractometer, Germany) for scanning. The machine operated at 40 kV and 30 mA, with a monochromatic Cu-Kα radiation at wavelength 1.542Å. The beam slit was 1°, receiving slits 0.05° and monochromator slit 0.15° for sharp focus, with a scanning rate of 2.32° min^{-1} (65°/28 min) between 5-70° 2θ. Duplicate measurements were done at an ambient temperature. Scattering data obtained were adaptively smoothened and background was subtracted before peak searching was carried out with Powderx software (Cheng Dong, Institute of Physics, National Laboratory for Superconductivity, Beijing). Multiple ASCII text files generated were imported as new column files and the curves were plotted as a function of relative intensity (I), versus scanning angle in degrees using "Origin 6.1" software (OriginLab Corporation, One Roundhouse Plaza, Northampton, MA 01060-4401 USA).

6.3.4 Determination of the degree of crystallinity (Xc)

The degrees of crystallinity were estimated following the standard method (Guinesi *et al*., 2006) as modified from Hulleman *et al*. (1999). The crystallinity index (Xc) was determined using the ratio of the intensity height of the crystallized portion of the X-ray diffractogram (Hc) to the total intensity height (Hc + Ha) (equation 1), with respect to the baselines drawn between 17° and 18° 2θ as shown in Figure 6.1.

(1).

where H_c and H_a are crystalline and amorphous intensities respectively.

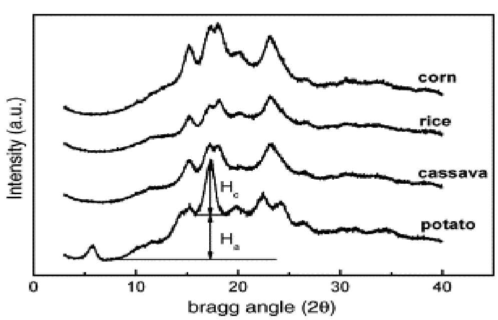


Figure 6.1 Intensities of amorphous (Ha) and crystalline (Hc) profiles (Guinesi *et al*., 2006)

6.3.5 Differential scanning calorimetry of starch gelatinization

Gelatinization properties of starch from control landraces and induced plants were determined using automated Differential Scanning Calorimetry (DSC822e, METTLER TOLEDO GmbH, Germany) following a modified method of Mweta *et al.* (2008). Approximately 3.0 mg of dry starch sample was placed into 40 µl DSC aluminium crucibles without pins (ME-26763), and 30µl distilled water was added to aquire a starch water ratio of 1:10. The mixture was stirred to achieve homogeneous moisture distribution after which the crucible was hermetically sealed.

The sample was left to stand for an hour to allow moisture equilibration before heating from 25°C to 110°C at a constant rate of 5°C/min to gelatinize the starch under nitrogen gas, flowing at 3.5 Pa pressure. A hermetically sealed empty crucible was used as a reference. A real time curve with gelatinization dip was plotted by the computer using instrumental STARe software version 9.0 (Mettler-Toledo, GmbH, 1993-2005). To obtain the characteristics of the dip, the limits of the gelatinization dip was integrated manually and the software used the limits to generate the data for the integral (mJ), normalised (Jg^{-1}), enthalpy of gelatinization (ΔH_G), onset (T_o), peak (T_p) and endset (T_e) temperatures (°C) relative to the T_p simultaneously. The temperature range (T_e - T_o) was extrapolated according to Peroni *et al.* (2006) and Mweta *et al.* (2008).

6.4 Results

6.4.1 X-ray diffraction spectroscopy of cassava starch granules

In Figure 6.2 it is shown that all induced HO008 plants produced an A-type X-ray diffraction pattern except N223P13 (d), N348P5 (i) and N166P3 (o) with C, C_A and C_A types respectively. The “C” type had peaks at 5°, 15°, 17°, 20°, 22° and 23° 2θ.

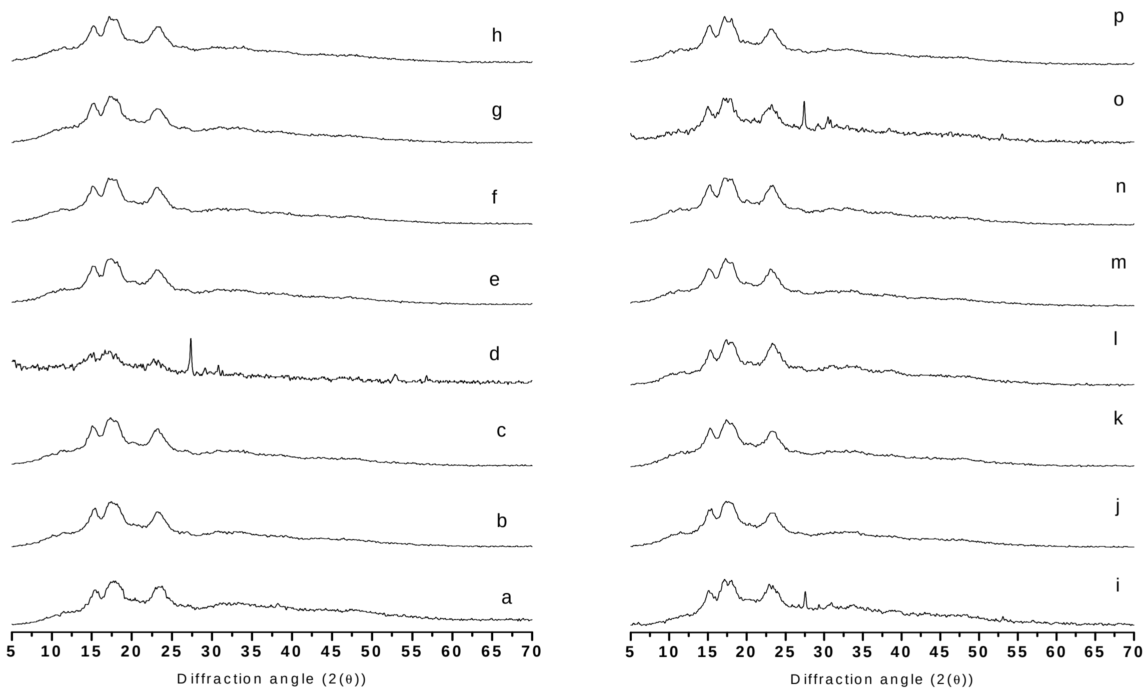
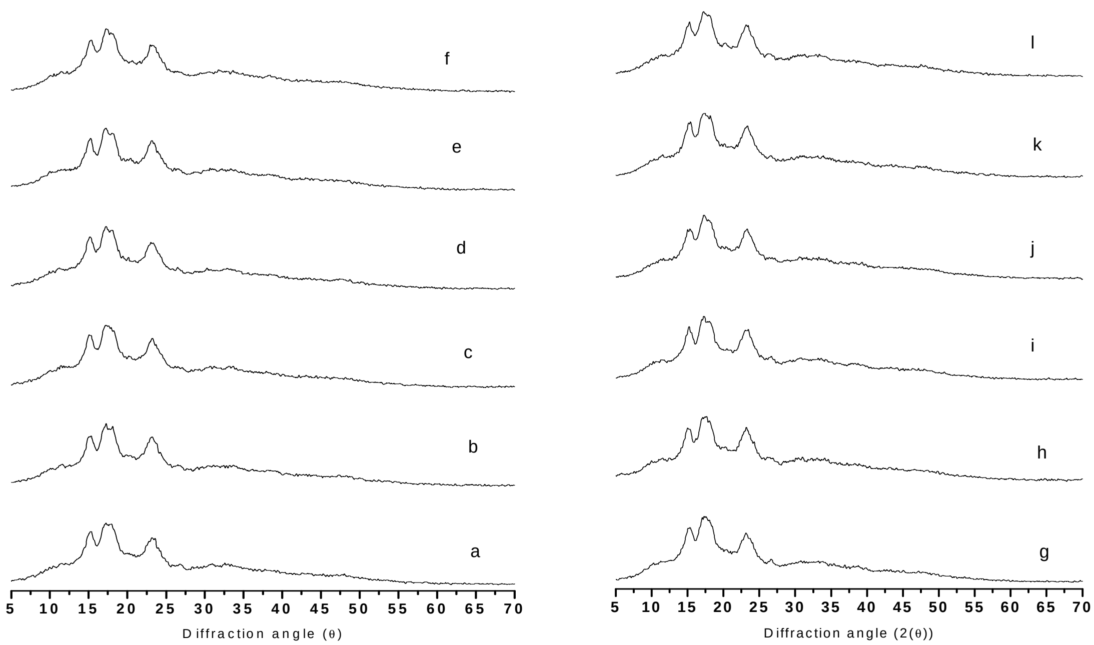


Figure 6.2 X-ray diffractograms of cassava starch from control landrace HO008 control and its induced plants at M_1V_2 stage. Diffractograms from control HO008 (a) and induced N330P2 (b), N223P12 (c), N223P13 (d), N329P4 (e), N329P6 (f), N329P11 (g), N348P4 (h), N348P5 (i), N96 P16 (j), N95P2 (k), N95P5 (l), N39P5 (m), N166P2 (n), N166P3 (o) and N166P5 (p) plant starches

All starches from the control landrace and induced HO001plants had only an A - type (Figure



6.3).

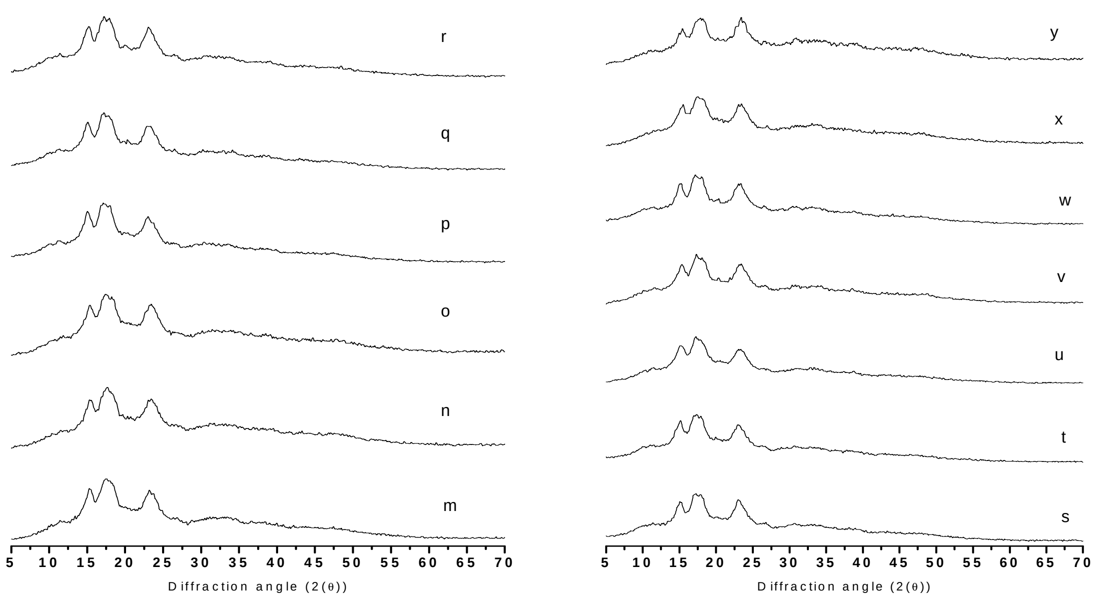


Figure 6.3 X-ray diffractogram of cassava starch from control landrace HO001control and its induced plants at M_1V_2 stage. Diffractograms from control HO 001 (a) and induced N387P3 (b), N387P4 (c), N387P5 (d), N383P2 (e), N383P10 (f), N383P11 (g), N373P8(h) N389P2 (i), N389P4 (j), N389P5 (k), N358P2 (l), N358P3 (m), N358P5 (n), N357P2 (o) N357P4(p), N357P54 (q), N357P5 (r), N354P3 (s), N354P5 (t), N353P3 (u), N353P34 (v), N353P5 (w), N353P6 (x) and N354P4 (y) plant starches

Based on the *2θ* angles, all the starches of the control UCC090 (a) and induced plants (Figure 6.4) display an A–type X- ray diffraction pattern, except the plants N419P4 (b), N412P4 (c) and N412P6 (d), which seem to be C_A- types.

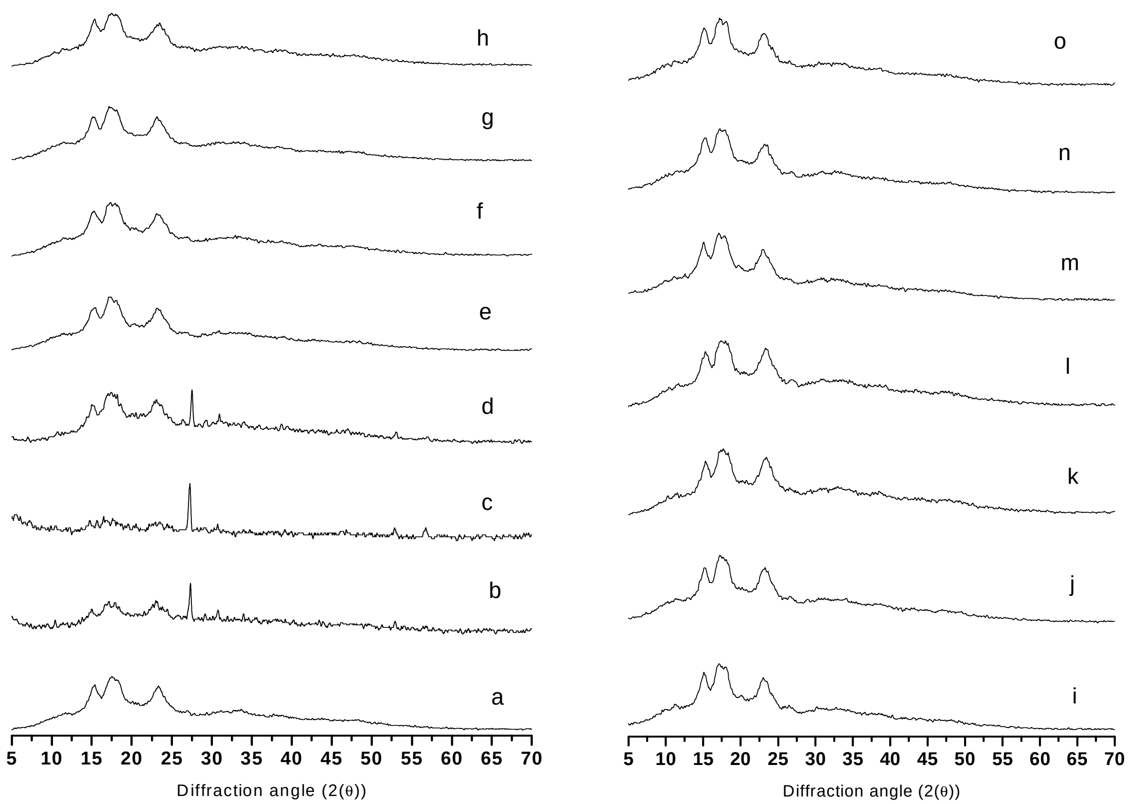


Figure 6.4 X-ray diffractogram of cassava starch from control landrace UCC090 and its induced plants at M_1V_2 stage. Diffractograms from control UCC090 (a) and induced N419P4 (b), N412P4 (c), N412P6 (d), N413P2 (e), N413P3 (f), 394P82 (g), N398P2 (h), N398P3 (i), N398P4 (j), N405P12 (k), N405P13 (l), N408P2 (m), N408P4 (n) and N429P3 (o) plant starches

Based on the X-ray spectra, all starches from control UCC026 (a) (not shown) and induced plants (Figure 6.5) display an A – type X- ray diffraction pattern, except plant N468P9 (f) which is of a C - type.

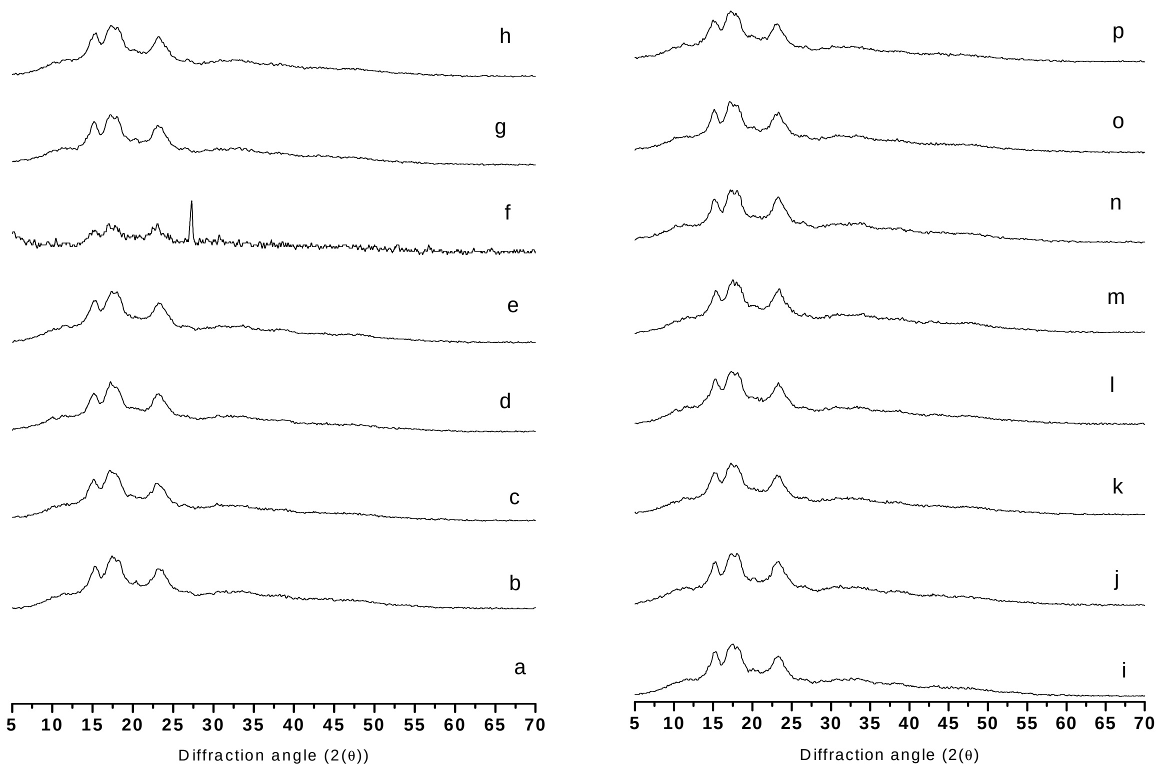


Figure 6.5 X-ray diffractogram of cassava starch from control landrace UCC026 and its induced plants at M_1V_2 stage. Diffractograms from control UCC026 (a) and induced N441P54 (b), N439P8 (c), N439P9 (d), N468P8 (e), N468P9 (f), N470P7 (g), N470P3 (h) N470P8 (i), N473P2 (j), N474P7 (k), N474P10 (l), N474P15 (m), N476P22 (n) N476 P23 (o) and N476P25 (p) plant starches

6.4.2 Amylose content and X-ray diffraction spectroscopy of control landrace and induced cassava plants at M_1V_2 stage

The ranking of plants were done according to amylose content, combinations of peak angles that characterize crystal pattern, the crystallinity index and the crystal pattern types (Tables 6.1 - 6.4). An A - type crystallinity was observed for the four highest (N441P54, N223P12, N373P2 and N329P11) and four lowest (N96 P16, N353 P6, N166 P2 and N476 P22) amylose producing plants selected across landraces. To locate a crystal type in Tables 6.1-4, one should follow the

A, B, C and V headings. For example a "C" type had all the columns under C (5°, 15°, 17°, 20°, 22° and 23° 2θ) filled. The B (not C) heading referred to the position filled to confirm the B - type but when empty, confirmed the C - type pattern. C_A referred to a C - type pattern which also has a peak at 18° exclusively reserved for A filled.

In Table 6.1, the crystalline lamellae regions of granules were scanned to produce an A - type X-ray pattern in all the induced HO008 plants, except C -, C_A - and C_A - types which were observed in N223P13 (d), N348P5 (i) and N166P3 (o) plants respectively. Moreover, some of the induced plants produced early peaks at 11° 2θ and late peaks at 27° or 31° 2θ which are not yet defined or reported in literature for starch. These extra peaks were observed in N329P6 (f), N348P4 (h), N96 P16 (j), N95P5 (l) and N39P5 (m) at 11° 2θ; in N223P13 (d), N329P11 (g), N348P5 (i) at 27°2θ and in N223P13 (d), N348P5 (i) N96 P16 (j) at 31°2θ for induced HO008 plants. In increasing order of the crystallinity indexes, the control landrace and induced HO008 plants were ranked from 0.44A N223P12 (c), 0.47A N330P2 (b), 0.47A N96 P16 (j), 0.48A N329P4 (e), 0.48A N95P2 (k), 0.48A N166P5 (p), 0.5A N329P6 (f), 0.5A N329P11 (g), 0.5A N348P4 (h), 0.51A HOOO8 (a), 0.51 N39P5 (m), 0.51A N166P2 (n), 0.51C_A N166P3 (o), 0.57C_A N348P5 (i), 0.58A N95P5 (l) to 0.66C N223P13 (d).

The X-ray diffraction pattern and crystalline properties of the control landrace and induced HO001 plants are presented in Table 6.2. All starches from the control and induced HO001plants had only an A - type of diffraction patterns. New minor peaks were observed at 11°, 27° or 31° 2θ which were not yet defined or reported in literature for starch. The ranking of the control and induced HO001 plants from lowest to highest degree of crystallinity were 0.44A N383P10 (f), 0.44A N353P6 (x), 0.45A N383P11 (g), 0.45A N389P5 (k), 0.45A N358P2 (l), 0.45A N357P5 (r), 0.46A HO001 (a), 0.46A N358P3 (m), 0.47A N389P4 (j), 0.47A N357P2 (o), 0.48AN387P4 (c), 0.48A N353P3 (u), 0.49A N389P2 (i), 0.49A N358P5 (n), 0.51A N387P5 (d), 0.51A N373P8 (h), 0.51A N357P4 (p), 0.51A N357P54 (q), 0.52A N354P3 (s), 0.52A N354P5 (t), 0.53C N354P4 (y), 0.53C_A N353P34 (v), 0.55A N383P2 (e), 0.56A N387P3 (b) and 0.56A N353P5 (w).

In Table 6.3, the crystalline lamellae region of granules diffracted an A - type X-ray pattern in the control landrace UCC090 and induced plants. A C_A pattern was observed in only three plants (N419P4 (b), N412P4 (c) and N412P6 (d)). Some of the induced plants produced peaks at 11° *2θ* and at 27° or 31° *2θ* which are not yet defined or reported in literature for starch. In increasing order of crystallinity index, the control and induced UCC090 plants ranked from 0.42A N398P2 (h), 0.45A N408P4 (n), 0.47A N413P3 (f), 0.48A N408P2 (m), 0.49A N413P2 (e), 0.49A N429P3 (o), 0.5A N405P13 (l), 0.51A UCC090 (a), 0.51A N394P82 (g), 0.51A N398P3 (i), 0.52A N398P4 (j), 0.54A N405P12 (k), 0.56 C_A N412P6 (d), 0.63C_A N412P4 (c) to 0.66C_A N419P4 (b).

The X- ray scanned the crystalline lamellae regions of granules and diffracted an A - type pattern in most control landrace and induced UCC026 plants (Table 6.4). Only N468P9 (f) showed a C - type X- ray diffraction pattern. Some of the induced plants produced early peaks at 11° *2θ* and late peaks at 27° or 31° *2θ* which were not yet reported in literature. In increasing order of degree of crystallinity with corresponding X-ray crystal pattern type, the control and induced UCC026 plants were ranked from 0.42 A N474P10 (l), 0.46A N470P3 (h), 0.47C N468P6 (c), 0.47A N468P8 (e), 0.47C_A N470P8 (i), 0.47A N474P15 (m), 0.47A N476P25 (p), 0.49A N441P54 (b), 0.49A N474P7 (k), 0.5A N470P7 (g), 0.52A N439P9 (d), 0.53A UCC026 (a), 0.53A N476P23 (o), 0.56C N468P9 (f), 0.56A N476P22 (n) to 0.56A N473P2 (j).

Table 6.1 Amylose content (%), crystallinity index (%) and crystal pattern of HO008 plants at M_1V_2 stage

Sample	Amylose	Combinations of angles of diffraction peaks at *2θ* diffraction that make up crystal pattern												Crystallinity index (%)	Crystal pattern
		B/C	V		V	A/C/B	ACB	A	A/C/B/V	B (not C)	A/C/ B(24°)				
		5°	7°	11°	13°	15°	17°	18°	20°	22°	23°	27°	31°		
(a) HOOO8	19.73	-		-		15.43	17.92	-	-	-	23.78	-	-	0.51	A
		-		-		(5.74Å)	(4.95Å)	-	-	-	(3.74Å)	-	-		
(b) N330P2	25.63	-		-		15.41	17.43	-	-	-	23.73	-	-	0.47	A
		-		-		(5.75Å)	(5.08Å)	-	-	-	(3.75Å)	-	-		
(c) N223P12	31.13	-		-		15.08	17.34	-	-	-	23.28	-	-	0.44	A
		-		-		(5.87Å)	(5.11Å)	-	-	-	(3.82Å)	-	-		
(d) N223P13	23.60	5.18		-		15.24	17.66	-	-	-	22.72	27.34	30.79	0.66	C
		(17.05 Å)		-		(5.81Å)	(5.02Å)	-	-	-	(3.91Å)	(3.26Å)	(2.90Å)		
(e) N329P4	26.28	-		-		15.12	17.46	18.18	20.44	-	23.03	-	-	0.48	A
		-		-		(5.85 Å)	(5.07 Å)	(4.88 Å)	(4.34 Å)	-	(3.86 Å)	-	-		
(f) N329P6	22.76	-		11.14		15.11	17.10	18.02	-	-	23.23	-	-	0.50	A
		-		(7.94 Å)		(5.86 Å)	(5.18 Å)	(4.92 Å)	-	-	(3.83 Å)	-	-		
(g) N329P11	26.83	-		-		15.29	17.21	-	-	-	23.16	26.56	-	0.50	A
		-		-		(5.79 Å)	(5.15 Å)	-	-	-	(3.84 Å)	(3.35 Å)	-		
(h) N348P4	26.77	-		11.54		15.93	17.17	18.04	-	-	23.13	-	-	0.50	A
		-		(7.66 Å)		(5.56 Å)	(5.16 Å)	(4.91 Å)	-	-	(3.84 Å)	-	-		
(i) N348P5	22.34	5.39		-		15.09	17.13	18.03	-	-	22.88	27.56	30.95	0.57	C_A
		(16.38 Å)		-		(5.87 Å)	(5.17 Å)	(4.92 Å)	-	-	(3.88 Å)	(3.23 Å)	(2.89 Å)		
(j) N96 P16	13.99	-		11.45		15.46	17.33	-	-	-	23.99	-	32.68	0.47	A
		-		(7.72 Å)		(5.73 Å)	(5.11 Å)	-	-	-	(3.71 Å)	-	(2.74 Å)		
(k) N95P2	14.44	-		-		15.26	17.36	-	-	-	23.32	-	-	0.48	A
		-		-		(5.80 Å)	(5.11 Å)	-	-	-	(3.81 Å)	-	-		
(l) N95P5	17.38	-		11.96		15.30	17.92	-	20.09	-	23.30	-	-	0.58	A
		-		(7.40 Å)		(5.79 Å)	(4.95 Å)	-	(4.42 Å)	-	(3.82 Å)	-	-		
(m) N39P5	15.36	-		11.25		15.09	17.27	-	-	-	23.06	-	-	0.51	
		-		(7.86 Å)		(5.87 Å)	(5.13 Å)	-	-	-	(3.85 Å)	-	-		A
(n) N166P2	13.20	-		-		15.25	17.12	17.78	20.01	-	23.38	-	-	0.51	A
		-		-		(5.80 Å)	(5.17 Å)	(4.98 Å)	(4.43 Å)	-	(3.80 Å)	-	-		
(o) N166P3	14.71	5.38		-		15.00	16.99	17.94	-	-	23.23	-	-	0.51	C_A
		(16.42)		-		(5.90 Å)	(5.22 Å)	(4.94 Å)	-	-	(3.83 Å)	-	-		
(p) N166P5	20.34			-		15.25	17.12	20.12	20.01	-	23.38	-	-	0.48	A
				-		(5.80 Å)	(5.17 Å)	(4.41 Å)	(4.43 Å)	-	(3.80 Å)	-	-		

Table 6. 2 Amylose content (%), crystallinity index (%) and crystal pattern of HO001 plants at M_1V_2 stage

Sample	Amylose	Combinations of angles of diffraction peaks at *2θ* diffraction that make up crystal pattern												Crystallinity index (%)	Crystal pattern
		B/C	V		V	A/C/B	ACB	A	A/B/V	B (not C)	A/C/B				
		5°	7°	11°	13°	15°	17°	18°	20°	22°	23°	27°	31°		
(a)HO001	22.02	-		-	-	15.27	17.23	17.94	-	-	23.19	-	-	0.46	A
		-		-	-	(5.80 Å)	(5.14 Å)	(4.94 Å)	-	-	(3.83 Å)	-	-		
(b) N387P3	26.13	-		11.60	-	15.31	17.25	17.98	-	-	23.22	-	-	0.56	A
		-		(7.62 Å)	-	(5.78 Å)	(5.14 Å)	(4.93 Å)	-	-	(3.83 Å)	-	-		
(c) N387P4	26.46	-		11.45	-	15.39	17.24	-	-	-	23.24	-	30.75	0.48	A
		-		(7.72 Å)	-	(5.75 Å)	(5.14 Å)	-	-	-	(3.82 Å)	-	(2.91 Å)		
(d) N387P5	26.36	-		11.23	-	15.14	17.25	-	20.13	-	23.04	-	-	0.51	A
		-		(7.88 Å)	-	(5.85 Å)	(5.14 Å)	-	(4.41 Å)	-	(3.86 Å)	-	-		
(e) N383P2	27.15	-		10.33	-	15.24	17.22	18.21	20.43	-	23.07	-	-	0.55	A
		-		(8.55 Å)	-	(5.81 Å)	(5.15 Å)	(4.87 Å)	(4.34 Å)	-	(3.85 Å)	-	-		
(f) N383P10	26.81	-		-	-	15.36	17.25	18.11	20.64	-	22.96	-	-	0.44	A
		-		-	-	(5.76 Å)	(5.14 Å)	(4.90 Å)	(4.30 Å)	-	(3.87 Å)	-	-		
(g) N383P11	23.29	-		10.27	-	15.36	17.33	-	20.46	-	23.08	26.55	30.79	0.45	A
		-		(8.61 Å)	-	(5.76 Å)	(5.11 Å)	-	(4.34 Å)	-	(3.85 Å)	(3.35 Å)	(2.90 Å)		
(h) N373P8	22.89	-		-	-	15.36	17.31	-	-	-	23.30	-	-	0.51	A
		-		-	-	(5.77 Å)	(5.12 Å)	-	-	-	(3.81 Å)	-	-		
(i) N389P2	27.2	-		11.35	-	15.23	17.14	-	-	-	23.47	-	-	0.49	A
		-		(7.79 Å)	-	(5.81 Å)	(5.17 Å)	-	-	-	(3.79 Å)	-	-		
(j) N389P4	21.83	-		-	-	15.36	17.31	-	-	-	23.30	-	-	0.47	A
		-		-	-	(5.77 Å)	(5.12 Å)	-	-	-	(3.81 Å)	-	-		
(k) N389P5	24.48	-		11.55	-	15.43	17.44	18.27	-	-	23.35	-	-	0.45	A
		-		(7.65 Å)	-	(5.74 Å)	(5.08 Å)	(4.85 Å)	-	-	(3.81 Å)	-	-		
(l) N358P2	20.13	-		-	-	15.29	17.26	17.93	20.27	-	23.20	-	-	0.45	A
		-		-	-	(5.79 Å)	(5.14 Å)	(4.94 Å)	(4.38 Å)	-	(3.83 Å)	-	-		
(m) N358P3	21.10	-		-	-	15.29	17.45	-	-	-	23.19	-	-	0.46	A
		-		-	-	(5.79 Å)	(5.08 Å)	-	-	-	(3.83 Å)	-	-		

(n) N358P5	21.24	-	10.39	-	15.28	17.69	-	-	-	23.44	-	-	0.49	A
		-	(8.51 Å)	-	(5.79 Å)	(5.01 Å)	-	-	-	(3.79 Å)	-	-		
(o) N357P2	22.62	-	11.84	-	15.35	17.45	18.23	-	-	23.45	-	-	0.47	A
		-	(7.47 Å)	-	(5.77 Å)	(5.08 Å)	(4.86 Å)	-	-	(3.79 Å)	-	-		
(p) N357P4	21.10	-	11.15	-	15.16	17.12	-	-	-	23.04	-	-	0.51	A
		-	(7.93 Å)	-	(5.84 Å)	(5.18 Å)	-	-	-	(3.86 Å)	-	-		
(q) N357P54	24.17	-	11.35	-	15.10	17.22	17.81	20.31	-	23.48	-	-	0.51	A
		-	(7.79 Å)	-	(5.86 Å)	(5.15 Å)	(4.98 Å)	(4.37 Å)	-	(3.79 Å)	-	-		
(r) N357P5	15.38	-	11.41	-	15.23	17.23	17.91	19.99	-	23.08	26.43	31.32	0.45	A
		-	(7.75 Å)	-	(5.81 Å)	(5.14 Å)	(4.95 Å)	(4.44 Å)	-	(3.85 Å)	(3.37 Å)	(2.85 Å)		
(s) N354P3	20.97	-	11.40	-	15.07	17.26	17.89	20.01	-	23.56	26.76	30.92	0.52	A
		-	(7.76 Å)	-	(5.87 Å)	(5.13 Å)	(4.95 Å)	(4.43 Å)	-	(3.77 Å)	(3.33 Å)	(2.89 Å)		
(t) N354P5	20.99	-	10.01	-	15.17	17.35	17.91	-	-	22.97	-	-	0.52	A
		-	(8.83 Å)	-	(5.84 Å)	(5.11 Å)	(4.95 Å)	-	-	(3.87 Å)	-	-		
(u) N353P3	21.03	-	11.49	-	15.21	17.26	-	-	-	23.06	-	-	0.48	A
		-	(7.69 Å)	-	(5.82 Å)	(5.13 Å)	-	-	-	(3.85 Å)	-	-		
(v) N353P34	22.69	-	11.67	-	15.44	17.48	18.08	20.33	-	23.41	-	-	0.53	A
		-	(7.58 Å)	-	(5.74 Å)	(5.07 Å)	(4.90 Å)	(4.37 Å)	-	(3.80 Å)	-	-		
(w) N353P5	22.68	-	11.54	-	15.25	17.15	17.54	20.36	-	23.33	-	30.94	0.56	A
		-	(7.67 Å)	-	(5.80 Å)	(5.17 Å)	(5.05 Å)	(4.36 Å)	-	(3.81 Å)	-	(2.89 Å)		
(x) N353P6	13.91	-	11.47	-	15.51	17.35	18.00	-	-	23.46	-	-	0.44	A
		-	(7.71 Å)	-	(5.71 Å)	(5.11 Å)	(4.93 Å)	-	-	(3.79 Å)	-	-		
(y) N354P4	19.04	-	-	-	15.42	17.84	-	-	-	23.41	-	30.92	0.53	A
		-	-	-	(5.74 Å)	(4.97 Å)	-	-	-	(3.80 Å)	-	(2.89 Å)		

Table 6. 3 Amylose content (%), crystallinity index (%) and crystal pattern of UCC090 plants at M_1V_2 stage

Sample	Amylose	Combinations of angles of diffraction peaks at *2θ* diffraction that make up crystal pattern												Crystallinity index (%)	Crystal pattern
		B/C	V		V	A/C/B	ACB	A	A/C/B/V	B (not C)	A/C/B				
		5°	7°	11°	13°	15°	17°	18°	20°	22°	23°	27°	31°		
(a) UCC090	22.55	-	-	11.74	-	15.38	17.53	-	-	-	23.33	-	-	0.51	A
		-	-	(7.53 Å)	-	(5.76 Å)	(5.05 Å)	-	-		3.8 Å)	-	-		
(b) N419P4	25.24	5.21	-	-	-	15.01	17.14	17.93	-	-	23.01	27.31	30.77	0.66	C_A
		(16.94 Å)	-	-	-	(5.90 Å)	(5.17 Å)	(4.94 Å)	-	-	(3.86 Å)	(3.26 Å)	(2.90 Å)		
(c) N412P4	26.77	5.55	-	-	-		16.48	17.63	-	-	23.50	27.24	30.72	0.63	C_A
		(15.90 Å)	-	-	-		(5.38 Å)	(5.03 Å)	-	-	(3.78 Å)	(3.27 Å)	(2.91 Å)		
(d) N412P6	22.00	5.11	-	10.72	-	15.02	17.48	18.19	-	-	23.09	27.46	30.90	0.56	C_A
		(17.27 Å)	-	(8.25 Å)	-	(5.89 Å)	(5.07 Å)	(4.87 Å)	-	-	(3.85 Å)	(3.25 Å)	(2.89 Å)		
(e) N413P2	25.38	-	-	11.49	-	15.44	17.39	18.08	-	-	23.38	-	-	0.49	A
		-	-	(7.70 Å)	-	(5.73 Å)	(5.10 Å)	(4.90 Å)	-	-	(3.80 Å)	-	-		
(f) N413P3	21.28	-	-	11.43	-	15.30	17.34	17.90	20.50	-	23.14	-	-	0.47	A
		-	-	(7.74 Å)	-	(5.79 Å)	(5.11 Å)	(4.95 Å)	(4.33 Å)	-	(3.84 Å)	-	-		
(g) N394P82	23.11	-	-	11.46	-	15.15	17.17	18.10	-	-	23.18	-	-	0.51	A
		-	-	(7.72 Å)	-	(5.84 Å)	(5.16 Å)	(4.90 Å)	-	-	(3.83 Å)	-	-		
(h) N398P2	19.41	-	-		-	15.43	17.48	17.97	-	-	23.49	-	-	0.42	A
		-	-		-	(5.74 Å)	(5.07 Å)	(4.93 Å)	-	-	(3.78 Å)	-	-		
(i) N398P3	23.83	-	-	11.22	-	15.09	17.12	18.05	20.09	-	23.04	-	-	0.51	A
		-	-	(7.88 Å)	-	(5.87 Å)	(5.18 Å)	(4.91 Å)	(4.42 Å)	-	(3.86 Å)	-	-		
(j) N398P4	18.89	-	-	11.34	-	15.26	17.22	18.07	20.18	-	23.28	-	-	0.52	A
		-	-	(7.80 Å)	-	(5.80 Å)	(5.14 Å)	(4.91 Å)	(4.40 Å)	-	(3.82 Å)	-	-		
(k) N405P12	17.69	-	-	11.53	-	15.31	17.68	18.35	-	-	23.31	-	-	0.54	A
		-	-	(7.67 Å)	-	(5.78 Å)	(5.0 Å)1	(4.83 Å)	-	-	(3.81 Å)	-	-		
(l) N405P13	21.80	-	-	11.70	-	15.28	17.35	17.99	-	-	23.34	-	-	0.5	A
		-	-	(7.56 Å)	-	(5.79 Å)	(5.11 Å)	(4.93 Å)	-	-	(3.81 Å)	-	-		
(m) N408P2	20.58	-	-	11.27	12.55	15.05	17.16	17.84	19.76	-	23.05	-	-	0.48	A
		-	-	(7.85 Å)	(7.05 Å)	(5.88 Å)	(5.16 Å)	(4.97 Å)	(4.49 Å)	-	(3.86 Å)	-	-		
(n) N408P4	19.62	-	-	-	-	15.30	17.84	-	-	-	23.24	-	-	0.45	A
		-	-	-	-	(5.79 Å)	(4.97 Å)	-	-	-	(3.82 Å)	-	-		
(O) N429P3	15.82	-	-	-	-	15.11	17.19	18.08	-	-	23.08	-	-	0.49	A
		-	-	-	-	(5.86 Å)	(5.15 Å)	(4.90 Å)	-	-	(3.85 Å)	-	-		

Table 6.4 Amylose content (%), crystallinity index (%) and crystal pattern of UCC026 plants at M_1V_2 stage

Sample	Amylose	Combinations of angles of diffraction peaks at 2θ diffraction that make up crystal pattern												Crystallinity index (%)	Crystal pattern
		B/C	V		V	A/C/B	ACB	A	A/C/B/V	B (not C)	A/C/B				
		5°	7°	11°	13°	15°	17°	18°	20°	22°	23°	27°	31°		
(a) UCC026	20.92	-	-	-	-	15.18	17.64	18.29	-	-	23.24	-	-	0.53	A
		-	-	-	-	(5.83 Å)	(5.02 Å)	(4.85 Å)	-	-	(3.82 Å)	-	-		
(b) N441P54	32.74	-	7.84	-	13.17	15.24	17.41	18.30	20.44	-	23.13	-	-	0.49	A
		-	(11.26 Å)	-	(6.72 Å)	(5.81 Å)	(5.09 Å)	(4.84 Å)	(4.34 Å)	-	(3.84 Å)	-	-		
(c) N468P6	22.85	-	-	-	-	15.12	17.98		19.71	-	23.06	-	-	0.47	A
		-	-	-	-	(5.85 Å)	(4.93 Å)		(4.50 Å)	-	(3.85 Å)	-	-		
(d) N439P9	25.80	-	-	11.61	-	15.16	17.22	17.94	-	-	23.22	-	-	0.52	A
		-	-	(7.62 Å)	-	(5.84 Å)	(5.15 Å)	(4.94 Å)	-	-	(3.83 Å)	-	-		
(e) N468P8	26.50	-	-	11.61	-	15.38	17.36	18.02	-	-	23.18	-	-	0.47	A
		-	-	(7.61 Å)	-	(5.76 Å)	(5.11 Å)	(4.92 Å)	-	-	(3.83 Å)	-	-		
(f) N468P9	25.82	5.26	-	-	-	15.28	17.02	17.76	-	22.42	23.11	27.30	-	0.56	C
		(16.79 Å)	-	-	-	(5.80 Å)	(5.21 Å)	(4.99 Å)	-	(3.96 Å)	(3.85 Å)	(3.26 Å)	-		
(g) N470P7	23.10	-	-	11.16	-	15.18	17.19	17.98	20.29	-	23.08	-	-	0.5	A
		-	-	(7.92 Å)	-	(5.83 Å)	(5.16 Å)	(4.93 Å)	(4.37 Å)	-	(3.85 Å)	-	-		
(h) N470P3	26.52	-	-	-	-	15.41	17.26	18.13	-	-	23.21	-	-	0.46	A
		-	-	-	-	(5.75 Å)	(5.13 Å)	(4.89 Å)	-	-	(3.83 Å)	-	-		
(i) N470P8	22.96	-	-	11.60	-	15.33	17.50	18.08	-	-	23.30	26.09	-	0.47	A
		-	-	(7.62 Å)	-	(5.78 Å)	(5.06 Å)	(4.90 Å)	-	-	(3.82 Å)	(3.41 Å)	-		
(j) N473P2	18.96	-	-	11.57	-	15.04	17.30	18.02	20.11	-	23.34	-	31.19	0.56	A
		-	-	(7.64 Å)	-	(5.89 Å)	(5.12 Å)	(4.92 Å)	(4.41 Å)	-	(3.81 Å)	-	(2.87 Å)		
(k) N474P7	18.17	-	-	11.23	-	15.11	17.13	17.97	-	-	23.09	-	-	0.49	A
		-	-	(7.87 Å)	-	(5.86 Å)	(5.17 Å)	(4.93 Å)	-	-	(3.85 Å)	-	-		
(l) N474P10	19.10	-	-	-	-	15.58	17.29	18.11	-	22.39	23.30	-	-	0.42	A
		-	-	-	-	(5.68 Å)	(5.12 Å)	(4.90 Å)	-	(3.97 Å)	(3.81 Å)	-	-		
(m) N474P15	17.70	-	-	-	-	15.26	17.49	17.97	20.18	-	23.24	-	-	0.47	A
		-	-	-	-	(5.80 Å)	(5.07 Å)	(4.93 Å)	(4.40 Å)	-	(3.82 Å)	-	-		
(n) N476P22	19.54	-	-	11.32	-	15.18	17.18	18.04	-	-	23.24	-	-	0.56	A
		-	-	(7.81 Å)	-	(5.83 Å)	(5.16 Å)	(4.91 Å)	-	-	(3.82 Å)	-	-		
(o) N476 P23	11.65	-	-	-	-	15.15	17.96	18.13	-	-	23.32	-	-	0.53	A
		-	-	-	-	(5.84 Å)	(4.94 Å)	(4.89 Å)	-	-	(3.81 Å)	-	-		
(P) N476P25	17.27	-	-	11.27	-	14.98	17.23	17.90	19.88	-	23.16	-	-	0.47	A
		-	-	(7.85 Å)	-	(5.91 Å)	(5.14 Å)	(4.95 Å)	(4.46 Å)	-	(3.84 Å)	-	-		

6.4.3 Differential scanning calorimetry of starch from control landrace and induced cassava plants

The real time DSC thermograph of the induced plant 357P4 and five control cultivated species are presented in Figure 6.6. Three thermal transitions were observed in the real time DSC curve, when cassava starch is heated in excess water from 25 to 150°C. These were the gelatinization transitions of cassava starch within the gelatinization zone (GZ), followed by the melting transition within the melting zone (MZ) and finally, the decomposition transition at higher temperature within the decomposition zone (DZ). Heating up to 150°C caused bursting of the 40μL Al-crucible in the furnace and robot failure to remove the broken crucibles. It also caused the gelatinization isotherm to be obscured and appeared flattened out when plotted on the same scale as the melting isotherm. A lower working temperature between 25 and 110°C was therefore chosen to emphasize the gelatinization of the starches. The heat transfer scale around 0.5 W g^{-1} and below is associated with the gelatinization transition, while around 50Wg^{-1} signifies melting of starch granules (Figure 6.6).

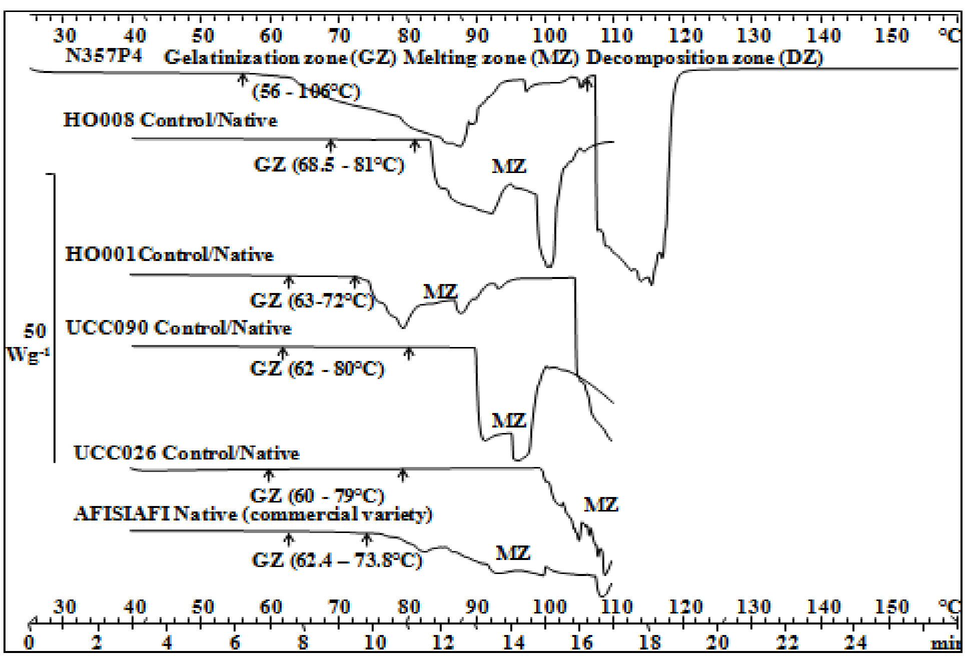


Figure 6.6 DSC thermographs of induced (N357P4) and five control landrace cassava starches at M_1V_2 stage

AFISIAFI cassava variety was included as reference against the four control landraces HO008, HO001, UCC090 and UCC026 (Figure 6.6). It is a control cassava variety which is used for a "Presidential Special Initiative" in Ghana to produce starch for export to European Markets under the African Growth and Opportunity Act (AGOA). Their respective gelatinization temperatures are indicated as GZ in Figure 6.6. The gelatinization temperature of the control cassava starches ranged from 62.4-73.8°C, 68.5-81°C, 61-72°C, 62-80°C and 60-79°C. The gelatinization temperature across the five control cultivated species therefore ranged from 60-81°C. Some induced plants, such as N357P4, showed both gelatinization and melting occurring within the gelatinization temperature range (Figure 6.6).

The real time DSC thermographs of some induced and control HO008 plants are shown in Figure 6.7 and 6.8 while the specific T_o, T_p, T_c and other gelatinization properties are given in Table 6.5. Starch granules in excess water absorb heat (endothermic) from the sample furnace (indicated on the thermographs as an exothermic peak with negative ΔH_G) to undergo gelatinization. Starch from plant N330P2 showed a narrow gelatinization temperature range (62.18-66.54°C) compared to the control HO008 . Induced N330P2, N169P7 and control

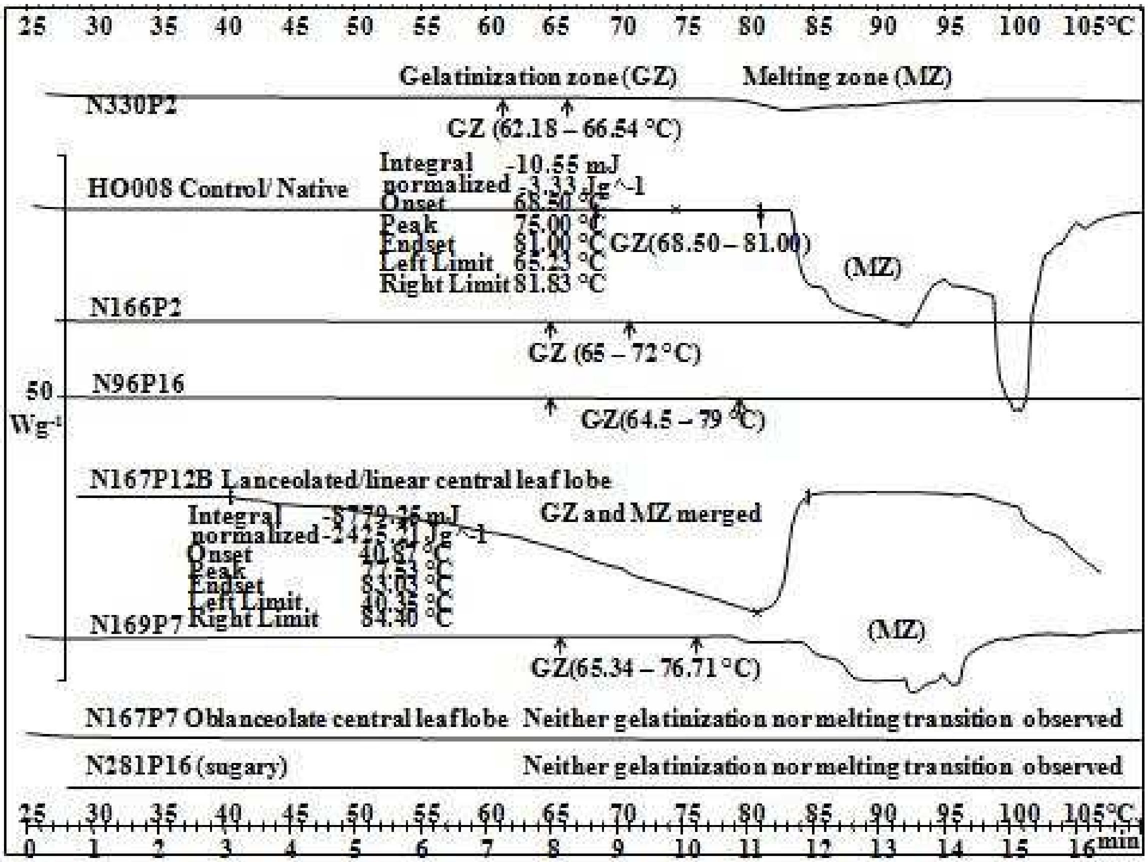


Figure 6.7 DSC thermographs of starch from induced HO008 cassava plants

HO008 plants had both the gelatinization and melting transitions before 110°C whilst N166P2, N96P16 only had gelatinization before 110°C. The lanceolated central leaf lobe mutant N167P12B had both gelatinization and melting isotherms merging into each other within the temperature range of normal gelatinization. Plant N169P7 and the putative free-sugar plant N281P16 did not show any gelatinization transition or melting transition before 110°C. In other words they showed only one isotherm between 25 - 110°C (Figure 6.7).

The DSC spectra continuation of gelatinization of induced plants derived from HO008 are shown in Figure 6.8. The shorter lengths of thermographs represent starch samples which started melting before 92°C whilst the full length thermographs represents plants which started melting after 92°C. Plants N223P13, N95P4, N166P3, N166P2, N166P5, N169P4 and N281P4 had two gelatinization peaks whilst the remaining plants had one.

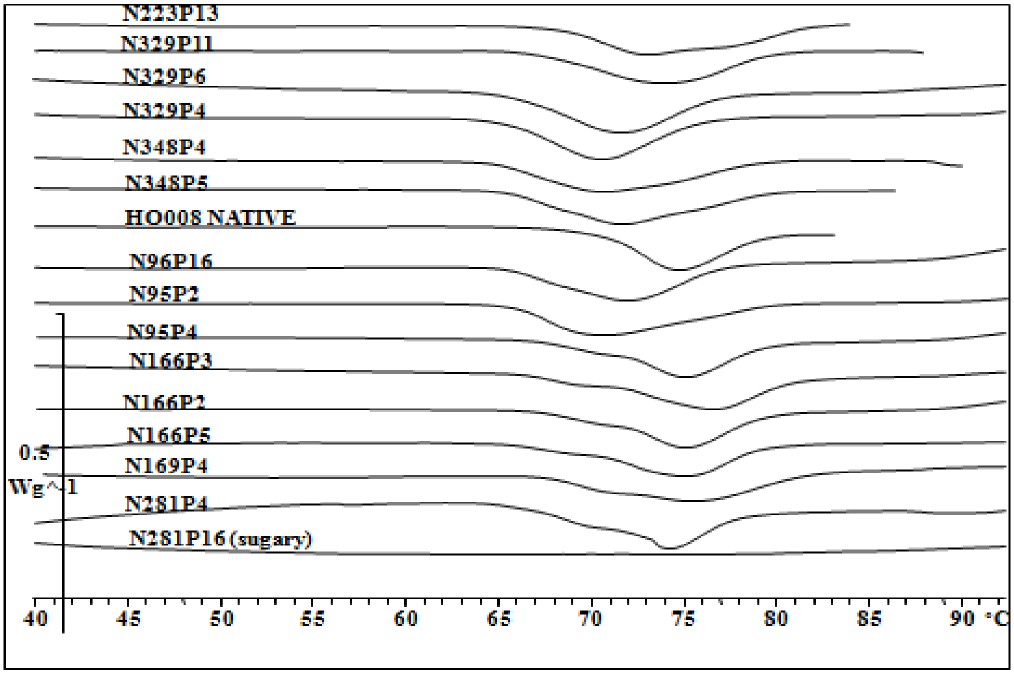


Figure 6.8. DSC thermograph continuation of starch from induced HO008 plants

The gelatinization properties of induced and control landrace plants of HO008 are presented in Table 6.5. The onset temperature (T_o) of gelatinization for the first peak ranged from 40.9 (N167BP12) to 83.7°C (N39P5), peak (T_p) from 64.01 (N330P2) to 85.3°C (N39P5), and endset (T_e) from 66.5 (N330P2) to 89.2°C (N39P5). The onset temperature (T_o) of gelatinization for the secnd peak ranged from 71.0 (N166P5) to 76.0°C (N223P13), T_p from 74 (N218P4) to 78 °C (N223P13) and T_e from 81.0 (N281P4) to 83.0°C (N233P13). The control HO008 had 68.5°C, 75.0°C and 81.0°C values for T_o, T_p and T_e transitions and a temperature range of 68.50 to 81.0°C and the temperature difference between the T_o and T_e (Range 1) of 12.5°C. The Range 1 values ranged from 4.4 (N330P2) to 42.1°C (N167BP12) and Range 2 from 7.0 (N223P13) to 11.0°C (N166P3). The full temperature range (Range3) (starting from the first onset to the last endset)

Table 6.5 The gelatinization properties of induced and control landrace HO008 plants

Name	Onset (T_o)	Peak1 (T_p)	Endset (T_e)	Range1 °C	Onset (T_o)	Peak2 (T_p)	Endset (T_e)	Range2 °C	ΔH (Jg^{-1})	Range3 °C
N330P2	62.2	64.1	66.5	4.4	-	-	-	-	0.0	4.4
N39P5*	83.7	85.3	89.2	5.5					47.9	5.5
N95P5*	73.3	77.1	80.5	7.2					77.2	7.2
N167BP13	68.5	75.3	79.0	10.5	-	-	-	-	3.8	10.5
N169P7	65.3	71.7	76.7	11.4	-	-	-	-	2.6	11.4
HO008	68.5	75.0	81.0	12.5	-	-	-	-	2.3	12.5
N167AObl	67.7	75.4	81.0	13.3	-	-	-	-	3.3	13.3
N96P16	64.5	71.5	79.0	14.5	-	-	-	-	3.6	14.5
N329P4	63.0	71.0	78.0	15.0	-	-	-	-	3.2	15.0
N166P2	65.0	70.0	72.0	7.0	72.0	75.0	81.0	9.0	3.0	16.0
N166P5	65.0	69.0	71.0	6.0	71.0	75.0	81.0	10.0	3.3	16.0
N329P11	65.0	74.0	81.0	16.0	-	-	-	-	3.4	16.0
N169P4	67.0	72.0	73.0	6.0	73.0	75.0	83.0	10.0	4.2	16.0
N281P4	65.0	70.0	71.0	6.0	71.0	74.0	81.0	10.0	4.3	16.0
N95P4*	58.9	71.4	75.5	16.6					1030.4	16.0
N95P2	64.0	70.5	81.0	17.0	-	-	-	-	3.4	17.0
N329P6	62.0	71.5	80.0	18.0	-	-	-	-	3.7	18.0
N166P3	65.0	69.0	72.0	7.0	72.0	77.0	83.0	11.0	5.8	18.0
N348P5*	61.5	71.0	81.0	19.5					51.9	19.5
N348P4	61.0	72.0	81.0	20.0	-	-	-	-	3.3	20.0
N223P13	62.0	66.5	76.0	14.0	76.0	78.0	83.0	7.0	3.9	21.0
N223P12*	62.0	82.4	88.9	26.9					1074.6	26.9
N167BP12*	40.9	77.5	83.0	42.1					2425.2	42.2
Total	1481.1	1673.1	1797.3	316.3	435.0	454.0	492.0	57.0	4764.2	372.8
Mean	64.4	72.7	78.1	13.8	72.5	75.7	82.0	9.5	207.1	16.2
STDEV	7.2	4.7	5.5	8.5	1.9	1.5	1.1	1.4	569.0	7.6

* referred to plants with high enthalpy of merged transition between gelatinization and melting.

also ranged from 4.4 (N330P2) to 42.2.0°C (N167BP12). The enthalpy of gelatinization (ΔH_G) respectively ranged from 0.03 (N330P2) to 2425.2 Jg^{-1} (N167BP12). Induced plants N330P2, N167BP13, N169P7, HO008, N167AObl, N96P16, N329P4, N166P2, N166P5, N329P11, N169P4, N281P4, N95P2, N329P6, N166P3, N348P4, N223P13 had normal gelatinization transition whiles induced plants N223P12, N348P5, N95P5, N95P4, N39P5, and N167BP12 had melting transition fused with gelatinization transition, thus making it impossible to separate the two transitions. The transition temperatures and enthalpies observed were therefore values for both gelatinization and melting which were higher than those having the normal gelatinization transitions. The two plants with the lowest T_o were N167BP12 (40.9°C) and N95P4 (58.9°C) whilst N95P5 (73.3°C) and N39P5 (83.7°C) were among the highest induced plants of control HO008 and these four plants were all having a merged transition between gelatinization and melting.

The real time DSC thermographs of induced and control HO001 plants at M_1V_2 stage are shown in Figure 6.9 and 6.10 together with gelatinization properties in Table 6.6. In Figure 6.9, induced plants N387P4, N387P5 and the control HO001 had a normal thermal transition in which a separate gelatinization transition was observed from melting transition whilst merged transition between gelatinization and melting were observed in the remaining plants (N357P4, N373P8, N353P6 and N389P5). Induced plants N357P4 and N373P8 had both gelatinization and melting transitions merged together within the gelatinization zone.

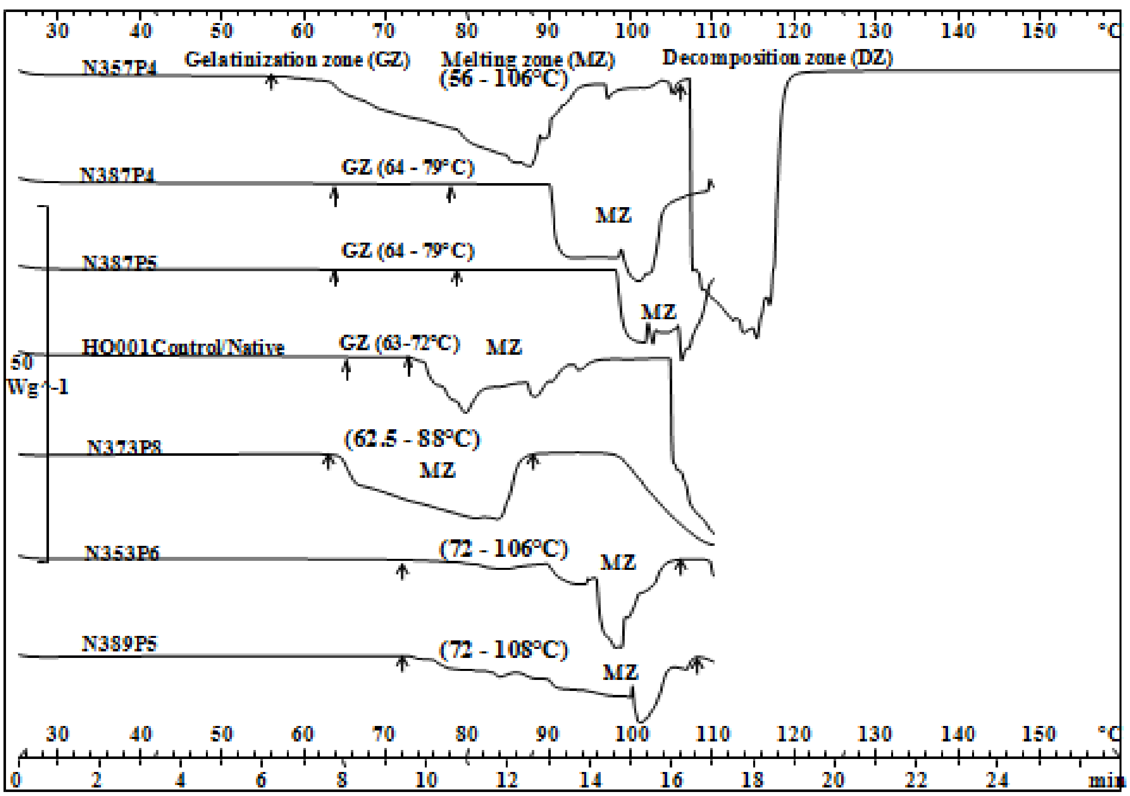


Figure 6.9. DSC thermographs of starch from induced HO001 cassava plants

In Figure 6.10, N383P2 differed from the rest by having two gelatinization transition peaks whilst the remaining plants had only one. T_o of most plants derived from HO001 are almost the same except for the putative free-sugar mutant N357P5 with significantly higher T_o and T_p than the rest. Plant N357P5 had a wider temperature range and delayed gelatinization transition than the control. The T_o and T_p of plant N387P3 were also lower than the rest. The shorter thermographs represent starch samples which had started melting before 92°C whilst the longer thermographs represent starch samples which started melting after 92°C.

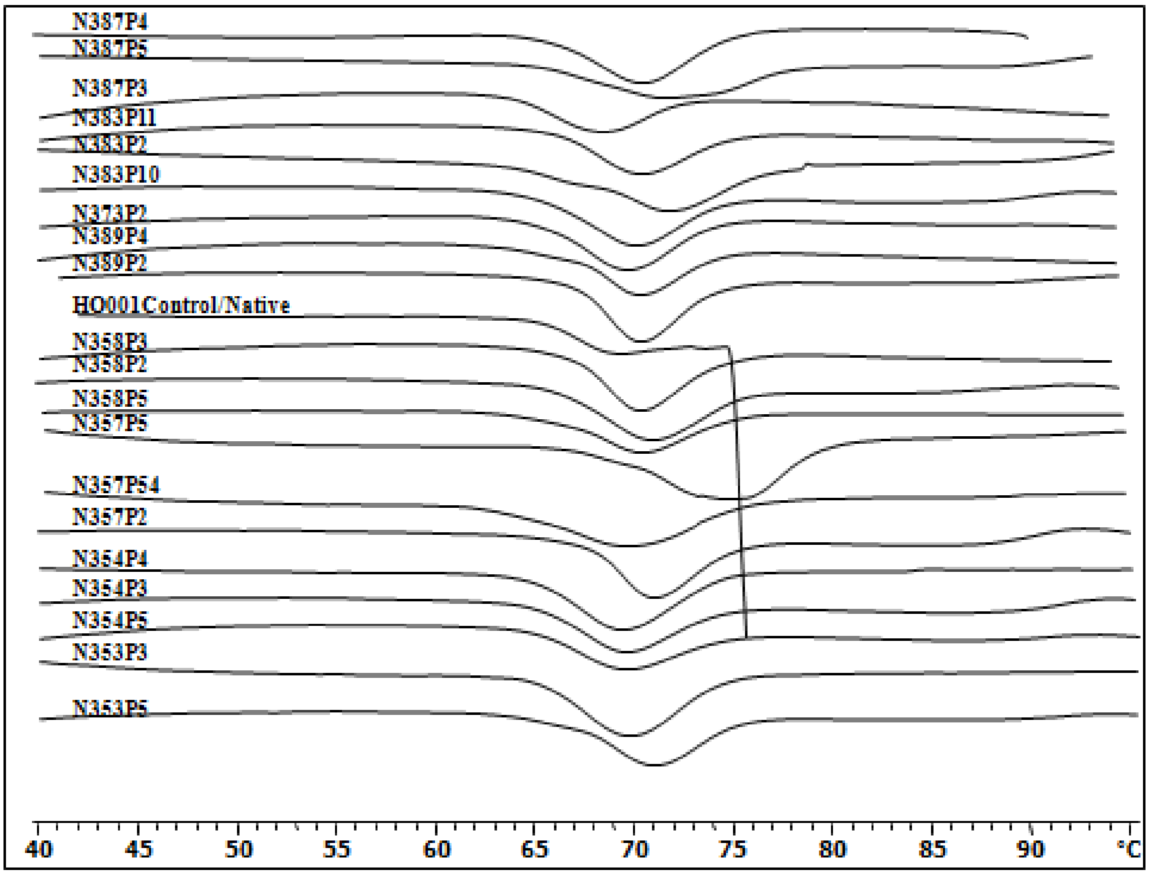


Figure 6.10. DSC thermographs continuation of starch from induced HO001 plants

The gelatinization properties of induced and control HO001 plants are presented in Table 6.6. The onset temperature (T_o) of gelatinization for the first peak (peak 1) ranged from 56.0 (N357P4) to 72.0°C (N389P5), T_p from 67.0 (N383P2) to 101.0 °C (N389P5) and T_e from 69.0 (N383P2) to 108.0°C (N389P5). The onset temperature (T_o) of gelatinization for the second peak

Table 6.6. The gelatinization properties of induced and control landrace HO001 plants

Name	Onset (T_o)	Peak1 (T_p)	Endset (T_e)	Range1 °C	Onset (T_o)	Peak2 (T_p)	Endset (T_e)	Range2 °C	ΔH (Jg^{-1})	Range3 °C
N387P4	63.0	70.0	78.0	15.0	-	-	-	-	2.9	15.0
N387P5	63.0	67.0	79.0	15.0	-	-	-	-	2.5	16.0
N387P3	62.0	67.0	75.0	13.0	-	-	-	-	2.0	13.0
N383P11	63.0	70.0	78.0	14.5	-	-	-	-	2.7	15.0
N383P2	62.0	67.0	69.0	7.0	69.0	72.0	79.0	11.0	3.8	17.0
N383P10	62.0	70.0	78.0	9.0	-	-	-	-	3.5	16.0
N373P2	62.0	69.5	78.0	14.0	-	-	-	-	3.2	16.0
N373P8*	62.5	81.4	88.0	25.5					1715.6	25.5
N389P5*	72.0	101.0	108.0	36.0					1401.7	36.0
N389P4	62.0	70.5	78.0	15.0	-	-	-	-	2.8	16.0
N389P2	62.0	70.5	78.0	15.0	-	-	-	-	12.9	16.0
HO001Nat.	63.0	67.0	72.0	9.0	-	-	-	-	1.5	9.0
N358P3	64.0	70.5	78.0	15.0	-	-	-	-	3.7	14.0
N358P2	64.0	71.0	78.0	16.0	-	-	-	-	3.7	14.0
N358P5	63.0	70.5	77.0	16.0	-	-	-	-	3.1	14.0
N357P54	62.0	70.0	77.0	16.0	-	-	-	-	2.8	15.0
N357P5	67.0	68.0	84.0	5.5	70.5	76.0	83.0	12.5	3.6	16.0
N357P4*	56.0	88.0	106.0	50.0					1890.8	50.0
N357P2	64.0	71.0	77.0	13.0	-	-	-	-	2.3	13.0
N354P4	63.0	70.0	77.0	15.5	-	-	-	-	3.7	14.0
N354P3	63.0	70.0	77.0	13.0	-	-	-	-	3.1	14.0
N354P5	63.0	70.0	77.0	14.0	-	-	-	-	2.8	14.0
N353P3	64.0	70.0	77.0	14.0	-	-	-	-	3.6	13.0
N353P5	63.0	71.0	77.0	16.0	-	-	-	-	3.6	14.0
N353P6*	72.0	98.0	106.0	34.0					895.4	34.0
Total	1586.5	1828.9	2027.0	426.0	139.5	148.0	162.0	23.5	5977.1	449.5
Mean	63.5	73.2	81.1	17.0	69.8	74.0	81.0	11.8	239.1	18.0
STDEV	3.1	9.1	10.2	9.7	1.1	2.8	2.8	1.1	572.1	9.1

* referred to plants with high enthalpy of merged transition between gelatinization and melting.

ranged from 69.0 (N383P2) to 70.5°C (N357P5), T_p from 72.0 (N383P2) to 76.0°C (N357P5) and T_e from 79 (N383P2) to 83.0°C (N357P5). The T_o, T_p and T_e values for the control HO001 plant were 63.0°C, 67.0°C and 72.0°C respectively. The temperature difference between the T_o and T_e (Range1) ranged from 5.5 (N357P5) to 50.0 (N357P4) and that of Range 2 ranged from 11.0 (N383P2) to 12.5 (N357P5) respectively. The ΔH_G ranged from 1.5 (HO001control landrace) to 1890.8 Jg^{-1} (N357P4). The full temperature range (range3) ranged from 2.01 (HO001 control landrace) to 50.0°C (N357P4). Plants N373P8, N389P5, N357P4 and N353P6 had a merged transision between gelatinization and melting within geleatenization zone as a result the energy obtained was definitely more than the small amount of energy required for gelatinization alone. The rest of the plants (N387P4, N387P5, N387P3, N383P11, N383P2, N383P10, N373P2, N389P4, N389P2, HO001Nat., N358P3, N358P2, N358P5, N357P54, N357P5, N357P2, N354P4, N354P3, N354P5, N353P3, N353P5) had a normal gelatinization transition with their T_o significantly lower than 67.0°C of the putative free-sugar plant N357P5. The two plants with the lowest T_o were N357P4 (56.0°C) and N387P3 (62.0°C) whilst N353P6 (72.0°C) and N389P5 (72.0°C) were among the highest induced plants of control HO001 and out of these plants N357P4 and N389P5 were among plants with the fused transition of gelatinization and melting.

Figure 6.11 shows the real time DSC thermographs of control landrace and induced UCC090 plants at the M_1V_2 stage. The control UCC090 and induced plants N419P4, N412P4, N413P2, N429P3 and N422P113 had two gelatinization peaks whilst the remaining plants had one. All plants showed a normal gelatinization transition but plant N398P3 showed melting alone or melting merging with the gelatinization instead of gelatinization transition within the gelatinization zone. Plants N398P3 and N398P4 had early gelatinization within the 25 - 110°C period. The T_o for control UCC090 and N405P12 were among the highest whilst the deep fissured granule producing plant N398P3 had the lowest. The putative free-sugar mutant N394P82 had a very wide gelatinization temperature range together with N413P2, N422P113 and N412P6 plants, indicating the presence of non-uniform granules.

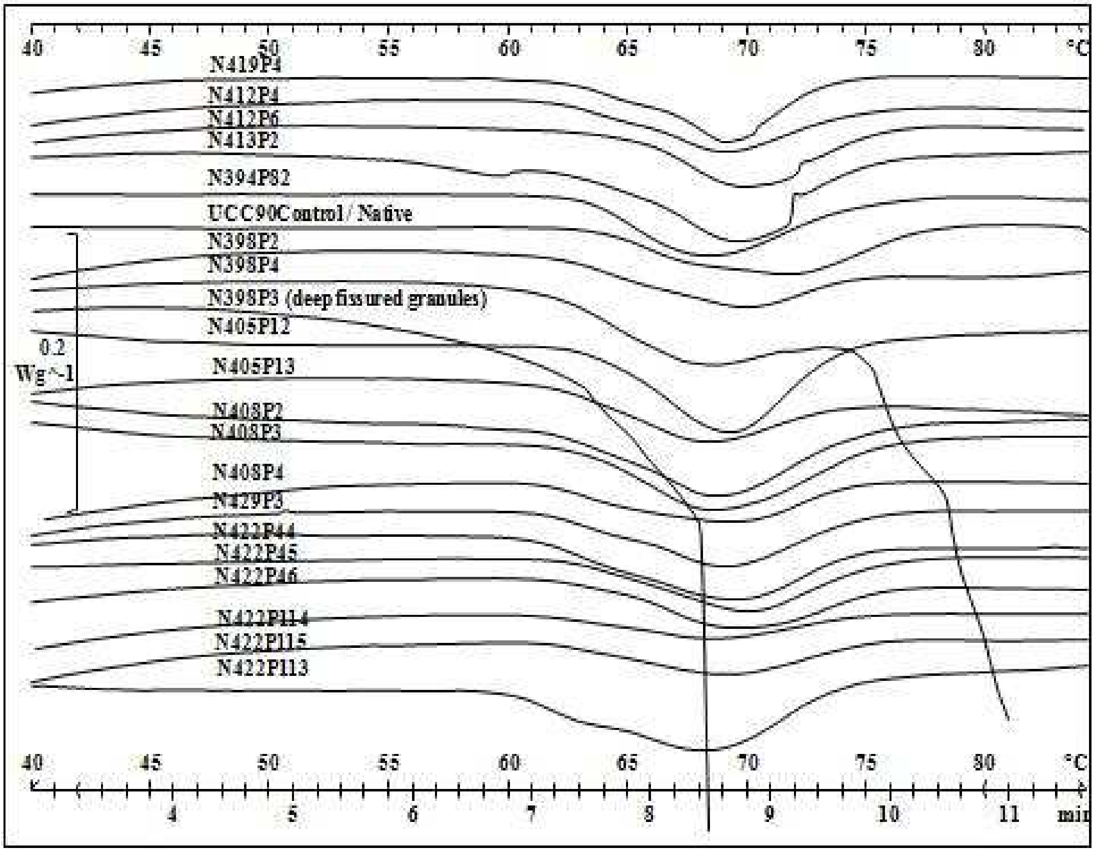


Figure 6.11 DSC thermographs of starch from induced UCC090 cassava plants

The gelatinization properties of induced and control landrace UCC090 plants are presented in Table 6.7. The onset temperature (T_o) of gelatinization for the first peak ranged from 47.0 (N398P3fissur) to 62.0°C (N405P12), T_p from 59.5 (N413P2) to 86.0°C (N398P3fissur) and T_e from 61.0 (N413P2) to 91.3°C (N398P3fissur). The onset temperature (T_o) of gelatinization for the second peak ranged from 61.0 (N413P2) to 70.0°C (UCC090Nat), T_p from 68.0 (N422P113) to 72.0°C (UCC090Nat) and T_e from 75.5 (N419P4) to 80.0°C (UCC090Nat). Plants N413P2, N419P4, N398P4 and N422P113 were among the lowest T_o group whilst the control UCC090 and N405P12 were among the highest. The T_o, T_p and T_e values for the control landrace were 62.0°C, 68.0°C peak low and 72°C peak high and 80°C respectively. The temperature difference between the T_e and T_o in the Range1 column and range 2 columns were 6.0 (N422P113) to 44.3°C (N398P3fissur) and 9.0 (N419P4) to 17.0°C (N413P2) respectively. The putative plant with fissured granules (N398P3fissur) had merged transition between gelatinization and melting and this is supported by significantly high enthalpy of thermal transition than the rest with normal gelatinization transitions. The ΔH_G ranged from 1.4 (UCC090Nat.) to 1403.8 Jg^{-1} (N398P3fissur). The full temperature range (range 3) had a range of values from 10.50 (N398P4) to 44.3°C (N413P2).

Table 6.7. The gelatinization properties of induced and control landrace UCC090 plants

Name	Onset (T_o)	Peak (T_p)	Endset (T_e)	Range1 °C	Onset (T_o)	Peak (T_p)	Endset (T_e)	Range2 °C	ΔH (Jg^{-1})	Range3 °C
N419P4	59.5	65.0	66.5	7.0	66.5	69.0	75.5	9.0	2.2	16.0
N412P4	60.0	65.0	66.5	6.5	66.5	69.0	76.0	9.5	2.3	16.0
N412P6	59.0	70.0	77.0	18.0	-	-	-	-	2.7	18.0
N413P2	54.5	59.5	61.0	6.5	61.0	69.5	78.0	17.0	4.1	23.5
N394P82	60.0	68.5	78.0	18.0	-	-	-	-	3.1	18.0
UCC090Nat	62.0	68.0	70.0	8.0	70.0	72.0	80.0	10.0	1.4	18.0
N398P4	59.0	68.5	69.5	10.5	-	-	-	-	1.7	10.5
N398P3* fissur	47.0	86.3	91.3	44.3					1403.8	44.3
N398P2	60.0	70.0	75.5	15.5	-	-	-	-	2.6	15.5
N405P12	62.0	69.0	74.5	12.5	-	-	-	-	4.0	12.5
N405P13	59.0	69.0	75.0	16.0	-	-	-	-	2.3	16.0
N408P3	61.0	69.0	78.0	17.0	-	-	-	-	3.6	17.0
N408P2	61.0	69.0	78.0	17.0	-	-	-	-	2.9	17.0
N408P4	61.0	69.0	78.0	17.0	-	-	-	-	2.5	17.0
N429P3	61.0	65.0	77.0	16.0	66.5	69.0	78.0	11.5	2.9	17.0
N422P45	61.0	70.0	78.0	17.0	-	-	-	-	10.3	17.0
N422P46	61.0	70.0	78.0	17.0	-	-	-	-	8.1	17.0
N422P44	61.0	69.0	76.0	15.0	-	-	-	-	10.5	15.0
N422P115	61.0	69.0	76.0	15.0	-	-	-	-	6.6	15.0
N422P114	61.0	69.0	77.0	16.0	-	-	-	-	5.7	16.0
N422P113	59.0	63.0	65.0	6.0	65.0	68.0	78.0	13.0	10.4	19.0
Total	1250.0	1440.8	1565.8	315.8	395.5	416.5	465.5	70.0	1493.7	375.3
Mean	59.5	68.6	74.6	15.0	65.9	69.4	77.6	11.7	71.1	17.9
STDEV	3.3	4.9	6.4	8.0	2.9	1.4	1.6	3.0	305.4	6.5

* referred to plants with high enthalpy of merged transition between gelatinization and melting.

The real time DSC thermographs of control landrace and induced UCC026 plants at M_1V_2 stage is presented in Figure 6.12. The shorter lengths of thermographs represent starch samples which started melting before 100°C whilst the full length thermographs represent plants which started melting after 100°C. Plants N439P9, N439P8, N470P8 and N476P22 had two gelatinization peaks whilst the remaining plants had one. The onset temperatures for most of the induced plants above the control UCC026 were lower than that of the control and vice versa. Plant N470P3 did not show any gelatinization or melting during transition periods. The putative free-sugar mutant (N441P54) and exceptional fissured granule producing plant (N441P26) were among plants with the wide gelatinization temperature range.

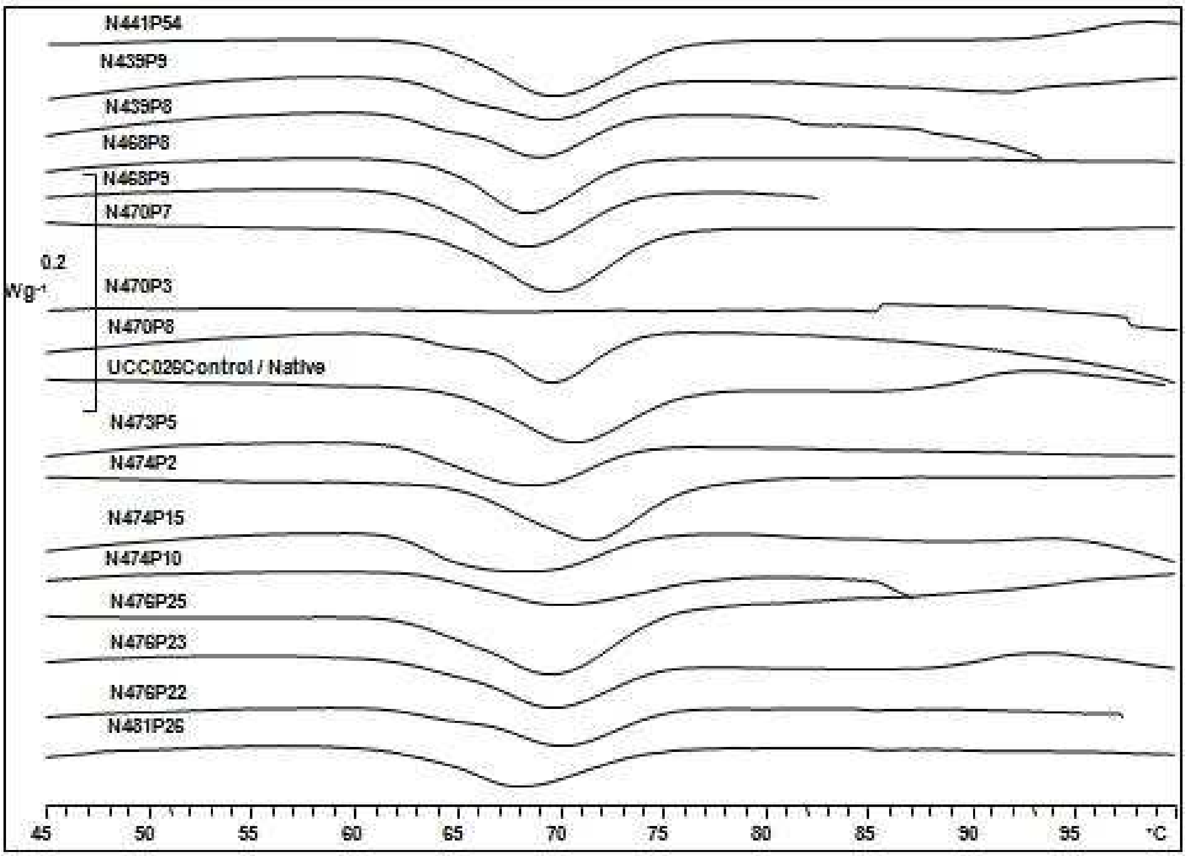


Figure 6.12 DSC thermographs of starch from induced UCC026 cassava plants

The gelatinization properties of induced and control UCC026 plants at M_1V_2 stage are presented in Table 6.8. There is no significant difference in differential scanning of control landrace and induced plants of UCC026 except N473P2 with the higest T_o and T_p values of 72.7°C and 74.0°C. The onset temperature (T_o) of gelatinization for the first peak ranged from 60.0 (N441P54, N439P9, N439P8, N468P8, N468P9, N481P26) to 72.7°C (N473P2), 65.0 (N439P9, N439P8, N470P8, N476P22) to 74.0°C (N473P2), 66.0 (N439P9, N439P8, N476P22) to 79.0°C (N481P26, N468P8, N468P9, N473P5, N474P10, N470P7, N441P54 (sug), UCC026Nat, N474P2). The onset temperature (T_o) of gelatinization for the second peak ranged from 66.0 (N439P9, N439P8, N476P22) to 67.0°C (N470P8), T_p from 69.0 (N439P9, N439P8) to 70.0°C (N476P22) and T_e from 78.0 (N476P22) to 79.0°C (N439P9, N439P8, N470P8). The onset temperature (T_o) of gelatinization for the control UCC026 was 62.0°C, T_p was 70.5°C and T_e was 79.0°C. The temperature difference between the T_o and T_e in the range 1 and range 2 columns ranged from 3.4 (N473P2) to 19.0°C (N481P26, N468P8, N468P9, N441P54 (sug)), and 12.0 (N476P22, N470P8) to 13.0 °C (N439P9, N439P8) respectively. The ΔH_G ranged from 0.69 (N474P10) to 11.47 Jg^{-1} (N473P2) whilst the control UCC026 had 3.05 Jg^{-1}. The full temperature difference (range3) ranged from 3.4 (N473P2) to 19.0°C (N439P8, N481P26, N468P8, N439P9, N468P9, N441P54 (sug)).

Table 6.8.The gelatinization properties of induced and control landrace UCC026 plants

Name	Onset (T_o)	Peak1 (T_p)	Endset (T_e)	Range1 °C	Onset (T_o)	Peak2 (T_p)	Endset (T_e)	Range2 °C	ΔH (Jg^{-1})	Range3 °C
N441P54(sug)	60.00	70.00	79.00	19.00	-	-	-	-	3.12	19.00
N439P9	60.00	65.00	66.00	6.00	66.00	69.00	79.00	13.00	2.76	19.00
N439P8	60.00	65.00	66.00	6.00	66.00	69.00	79.00	13.00	1.88	19.00
N468P8	60.00	68.50	79.00	19.00	-	-	-	-	2.75	19.00
N468P9	60.00	68.50	79.00	19.00	-	-	-	-	2.77	19.00
N470P7	61.00	69.50	79.00	18.00	-	-	-	-	3.46	18.00
N470P8	61.00	65.00	67.00	6.00	67.00	69.50	79.00	12.00	0.97	18.00
UCC026Nat	62.00	70.50	79.00	17.00	-	-	-	-	3.05	17.00
N473P2	72.74	74.00	76.14	3.40	-	-	-	-	11.47	3.40
N473P5	63.00	68.50	79.00	16.00	-	-	-	-	2.58	16.00
N474P2	64.00	71.50	79.00	15.00	-	-	-	-	3.35	15.00
N474P15	61.00	68.00	76.00	15.00	-	-	-	-	2.41	15.00
N474P10	62.00	69.00	79.00	17.00	-	-	-	-	0.69	17.00
N474P7	62.28	68.12	73.84	11.56	-	-	-	-	2.31	11.56
N476P25	61.00	69.00	78.00	17.00	-	-	-	-	3.20	17.00
N476P23	61.00	69.50	78.00	17.00	-	-	-	-	2.76	17.00
N476P22	61.00	65.00	66.00	5.00	66.00	70.00	78.00	12.00	1.50	17.00
N481P26	60.00	68.00	79.00	19.00	-	-	-	-	2.11	19.00
Total	1112.02	1232.62	1357.98	245.96	265.00	277.50	315.00	50.00	53.14	295.96
Average	61.78	68.48	75.44	13.66	66.25	69.38	78.75	12.50	2.95	16.44
STDEV	2.97	2.40	5.25	5.67	0.50	0.48	0.50	0.58	2.26	3.79

6.5 Discussion

6.5.1 Amylose content and crystalline properties of cassava plants at M_1V_2 stage

Starch is mainly synthesized in the storage root organs, such as seeds, tubers and roots. To store glucan molecules in an effective and stable condition in these organs, starch is packed in a semicrystalline granular form (French, 1984). Starch biosynthesis is not consistent throughout the development of starch granules. The structure of amylose and amylopectin varies in the radial locations, from the hilum to the periphery (Pan and Jane, 2000; Jane and Shen, 1993). To date, the only available non-invasive probe of starch granule organization has been X-ray diffraction, a technique which reports on an ordered arrangement of double helical amylopectin forming the crystalline region but it does not detect irregularly packed structures in the amorphous region (Gidley and Bociek, 1985; Tukomane *et al.*, 2007; Rodríguez-Sandoval *et al.*, 2005). The classification of the diffractograms into crystal patterns of cassava starch was based on the location of major and minor peaks identified with individual diffractograms in degrees of Brag's angle (2θ). It can be deduced from reports of Shujun *et al.* (2005), Cheetham and Tao (1998) and Chandrasekaran (1998) that peaks at 5°, 15°, 17°, 18°, 20°, 22° and 23° 2θ are used to classify the starches. The X- ray diffraction pattern of an A-type crystal, lacks the 5° and 22° 2θ peaks; the X-ray diffraction pattern of a C-type crystal lacks 18° and 22° 2θ peaks and that of a B-type crystal lacks only the 18° 2θ peak from the above combinations of peak angles. Each peak comes with the inter-atomic distance (d-distance) between the crystalline lamellae planes within the crystalline regions of the starch granules. New and minor peaks were observed in the current study at 11°, 27° or 31° 2θ in some induced plants (Figure 6.2:d, i, o and Table 6.1:d, i, o) which were not yet reported in literature. It may be due to irradiation response of cassava plants at the amylopectin level.

The range of amylose content in control cassava starch is 10 – 30%, amylopectin is 70-90% and the range of the degree of crystallinity in a typical control cassava starch is between 15% and 45% (Zobel, 1988, Christopher, 1997; Tang *et al.*, 2004). Since amylopectin is solely responsible for the crystallinity of granules (Manners, 1969; Nikuni, 1969; Hizukuri, 1986) but the percentage amylopectin is far greater than the degree of crystallinity. It means that some amylopectin is not in the crystalline form. Such “excess” amylopectin is not only found in the amorphous regions, but also in the form of double helices (Cooke and Gidley, 1992). The excess amylopectin double helices and amylose tie-chains which are not part of the crystallites are arranged between the crystalline lamellae acting as so-called defects (Matveev *et al.*, 1998; Yuryev, 1998). The non crystalline portion of amylopectin molecules exists outside the crystalline regions and that portion is responsible for faster chemical reactivity and gelatinization. It is less dense than the crystalline region and makes up between 15 – 75 % of the total amylopectin portion of starch granules, depending on the botanical origin (Gallant *et al.*, 1992, Matveev *et al.*, 2001). The new minor X-ray diffraction peaks observed at 11°, 27° or 31° 2θ for some of the starches from induced plants might be as a result of converting excess double helixes of amylopectin into more organized crystalline form. More work will have to be carried out to investigate the origin of those peaks (Tables 6.1 – 6.4). In some induced plants, extra peaks were observed in N329P6 (f), N348P4 (h), N96 P16 (j), N95P5 (l) and N39P5 (m) at 11° 2θ; in N223P13 (d), N329P11 (g), N348P5 (i) at 27°2θ and in N223P13 (d), N348P5 (i) N96 P16 (j) at 31°2θ for induced HO008 plants. The emergence of these peaks in induced HO008 plants and others caused drastic reduction in the amorphous region of granules; therefore the ratio of crystalline intensity to the total intensity resulted in higher crystallinity indexes observed. Chemical industries recognize the amorphous region in amylopectin as a good acceptor site for active chemicals (Gallant *et al.*, 1992). However, with relatively higher crystalline indexes observed, it implies that such starches would rather be preferred for binding chemicals in tablet forms since the chemical active portion is much smaller than those with lower crystallinity indexes.

All of the controls and most induced plants had an A - type X- ray diffraction pattern. Only seven induced plants showed a transition from an A-type to C-type diffraction pattern. These are three induced plants of HO008 (N223P13 (d), N348P5 (i) and N166P3 (o) with C, C_A and C_A) respectively; three induced plants of UCC090 (N419P4 (b), N412P4 (c) and N412P6 (d) plants with C_A – pattern each; and one induced plant of UCC026 (N468P9 (f) with a C – pattern. None of the HO001 plants showed the C-type pattern. The occurrence of the A-type X-ray diffraction pattern in control landrace and most induced cassava plants implies that cassava cultivars generally show an A-type pattern as reported in most of the literature. Cassava, which is a storage root is known not to contain starch of a B-type diffraction pattern under natural condition like tuber crops (such as potato, sweet-potato and yam), unless mutated or modified (Defloor *et al.,* 1998a;b; Eberstein *et al.,* 1980; Lorenz and Kulp, 1992 and Nuwamanya *et al.,* 2010). The irradiation of cassava stakes before planting was therefore observed to change the diffraction pattern of seven induced plants *in planta* from A-type to C-types with respect to HO008, UCC090 and UCC026 controls. This observation indicated that A-type to C-type transformation requires reorientation in the packing of the double helical starch molecules and redistribution of water within the crystallites. For example, a change from eight water molecules per unit cell in the A structure to 36 per cell in the B was reported (Sarko and Wu, 1978). The C-type may contain an intermediate level of water molecules per unit cell between A and B types. The implications are that the C- type is more resistant to hydrolysis than the A-type. It was reported that the extent of final hydrolysis of starch was less than 35% for predominantly B-type starches, 60% for C-type and more than 70% for predominantly A- or V-types, thus suggesting that the B-type starch is more resistant than the C-type, followed by A - types (Gérard *et al.,* 2001).

Cassava, rice, corn and potato were reported to have crystallinity indexes of 0.50, 0.53, 0.44 and 0.55 respectively (Guinesi *et al.,* 2006). The crystallinity indexes observed ranged from 0.44 (N223P12) to 0.66 (N223P13) compared to 0.51 for the control HO008; 0.44 (N383P10) to 0.56 (N353P5) compared to 0.46 for the control HO001; 0.42 (N398P2) to 0.6 (N419P4) compared to 0.51 for the control UCC090 and 0.42 (N474P10) to 0.56 (N476P22) as related to 0.53 of the control UCC026. The range of crystallinity indexes observed for induced plants were similar to that reported in literature, but a few induced plants were significantly higher than respective controls and reported figures. Similarly, the overall range of the crystallinity indexes observed across the four induced landraces (0.42 to 0.66) was broader than across the four controls (0.46 to 0.53). It also showed that some induced plants were higher in crystallinity indexes than the range reported by Zobel (1988) of 0.15-0.45 (15-45% degrees of crystallinity) and Tang *et al.* (2006) of 0.17-0.51 (17-51 % degrees of crystallinity) in normal cassava starches. The crystallinity indexes of 0.51 was observed for control HOOO8 but higher indexes of 0.57 (N348P5), 0.58 (N95P5) and 0.66 (N223P13) were observed for induced HO008 plants. Lower degree of crystallinity (0.46) was displayed by control HO001, but higher degrees of crystallinity such as 0.52 (N354P3), 0.52 (N354P5), 0.53 (N354P4), 0.53 (N353P34), 0.55 (N383P2), 0.56

(N387P3) and 0.56 (N353P5) were displayed by induced HO001 plants. Similarly, crystallinity index of 0.51 was observed for control UCC090 but higher values of 0.52 (N398P4), 0.54 (N405P12), 0.56 (N412P6), 0.63 (N412P4) and 0.66 (N419P4) were observed for induced UCC090 plants. The control UCC026 also had degree of crystallinity of 0.53 (UCC026) and higher values in induced plants of 0.53 (N476P23), 0.56 (N468P9), 0.56 (N476P22) and 0.56A (N473P2). The higher crystallinity indexes observed were similar to the 0.4 to 0.58 (39.53 to 57.75% degrees of crystallinity) reported for acid modified cassava starch (Atichokudomchai *et al.*, 2001) which is suitable for compressing chemicals into tablets in the pharmaceutical industry (Sanguanpong *et al.*, 2003; Chun, 1997). The induced plants with the highest crystallinity indexes observed may therefore replace acid modified starches in pharmaceutical industries because they already have far less amorphous regions compared to their controls (Figures 6.2.1: d, i, o).

The seven induced plants with C - type patterns were among the highest ranking crystallinity indexes. Hizukuri (1985) reported that the crystal type is strongly influenced by the amylose content and average chain length of the respective amylopectin. Whether A -, B - or C - types of patterns are formed is thought to be related to the average chain lengths within amylopectin clusters. Short A and B_1 chains display A-type crystallinity, while long B_2-B_4 chains make up the B-type crystallinity, while intermediate chain length is associated with C-type crystallinity (Cheetham and Tao, 1998; Hizukuri, 1985; Hizukuri *et al.*, 1983). However, wrong impressions should not be created about higher crystallinity indexes observed and their resistance status. The crystallinity levels and amylose content has no marked direct influence on final hydrolysis or resistance of starches as previously reported. New evidence reported by Gérard and co-workers (2001) showed that the extent of final hydrolysis of starches was directly related to the proportion of B-type of chains in the whole granule. The seven plants with C-type patterns and crystallinity indexes might have various proportions of B-types of chains in the whole granule that may impart various degrees of resistance to enzymatic hydrolysis. Longer chains of B (B2-B4) offer more resistant than shorter B1-chains. Thus, the effect of B-crystalline type on the susceptibility of starch to hydrolysis prevailed over crystallinity level and amylose content (Gérard *et al.*, 2001).

In decreasing order of $T_{o,}$ the seven plants with C-types patterns had values of 65.0°C (N166P3), 61.5°C (N348P5), 62.0°C (N223P13), 59.0°C (N412P6), 60.0°C (N412P4), 59.5°C (N419P4) and 60.0°C (N468P9). The respective crystallinity indexes were 0.51, 0.66, 0.57, 0.63, 0.53, 0.66 and 0.56. The variation in both gelatinization and crystallinity indexes observed were influenced by both the varietal and gamma irradiation effects. There was a strong varietal effect because plants from four different controls responded differently to gamma irradiation effects at the molecular level. Amylopectin chain length differs between the X-ray crystallographic starch types. The longer B-chain is characteristic of starches with the B crystal pattern as found in potato whilst the shorter A-chain is a characteristic of A-crystal pattern of cereals and cassava (Hizukuri, 1987). Therefore the seven C-type plants had an intermediate chain length between A-type and B-type patterns. According to Chiotelli and Meste (2002) starch with more of the shorter A and B1 chains were weaker than B2 - B4 chains because they are not long enough to connect and bind two or more cluster units together. The A-type with A and B1 chains would therefore register lower onset gelatinization temperature and lower resistance to hydrolysis than the seven C-type granules with more combinations of A, B1 and B2-B4 chains. Similarly, A-type X-ray pattern starches are more compact and crystalline than B-type and C-type starches but are less resistant to amylase hydrolysis than B-type and C-types (Planchot *et al.*, 1997; Williamson *et al.*, 1992; Gallant *et al.*, 1992; Gérard *et al.*, 2001). According to Cheetham and Tao (1998), one would expect that crystalline regions and excess double helices of amylopectin in an A-type to resist amylase hydrolysis but in practice it is not so. This is why less crystalline starches with high amylose or amorphous regions resist amylase digestion more than waxy starches with more crystalline regions (Padmanabhan and Lonsane, 1992). The higher crystallinity index alone is therefore not enough to explain the higher gelatinization properties. The ratio of shorter to longer chain lengths of amylopectin packed in the granule and other physical characteristics of granules might probably play a role. The actual length of central C chains is still not known but it is longer than A and B chains. It connects more clusters to give more strength to granules. Therefore, variation in C chains of amylopectin in C-types of diffraction patterns may also add to the variation in strength, resistivity to hydrolysis and stability of starch granules.

Assuming all other factors affecting gelatinization and crystallinity remain constant, the higher the degree of crystallinity the higher the ΔH_G (Krueger *et al.*, 1987). The ΔH_G for the seven plants with C-types of X-ray pattern were 5.83 Jg^{-1} (N166P3), 51.85 Jg^{-1} (N348P5), 3.91 Jg^{-1} (N223P13) of HOO8 plants, 2.66 Jg^{-1} (N412P6), 2.28 Jg^{-1} (N412P4), 2.23 Jg^{-1} (N419P4) of UCC090 plants and 2.77 Jg^{-1} (N468P9) of UCC026 plant. The ΔH_G for N348P5 was significantly higher than N166P3, N223P13 and the rest. However, ΔH_G for N223P13 was similar to N468P9, N412P6, N412P4 and N419P4. Plants with higher ΔH_G and crystallinity are suitable for pharmaceutical industries whilst plants with lower gelatinization and crystallinity can compete with other starches for the production of alcohol, starch for sizing paper and textiles, glues, sweeteners, bio-degradable products, butanol and acetone, manufacturing of explosives, and coagulation of rubber latex (FAO, 2001).

6.5.2 Gelatinization properties of the four control landraces of cassava

Starch granules heated in excess water was observed to absorbed heat (endothermic) lost by the DSC sample furnace relative to the reference furnace (exothermic) to undergo gelatinization transition before melting and decomposition transitions. Three thermograph transitions were observed in the real time DSC curve, when induced cassava starch was heated in excess water up to 150°C. The gelatinization transition of cassava occurred first, between 60-85°C (Paes *et al.*, 2008), 65-70°C in 45% water (Lacerda *et al.*, 2009) followed by melting around 170°C (Sriroth *et al.*, 2000) and 169.2ºC (of dry unhydrous cassava starch) (Lacerda *et al.,* 2009) and decomposition transitions at higher temperatures. The enthalpy required for melting dry cassava starch was estimated as 129.16 $J.g^{-1}$ (Lacerda *et al*., 2009) which is far higher than the energy for gelatinization of cassava 11.41-154.04 $J.g^{-1}$ (Mweta *et al*., 2008) 10.4 and sweet potato 12.9 $J.g^{-1}$ (Peroni *et al*., (2006).

Heating samples in 40 μm aluminium crucibles up to 150°C was too high and as a result, pressure was generated which caused the bursting of the crucibles. As a precaution, a lower but safer working temperature between 25 and 110°C was chosen to place more emphasis on the gelatinization of the starches with heat transfer scale around 0.5 $W.g^{-1}$ instead of $50W.g^{-1}$ associated with melting and decomposition transitions (Figure 6.6).

T_o is similar for most of the control landraces except HO008, however T_p and T_e differed significantly. In increasing order of T_o, the five landraces were ranked as 62°C (UCC090), 62°C (UCC026), 62.4°C (AFISIAFI), 63°C (HO001) and 68.5°C (HO008) with corresponding T_p as 68°C, 70.5°C, 66°C, 67°C, 75°C and the final T_e as 80°C, 79°C, 73.8°C, 72°C and 81°C respectively. The full gelatinization temperature ranges observed were 18, 17, 11.4, 9.0 and 12.5°C respectively. T_o values vary depending on the concentration of starch in water. A 45% of cassava starch water mixture was reported to gelatinize between 65-70°C (Lacerda *et al.*, 2009) or between 60-85°C (Paes *et al.*, 2008) and a 50% of starch water mixture gelatenizes between 57.6-62.0°C (Mweta *et al.*, 2008). Similar T_o's were observed for landrace starches from UCC090, UCC026, AFISIAFI, and HO001, but not for HO008, which is higher at 75°C. Apart from obvious genotypic differences, the higher T_o and T_p are characteristic of starches with lower proportions of short amylopectin branch chains (Jane *et al.*, 1999). Since ontrol HO008 had the highest T_o, it was expected to also have the highest ΔH_G. The fact that the ΔH_G of control HO008 (2.3 Jg^{-1}) was second to control UCC026 (3.05 Jg^{-1}) implies that other factors were responsible for the relative decrease in the ΔH_G. Among all the control landraces, it was only Control HO008 granules that had dents/holes in the granules whilst control UCC026 lacked them (Chapter 5: Table 4.2). This could be responsible for the reduction in enthalpy required by the control UCC026. Control HO008 had a rough surface which may aid water absorption whilst a smooth surface without dents or holes was observed in control UCC026 (Chapter 5: Table 4.2)., which might prevent early absorption of water and lead to the delay of the gelatinization process and subsequent higher enthalpy demand for the control UCC026 compared to HO008.

All the control landraces had A-type X-ray diffraction with crystallinity indexes of 0.46, 0.51, 0.51 and 0.53 for HO001, HO008, UCC090 and UCC026 respectively. The breakdown of hydrogen bonds in double helixes within the relatively higher crystalline regions of control UCC026 compared to HO008 might also be responsible for the higher ΔH_G observed for control UCC026 than control HO008. These structural differences of amylose and amylopectin have varying effects on the starch functionality such as gelatinization temperatures, pasting viscosity, gel elasticity and strength, swelling power and solubility (Lu *et al.*, 2008; Tattiyakul *et al.*, 2007; Lu *et al.*, 2005; Charles *et al.*, 2005; Jane *et al.*, 1999; Sasaki and Matsuki, 1998; Noda *et al.*, 1998; Shibanuma *et al.*, 1996). Starches with higher levels of short chains had lower gelatinization T_o, T_p and T_c or T_e temperatures, whereas higher contents of longer chains (DP 18-25 and DP 25-80) led to higher gelatinisation temperatures (Yamin *et al.*, 1999; Sang-Ho and Jay-Lin, 2002; Albert *et al.*, 2005).

6.5.3 Gelatinization properties of starch from induced cassava plants at M_1V_2 stage

All the control landraces showed single gelatinization peaks for starch except starch from control UCC090. After irradiation double gelatinization peaks of starch were observed in some plants which showed a lower first T_p than T_p of respective control landrace except N357P5. Example, lower first T_p values were observed in induced N223P13 (67, 78°C), N166P3 (69, 77°C), N166P2 (70, 75°C), N166P5 (69, 75°C), N169P4 (72, 75°C) and N281P4 (70, 74°C) plants than T_p of 75.0°C from the control HO008. Lower first peaks were observed in N419P4 (65, 69°C), N412P4 (65, 69°C), N413P2 (60, 70°C), N429P3 (65, 69°C) and N422P113 (63, 68°C) than first T_p of the control UCC090 (68, 72°C). Again lower first peaks in N439P9 (65, 69°C), N439P8 (65, 69°C), N470P8 (65, 70°C) and N476P22 (65, 70°C) than first peak in the control landrace UCC090 (68, 72°C). Control HO001 had the same T_p as the first T_p of N383P2 (67, 72°C) but produced a higher first peak in N357P5 (68, 76°C). The putative free-sugar mutant N357P5 therefore showed a deviation in the general lower trends observed for others.

It was noted that thirteen double T_p plants (N223P13, N166P2, N412P4, N413P2, N439P9, N476P22, UCC090, N166P5, N169P4, N281P16, N419P4, N422P113, N470P8) had non-uniform granule sizes whilst only three of the double T_p plants (N166P3, N429P3, N439P8) had uniform granules. These suggest that the observation of double gelatinization isotherms were due to non-uniform granules more than uniform granules. Small granules gelatinize at a higher temperature and over a wider temperature range than larger granules (Vermeylen *et al.*, 2005; Jane, 2007). In the pool of large and small granules the large granules would preferentially gelatinize at a lower temperature to produce the first peak before the small granules produce the second peak. Therefore the two peaks observed were largely due to gelatinization of large granules occurring before that of small ones. Contrary to this, a few starches with non-uniform granule sizes did not show double gelatinization peaks, while some plants with uniform granules did. This might suggest that additional minor factors might also contribute to the occurance of double T_p peaks.

Starch from the control landraces HO008, HO001 and UCC026 presented one peak at 75, 72 and 72°C respectively, while control UCC090 presented two peaks at 68 and 72°C. Most of the induced plants showed single peaks around 68, 72 or 75°C, however, T_o and T_p may differ from their respective control landraces. Since gelatinization by DSC was only carried out once because of lack of logistics required for evaluation of large replicated samples, a comparison between the control landrace and respective induced plants was the most probable option left for selecting putative mutants. Furthermore, most mutants in vegetatively propagated crops starts with DNA changes in a single cell, which later on developed into a full grown mutant. Therefore mutation screening lays more emphasis on individual evaluation of induced plants rather than means based on pool results. Since work done by Mweta and others (2008) using identical DSC equipment did not show any significant difference between repeated calorimetric scanning of the same sample, the results of the current study might therefore be largely due to the effects of irradiation. It may therefore be concluded in general that gelatinization occurs between 60 and 80°C with the T_p of cassava starch gelatinization observed between 68 and 75°C.

The four induced plants that displayed the overall lowes T_o temperatures were N167BP12 (40.9°C), N398P3fissur (47.0°C), N413P2 (54.5°C) of control HO008 (68.5) descent and N357P4 (56.0°C) of control HO001 (63°C) descent whilst the four plants that displayed the highest T_o temperatures were N389P5 (72.0°C) of control landrace HO001 (63.0°C) descent, N473P2 (72.7°C) of control UCC026 (62.0°C) descent and N95P5 (73.3°C), N39P5 (83.7°C) of control HO008 (68.5) descent. Out of these eight plants five of them (N167BP12, N398P3fissur, N389P5, N95P5 and N39P5) were having merged gelatinization and melting transitions.

The drastic enthalpy changes in the 11 plants were observed in the ΔH_G values of 47.9 Jg^{-1} (N39P5), 77.2 Jg^{-1} (N95P5), 1030.4 Jg^{-1} (N95P4), 51.9 Jg^{-1} (N348P5), 1074.6 Jg^{-1} (N223P12), 2425.2 Jg^{-1} (N167BP12) of HO008 descent; 1715.6 Jg^{-1} (N373P8), 1401.7 Jg^{-1} (N389P5), 1890.8 Jg^{-1} (N357P4), 895.4 Jg^{-1} (N353P6) of HO001 descent; and 1403.8 Jg^{-1} (N398P3fissur) of UCC090 descent. The altered ΔH_G values observed for 11 induced plants were significantly higher than ΔH_G values of the control landraces (2.3 Jg^{-1} for HO008, 1.5 Jg^{-1} for HO001, 1.4 Jg^{-1} for UCC090 and 3.1 Jg^{-1} of UCC026). The drastic enthalpy changes in the 11 plants were also extremely higher than 10.4 $J.g^{-1}$ (Peroni *et al.,* 2006) and 11.4-15.0 Jg^{-1} (Mweta *et al.,* 2008). Such differences could be attributed to cassava genotype, environment and starch water ratios used for DSC determinations. The altered ΔH_G values of the 11 plants were due to merged thermal transition between gelatinization and melting within gelatinization zone. The resultant merged enthalpy of thermal transition was significantly higher than plants with normal gelatinization isotherms. Dry cassava starch (almost anhydrous) was reported to melt around 170°C (Sriroth *et al.,* 2000) and 169.2ºC with an enthalpy of melting estimated as 129.16 Jg^{-1} (Lacerda *et al.,* 2009) which falls within the range of transition enthalpy observed from 47.9 to 2425.2 Jg^{-1}. This further confirmed that the observed higher enthalpy of thermal transition was either for melting alone or melting and gelatinization. As a result of the fused enthalpy transition, it is impossible to separate the gelatinization isotherm from the melting isotherm.

Notable among the 11 plants were descendants of the HO008 that display multiple changes due to irradiation such as plant N223P12 which displayed a higher T_p at 82.4°C and also with the lowest crystallinity index (0.42), while plant N348P5 displayed a lower T_p at 71°C and also with a transition from A-type to C-type of X-ray diffraction. Plant N167BP12 also displayed a transition from oblanceolate to lanceolate middle leaf lobes and possessed fissured granules. Plant N39P5 was among the least four amylose producing plants (15.4%) of HO008. Among the four highest amylose producing plants of HO001 was plant N389P5 which displayed a higher T_p at 101.0°C whilst plant N353P6 was also among the four lowest amylose producing plants (13.9%) of HO001. Descendants of UCC090 that displayed multiple changes includes plant N398P3 (which is similar to N398P4) displayed an overall lowest T_o at 47.0°C, higher T_p at 86.3°C and exceptionally deep fissured granules that easily produce flakes (Chapter 4 section 4.5.2.5). Plants with fissured granules are bound to have such abnormal lower gelatinization and melting transitions or possible merging of the two transitions. This agreed with a report that a novel sweet potato (Kanto 116) with cracked granules had an approximately 20°C lower pasting temperature and lower energy of gelatinization (8.8 Jg^{-1}) than the control and parent starches (Katayama *et al.*, 2002). The significance of the early completion of merged gelatinization and melting transition is that short processing time is required for commercial applications in various industries.

Apart from the 11 plants with extremely high ΔH_G, 3 plants were also observed without ΔH_G or any form of thermal transition during differential scanning calorimetry of starch from 25 to 110°C. These novel plants were N281P16 and N167P7 of HO008 descent and N470P3 of UCC090 descent. Plant N281P16 is a putative free-sugar mutant, while N167P7 has oblanceolate central leaf lobes, which indicate that these two plants have been affected by irradiation. Plant N470P3 is rich in amylose (26.5%) but relatively low in crystallinity indexes (0.46) and with an A-type of X-ray diffraction pattern. The commercial implication is that they are already in a gelatinized state after extraction and drying and no significant heating cost would be incurred in water processing for industries.

Finally, two plants were observed to have a normal gelatinization thermograph and a normal low ΔH_G but with relatively high T_o than their control landrace. These were N357P5 of HO001 descent and N473P2 of UCC026 descent. The putative free-sugar mutant N357P5 was significantly higher in T_o (67°C) and crystallinity index (0.51) than T_o (63°C) and crystallinity index (0.46) of the control landrace. This means that water-soluble sugars contained in the putative free-sugar mutant N357P5 probably caused an increase in T_o and crystallinity index observed. This agreed with the report that sugars in storage roots reduce the water replacement of hydrogen bonds during swelling of granules and stabilize the amorphous regions of the starch granules through interaction with starch chains (Spies and Hoseney, 1982; Kohyama and Nishinari, 1991), thereby delaying gelatinization and increasing the T_o and ΔH_G of starch (Spies and Hoseney, 1982; Sumnu *et al.*, 1999). This implies that macro molecule composition and packing in the starch granules of plant N357P5 differed as evidenced in gelatinization properties.

Plant N473P2 with T_o (72.7°C) and T_p (74°C), narrow temperature range value (3.4°C), a high ΔH (11.5 Jg^{-1}), a high degree of crystallinity of 0.56% which is significantly different from T_o (62°C), T_p (70.5°C), temperature range (17.0), ΔH (3.0 Jg^{-1}) and crystalinity of 0.53% of the control UCC026. Since other prominent changes in starch phenotypic properties were also noted in previous chapters, the drastic changes observed for N398P3, N398P4, N281P16 and N167P7, N357P5 could also be ascribed to the effect of irradiation. Drastic changes ascribed to irradiation effect without changes in phenotypic properties could also be re-examined during M_1V_3 stage.

6.5.4 X- ray diffraction spectroscopy and gelatinization properties of the overall four highest and four lowest amylose producing plants

The overall four highest (N441P54, N223P12, N373P2 and N329P11) and four lowest (N96 P16, N353 P6, N166 P2 and N476 P22) amylose producing plants were found to have A-type crystallinity. This is similar to the report that both waxy maize mutants (very low or zero amylose content) and normal maize showed either A-type or intermediate C-type crystallinity (Padmanabhan and Lonsane, 1992). This implies that amylose levels may influence, but not determine, the crystal type of the starch. It is rather determined by the proportion of longer B chains of amylopectin (Gérard *et al.,* 2001). The observed A-type pattern in both four highest and four lowest amylose producing plants therefore agreed with literature. The A-type of X-ray pattern is more crystalline and more compacted than B- and C- types (Burton *et al.,* 1995), but A – types are less resistant to amylase hydrolysis than B- and C-types (Riley *et al.,* 2004; El-Harith *et al.,* 1976; Planchot *et al.,* 1997, Williamson *et al.,* 1992, Gallant *et al.,* 1992; Gérard *et al.,* 2001; Gallant *et al.,* 1982). Therefore high crystallinity alone is not reason enough to explain the resistance of starch granules and its application in the bio-ethanol, food and chemical industries. Abundance of small granules, presence of channels, pores, the dilution effect of amylose on amylopectin crystallinity and the location of amylose within the granule are among the major factors that influence local crystallinity and resistance (Fannon *et al.,* 1993; Gallant *et al.,* 1997; Ciésla *et al.,* 1992; Zobel, 1988; Morrison *et al.,* 1994; Jenkins and Donald, 1995). There is a less ordered arrangement of the polysaccharide chains in the smaller granules compared to the larger ones (Chiotelli and Meste, 2002).

Gelatinization properties are characteristic of genotype of the plant and it is reported to vary with amylose/amylopectin ratio, crystallinity, granule size and distribution, crystalline/amorpous ratio and quantity of smaller components like phosphorus, lipids and proteins (Gunaratne and Hoover, 2002; Goñi *et al.,* 2008; Pérez *et al.,* 2005; Mweta *et al.,* 2008). When all other factors that affect gelatinization are assumed to remain constant, the higher degree of crystallinity was associated with higher gelatinization properties (Defloor *et al.,* 1998b). Most induced plants with significantly high amylose contents (see Chapter 5) were observed to have lower T_o and T_p than those with low amylose content and vice versa. The four highest amylose producing plants had a lower onset gelatinization temperature compared to the four lowest amylose producing plants. The T_o for the four highest amylose containing plants were not significantly different from each other (60.0°C N441P54, 62.0°C N223P12, 62.0°C N373P2), except 65.0°C (N329P11), with significantly higher T_o than the rest. The higher T_o observed for N329P11 was therefore due to the breaking down of the relatively ordered structure of amylopectin and hydrogen bonds during pasting than the rest as supported by relatively lower amylose content and higher crystallinity index than the rest.

The same reciprocal relation was observed for the amylose contents of the overall four highest amylose producing plants, 32.7% (N441P54), 31.1% (N223P12), 27.2% (N373P2), 26.83% (N329P11) amylose content and crystallinity indexes of 0.49, 0.44, 0.49, 0.50 respectively. Similarly, the crystallinity indexes of 0.47, 0.44, 0.51 and 0.56 showed an inverse relation to amylose contents in the overall four lowest amylose producing plants of 14.0% (N96P16), 13.9% (N353P6), 13.2% (N166P2) and 11.7% (N476P22). The four highest amylose containing plants might have had an increased interaction between amylose and amylopectin double helixes in the semi-crystalline shells which caused their decreased crystallinity and vice versa (Gallant *et al.*, 1997; Morrison *et al.,* 1994; Jenkins and Donald, 1995). This observation agreed with Hough (1985) who had reported that the degree of crystallinity was inversely proportional to the amylose content as waxy maize starch, with no amylose, was reported to have the highest percentage crystallinity (41.8%), while high amylose maize with 84% amylose content had the lowest percent crystallinity (17.2%).

With other factors being constant, the decrease in crystallinity could lead to low gelatinization temperature and endothermic enthalpies (Gallant *et al*., 1997; Sasaki, 2005). The ΔH_G for the N441P54, N223P12, N373P2 and N329P11 were 3.12 Jg^{-1}, 1074.6 Jg^{-1}, 3.16 Jg^{-1}, 3.40 Jg^{-1} respectively. The high ΔH_G for the N223P12 was significantly higher than the rest of the four highest amylose producing plants. This might be due to melting transition alone or merging of melting with gelatinization transition within the gelatinization zone as explained earlier.

6.6 Conclusions and recommendations

By X- ray diffraction spectroscopy it was shown that almost all induced and control landrace cassava plants produced an A - type X-ray diffraction pattern. Only seven plants were found to be of the C - type. This was accompanied by a significant variation in crystallinity indexes and gelatinization properties. Plants with higher ΔH_G and crystallinity indexes observed are suitable for pharmaceutical industries, whilst plants with lower gelatinization and crystallinity may possibly find application in the production of alcohol, starch for sizing paper and textiles, glues, sweeteners, bio-degradable products, butanol and acetone, manufacturing of explosives, and coagulation of rubber latex. The four cassava plants with the overall lowest T_o temperatures were N167BP12 (40.9°C), N398P3fissur (47.0°C), N413P2 (54.5°C) of control HO008 (68.5) descent and N357P4 (56.0°C) of control HO001 (63°C) descent whilst the overall four highest plants were N389P5 (72.0°C) of control HO001 (63°C) descent, N473P2 (72.7°C) of control UCC026 (62.0°C) descent and N95P5 (73.3°C) and N39P5 (83.7°C) of control HO008 (68.5) descent. Out of these eight plants five of them (N167BP12, N398P3fissur, N389P5, N95P5 and N39P5) dislpayed merged gelatinization and melting transitions. Therefore, all induced plants could be classified into three gelatinization categories. The first group consisted of three induced plants (N167P7, N281P16, and N470P3) which did not show a gelatinization transition. The second group included 99% of the induced plants with normal gelatinization transition properties similar to control landrace. The third group consisted of 11 plants (N223P12, N348P5, N95P5, N95P4, N39P5, N167BP12, N373P8, N389P5, N357P5, N353P6 and N398P3) which displayed merged gelatinization and melting transitions, occurring within the gelatinization zone during calorimetric scanning from 25 – 110°C. The first and third categories included most of the putative mutants described in the previous research chapters. These starches might be suitable for shortening the starch processing time required in most commercial starch application industries.

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CHAPTER 7

General conclusions and recommendations

Immediately after irradiation, mutation is induced in the planting material. The use of inappropriate chemical screening tests for a large population of induced plants is the major reason for the unnecessary delay in noticing a mutant plant with desired traits. To increase the efficiency of mutation breeding, improvement of screening methods is more important than length of trials used for raising mutation probabilities. The quest for cutting down cost and finding useful amylose mutants early enough in mutation breeding for increasing market demands was a driving factor behind early evaluation of individual induced plants in this study. The early characterization of M_1V_1 plants developed from acute 35 Gy gamma irradiation of cassava stakes from a Co^{60} source was helpful in the identification of solid mutant with linear leaf shape of central leaf lobes and chimerical mutants in both root and shoot systems. Early identification of these mutants was a sign that the irradiation dose applied was effective to cause *in planta* modification of starch granules and amylose contents. Eventually, four putative mutant plants were observed to possess altered starch qualities, suspected to be free-sugar plants at the M_1V_2 stage. It was recommended that apart from the field iodine test, the field glucose oxidase–peroxidase strip test should be conducted on the N281P16, N357P5, N394P82 and N441P54 free-sugar plants for confirmation of their free-sugar status in the subsequent mutation breeding stages which was not covered here.

Starch granule descriptors were developed to guide users to characterize starch granules effectively under both near *in-situ* and *ex-situ* conditions in control and induced cassava plants. The ranking from relatively scarce to the most common traits of starch granules observed was as follows: small size (108, 8%), presence of multi-heads (108, 8%), absence of dents/holes (181, 14%), presence of fissures (216, 17%), presence of compound granules (325, 25%) and absence of smooth surface (469, 36%). The ranking of descriptor scores was helpful in knowing which traits were relatively very scarce. Mutants are more likely to be found among scarce traits than common ones. If a less scarce trait is to be considered as a deciding factor for a mutant, there must be an exceptional difference between it and the controls. For instance, none of the control landrace plants produced up to tetravalent compound granules. The production of up to dodecavalent (12) compound granules by the free-sugar plant (N357P5) is an exceptional *in planta* modification compared to control landrace starch. In addition, linear central leaf lobe mutants (N167B, N169P4), and putative free-sugar mutants (N281P16, N357P5, N394P82 and N441P54) also produced fissured granules, but plants N398P3 and N481P26 showed extremely deep fissures. Compound granules were found in every cassava root cortex (rind) but not in the majority of the storage root flesh. It therefore seems to be an indication of an important mutation if such granules are also found in the storage root flesh.

The mutagenesis of the four control landraces resulted in the broadening of the genetic base of amylose, which was also observed to be associated with the variation in the viscosity of starch. The amylose content of the control landraces ranged from 19.7% (HO008) to 22.6% (UCC090) which was significantly narrower than the amylose range from 11.7% (N476 P22) to 32.7% (N441P54) observed in the four highest amylose producing plants N441P54 (32.7%), N223P12 (31.1%), N373P2 (27.2%), N329P11 (26.8%) and four lowest amylose producing plants N96P16 (14.0%), N353P6 (13.9%), N166P2 (13.2%) and N476 P22 (11.7%), which were significantly different from the four control landraces.

By X- ray diffraction spectroscopy it was shown that almost all control landrace and induced cassava plants produced have an A - type X-ray diffraction pattern. Only seven plants were found to be of the C - type. This was accompanied by a significant variation in crystallinity indexes and gelatinization properties. Plants with higher ΔH_G and crystallinity indexes observed are suitable for pharmaceutical industries, whilst plants with lower gelatinization and crystallinity may possibly find application in the production of alcohol, starch for sizing paper and textiles, glues, sweeteners, bio-degradable products, butanol and acetone, manufacturing of explosives, and coagulation of rubber latex. The four cassava plants with the overall lowest T_o temperatures were N167BP12 (40.9°C), N398P3fissur (47.0°C), N413P2 (54.5°C) of control HO008 (68.5) descent and N357P4 (56.0°C) of control HO001 (63°C) descent whilst the overall four highest plants were N389P5 (72.0°C) of control HO001 (63.0°C) descent, N473P2 (72.7°C) of control UCC026 (62.0°C) descent and N95P5 (73.3°C) and N39P5 (83.7°C) of control HO008 (68.5) descent. Out of these eight plants five of them (N167BP12, N398P3fissur, N389P5, N95P5 and N39P5) were having merged gelatinization and melting transitions. Therefore, all induced plants could be classified into three gelatinization categories. The first group consisted of three induced plants (N167P7, N281P16, and N470P3) which lacked gelatinization transition. The second group included 99% of the induced plants with normal gelatinization transition properties similar to controls. The third group consisted of 11 plants (N223P12, N348P5, N95P5, N95P4, N39P5, N167BP12, N373P8, N389P5, N357P5, N353P6 and N398P3) which displayed merged gelatinization and melting transitions, occurring within gelatinization zone during calorimetric scanning from 25 – 110°C. The first and third categories included most of the putative mutants described in the research chapters. For example, in the 11 plants, some of the descendants of HO008 such as plant N223P12 displayed a higher T_p at 82.4°C and also with the lowest crystallinity index (0.42), while plant N348P5 displayed a lower T_p at 71°C and also with a transition from A-type to C-type of X-ray diffraction. Plant N167BP12 also displayed a transition from oblanceolate to lanceolate middle leaf lobes and with the fissured granules. Plant N39P5 was among the least four amylose producing plants (15.4%) of HO008. Among the four highest amylose producing plants of HO001 was plant N389P5 which displayed a higher T_p at 101.0°C whilst plant N353P6 was also among the four lowest amylose producing plants (13.9%) of HO001. Also descendants of UCC090 includes plant N398P3 (which is similar to N398P4) displayed an overall lowest T_o at 47.0°C, higher T_p at 86.3°C and exceptionally deep fissured granules that easily produce flakes (Chapter 4 section 4.5.2.5). The significance of the early completion of merged gelatinization and melting transition is that short processing time is required for commercial applications in various industries. It is therefore recommended that more research attention should be paid on these plants during M_1V_3 stages.

Summary

Cassava provides more dietary energy per unit area than any staple crop but native cassava starch has limited food and industrial applications. Different transgenic biotechnology approaches and *ex situ* physical and chemical starch modifications have been used to obtain desirable traits suitable for various food applications. But consumers are skeptical about the safety of the genetically modified foods and chemical residues. To meet the demand, gamma irradiation (this is energetic electromagnetic wave) which does not leave any residue, was used as a tool to induce *in planta* variation in amylose production. The four highest amylose producing plants N441P54 (32.7%), N223P12 (31.1%), N373P2 (27.2%), N329P11 (26.8%) and four lowest amylose producing plants N96P16 (14.0%), N353P6 (13.9%), N166P2 (13.2%), N476 P22 (11.7%) observed in the M_1V_2 generation were found to be significantly different from any one of the four controls HO008 (19.7%), HO001 (22.0%), UCC090 (22.6 %) and UCC026 (20.9%). Mutation is uncontrollable and could also lead to unexpected useful mutants. From this study four unexpected putative free-sugar mutants were identified, which could be used for bioethanol and glucose syrups production; two exceptional deep fissured and flaky mutants for the food industry; seven mutants with transition from A- to C-types of X-ray diffraction patterns with very high levels of crystallinity indexes suitable for replacing acid modified starches in pressing tablets in the pharmaceutical industries were discovered. Based on DSC classification, three gelatinization categories were observed. These were plants without gelatinization transition, plants with gelatinization transition and plants with merged gelatinization and melting transitions, occurring within gelatinization zone during calorimetric scanning from 25 – 110°C. The first and third categories included most of the putative mutants described in the research chapters and they were suitable for shortening the starch processing time required in most commercial starch applications in industry.

Opsomming

Cassava voorsien meer voedingsenergie per eenheid area as enige ander stapelvoedsel, maar natuurlike cassava stysel het beperkte voedsel en industriële toepassings. Heelwat transgeniese biotegnologie metodes en *ex situ* fisiese en chemiese stysel modifikasies is al gebruik om eienskappe te kry wat in aanvraag is vir verskillende voedsel toepassings. Verbruikers is egter skepties oor die veiligheid van geneties gemodifiseerde voedsel en chemiese residue. Om hierdie probleem aan te spreek is gamma bestraling, wat suiwer energie is en geen residu agterlaat nie, gebruik as ‘n metode om *in situ* variasie in amilose produksie *in planta* te genereer. Die vier klone met die hoogste amilose waardes N441P54 (32.7%), N223P12 (31.1%), N373P2 (27.2%), N329P11 (26.8%) en die vier klone met die laagste waardes N96P16 (14.0%), N353P6 (13.9%), N166P2 (13.2%), N476 P22 (11.7%) wat gevind is in the M_1V_2 generasie, was betekenisvol verskillend van die ongemuteerde kontroles HO008 (19.7%), HO001 (22.0%), UCC090 (22.6 %) en UCC026 (20.9%). Mutasie is ongekontroleerd en kan dus ook lei tot onverwagse bruikbare mutasies. Uit hierdie studie is vier onverwagse voorlopige hoë suikerinhoud mutasies geïdentifiseer wat gebruik kan word vir bio-etanol, en glukose stroop produksie; twee mutasies met diep groewe en goeie flokkuleringseienskappe van stysel vir die voedselindustrie; en sewe mutasies met transisie van A - na C - tipe X-straal diffraksiepatrone met hoë vlakke van kristaliniteitsindekse wat geskik is vir die vervanging van suur gemodifiseerde stysel vir die maak van tablette in die farmaseutiese industrie. Gebasseer op DSC klassifikasie is daar drie kategorië van gelvorming gesien. Eerstens was daar klone sonder gelvorming transisie, daar was die met gelvorming transisie en dan die klone wat gesamentlike gelvorming transisie en smelting transisie gehad het, binne die gelvormingssone met skandering tussen 25 – 110°C. Die eerste en derde kategorië het meeste van die voorlopige mutasies ingesluit wat in hierdie studie bespreek is. Hulle was geskik vir verkorte stysel prosseseringstyd soos wat deur meeste kommersiële stysel produkte in die industrie vereis word.

Appendix

Letter	Plants represented								
A	N93P2	N99P4	N14P3	N67P4	N85P4	N57P17	N59P5	N304P3	N313 P6
	N28 P2	N300 P7	N223 P6	N225P9	N179P8	N205P2	N153P24		
B	N72P7	N85P2	N169P4new	N321P6	N69P5	N314P6			
C	N66P4	N68P2	N60P3	N60P4					
D	N84P6	N89P8	N109P2	N73P8	N76P3	N72P5	N73P5	N62P10	N66 P7
	N57 P12	N342 P7	N39P2	N333P5	N338P8	N328P9	N33P4	N320P6	N322P8
	N319 P1	N302 P20	N305 P2	N280 P3	N297P2	N260P3	N261P16	N253P2	N259P3
	N236P5	N217P14	N220 P16	N206 P2	N215 P2	N20 P4	N202P11	N173P5	N198P13
	N17P2	N149P3	N16P2	N11P2	N136 P3	N49 P8	N312P3	N222P5	N164P3
E	N78P8	N94P5	N123P7	N74P2	N78P2	N67P6	N70P5	N55P12	N59 P2
	N50 P13	N48 P9	N49P5	N40P4	N47 P13	N186P2	N240P4	N164P3	N168P5
F	N264P8	N328P3	N116P8	N218P8	N222P4	167P8old	N144 P10	N139P5	N5 P6
G	N31P5	N46P11	N162P5	N2P1	N2P2	N167A OB			
H	N87 P3	N92 P6	N1P2	N79P9	N8P3	N5P1	N79P10	N45P13	N5 P3
	N41 P2	N3P2	N3P1	N25 P1	N276P3	N201P4	N228P5	N143P4	N201P2
I	N54P7	N88 P2	N158P15	N24P2	N242P3	N210P7	N112P3	N4 P8	
J	N71P14	N118P25	N46P12	N53P2	N341P6	N40P6	N289P5	N308P5	N266 P3
	N200 P3	N212 P10	N148P2	N164P12	N142P8	N280P6	N283 P16		
K	N328P4	N49P9	N10P2	N31P6	N322P2	N264P4	N277P7	N20P10	N250 P6
	N2 P2	N171 P9	N187P11	N168P7	N17P3	N152P2	N167P7NEW	N136P2	N146P6
L	N93P5	N98 P2	N1P3	N9P3	N92P7	N8P3	N9P2	N78P7	N8 P2
	N71 P4	N56 P6	N6P1	N55P4	N55P6	N52P8	N53P6	N40P6	N5P2
	N4 P3	N342 P2	N347 P6	N322 P7	N338P11	N32P4	N32P4	N316P2	N32P2
	N312P5	N301P7	N31 P4	N3 P2	N3 P3	N29 P1	N29P3	N28P3	N285P11
	N28P1	N270P9	N271P2	N25P3	N27 P3	N240 P2	N246 P32	N234 P2	N24P3
	N23P3	N220P2	N224P4	N219P8	N22P2	N214P7	N215 P7	N21 P3	N212 P7
	N21P2	N200P16	N202P13	N196P6	N20P1	N185P13	N186P2	N173P7	N18 P3
	N171 P9	N165 P6	N167P6old	N161P7	N165P4	N160P9	N161P7	N152P4	N16P1
	N151 P2	N143 P2	N146 P3	N140 P4	N140P5	N133P2	N14P2	N130P5	N131P27
	N13P2	N126P4	N127 P5	N110 P8	N123 P11	N1 P4	N102P13	N197 P20	N115P4
	N6 P2	N349P11	N312P4	N275P3	N226P5	N206 P10	N167 P7old	N148P6	N13P1
M	N187P2	N274P2	N15P3	N164P12					
N	N298P3	N335P7	N132P3	N152P2	N158P2				
O	N320P3	N61P2	N114P7	N135P6	N214P8				
P	N321P2	N339P3	N101P3	N180P8	N222P5	N127P4	N130P2	N113P2	N189 P3
Q	N254P6	N318P3	N142P3	N229P4	N232P2	N218P4	N223P12	N213P2	N216 P5
R	N263P3	N345P3	N181P14	N225P8	N235P2	N192P13	N192P13	N181P15	N191 P6

S	N128P3	N227P7	N110P2	N120P5	N127P3	N23P2	N336P10	N169P2old	
T	N41P6	N94P5	N16P4	N345P2	N349P10	N332P5	N344P5	N325P4	
U	N313P9	N305P4	N308P3	N30P1	N304P10	N287P7	N290P6	N282P4	N285 P9
	N27P1	N207P2	N253P3	N177P5	N188P11	N17P1	N26 P1		
V	N91P2	N91P4	N13P3	N59P11	N87P2	N36P6	N40P3	N348P4	N36 P3
W	N339P5	N344P9	N175P9	N331P5	N165P4				
X	N306P5	N55P12	N144P20	N291P3	N305P5	N272P3	N291P2	N203P2	N239 P2
	N193P19	N147P3	N151 P4	N164 P13					
Y	N309P3	N64P9	N161P8	N302P18	N304P11	N198P11	N207P4	N168P7old	N183 P13
Z	N299P4	N81P4	N132P2	N245P5	N258P2	N173P7	N190P6	N133P3	N168 P7
ZA	N311P7	N337P4	N168P13	N291P9	N292P3	N180P4	N248P3		
ZB	N341P11	N346P2	N131P25	N333P3	N333P4	N309P2	N314P2	N242P7	N283P17
	N220P17	N145P5	N180P4	N137P2	N196 P20	N285 P8	N179 P13	N263 P8	
ZC	N43P5	N81P3	N164P13	N316P10	N336P7	N303P19	N303P2	N297P11	N301P10
	N288P7	N267P3	N273P7	N23P1	N235P3	N194P8	N198P11	N183P3	N188 P8
ZD	N333P9	N294P5	N298P2	N284P5	N293P2	N278P4	N280P7	N276P4	N278 P3
	N274P5	N223P2	N258P14	N193P2	N204P4	N188P7	N188P7	N184P2	N186 P5
	N172P2	N157P9	N159P12	N146P7	N153P22	N140P8	N141P9	N139P2	N160 P7
ZE	N337P3	N338P9	N172P2	N270P3	N308P7	N189P3	N242P5		
ZF	N58P5	N99P3	N14P1	N243P5	N58P2	N165P5	N22P3		
ZG	N4P1	N48P8	N182P10	N33P5	N168P6OLD	N172P3			
ZH	N308P6	N346P3	N296P4	N298P5	N204P4	N284P12	N169P4 OLD	N74 P3	
ZI	N310P7	N22P2	N274P4	N174P9	N179P7	N169P3	N169P3OLD	N114P5	N116 P5
ZJ	N75P3	N225P20	N314P3	N340P2	N272P2				
ZK	N42P2	N46P10	N247P6	N267P5	N162P15	N187P2	N155P3	N162P15	N125 P8
	N105P2	N120P7	N104P5	N138 P5					
ZL	N102P16	N63P4	N80P3	N346P4	N4P1	N326P2			
ZM	N300P13	N221P8	N270P7	N178P15	N198P10	N111P4	N112P2		
ZN	N89P10	N90P3	N100P11	N75P3	N88P6	N64P11	N67P7	N43P7	N61 P6
	N35P2	N27P2	N279P4	N254P4	N254P9	N244P3	N244P31	N236P3	N243 P7
	N207P4	N186P5	N193P11	N185P12	N185P12	N174P9	N180P6	N134P7	N167P13new
	N134P4	N105P6	N119P7	N349 P15	N203 P3	N129 P3	N110 P5	N189 P5	N250 P5
ZO	N257P6	N266P5	N122P7	N227P10	N238P1	N184P12	N290 P4	N38 P7	N205 P6
ZP	N318P4	N322P6	N135P5	N309P7	N310P6	N296P2	N296P5	N284P3	N295 P2
	N273P2	N197P3	N264P5	N175P4	N181P10	N157P4	N169P4	N14P1	N268 P2
ZQ	N261P12	N84P3	N100P10	N216P7	N246P35	N172P6	N19P1	N153P14	N162 P5
ZR	N94P4	N98P6	N127P2	N64P3	N91P5	N62P2	N62P5	N34P4	N51 P5
	N321P58	N283P20	N287P4	N265P2	N282P2	N221P10	N262P2	N1P1	N1P2
ZS	N10P3	N79P7	N85P5	N75P4	N77P7	N68P3	N70P4	N6P1	N62 P8
	N47P10	N318P6	N38P11	N29P3	N295P3	N261P13	N271P4	N258P12	N260 P2
	N256P8	N246P27	N250P4	N244P32	N245P3	N237P2	N240P5	N209P5	N222 P3

	N206P21	N171P7	N190P6	N145P3	N167P8	N141P5	N143P1	N136P4	N138 P3
	N131P24	N122P5	N124P20	N11P2	N121P56	N106P4	N11P1	N100P13	N102 P3
ZT	N63P7	N81P2	N275P4	N316P3	N94P3	N103P4	N238P9	N287P2	N157 P6
	N137P4	N142P8	N126P5	N129P4	N116P9	N123P17	N128 P5	N224 P6	
ZU	N339P6	N35P5	N103P3	N118P23	N151P3	N107P7	N113P3	N104P4	
ZV	N277P4	N36P3	N128P2	N241P5	N267P4	N154P8	N200P2	N143P5	N154 P4
ZW	N90P5	N98P5	N121P36	N80P2	N86P11	N38P5	N47P8	N299P2	N38 P2
ZX	N334P3	N42P5	N149P4	N256P10	N300P6	N228P10	N245P6	N156P2	N260P7
ZY	N101P5	N335P6	N63P2	N169P2	N302P19	N154P2	N161P9		
ZZ	N212P8	N347P8	N132P4	N152P6	N159P9				
ZZA	N275P2	N278P2	N224P3	N266P6					
ZZB	N213P3	N337P2	N175P4	N186P6					
ZZC	N289P7	N71P7	N130P7	N223P6	N259P5	N147P4	N188P8	N135P4	N138 P4
ZZE	N295P4	N300P9	N292P5	N294P6					
ZZF	N322P4	N344P8	N101P4	N175P9					
ZZG	N326P3	N4P3	N12P2	N172P5	N216P3				
ZZH	N109P14	N68P5	N83P6	N334P4	N340P7	N325P7	N329P6	N289P3	
ZZI	N133P4	N156P4	N281P3	N148P5	N156P3				
ZZJ	N182P9	N53P6	N111P2	N117P4	N12P1				
ZZK	N332P4	N34P1	N144P12	N187P8	N219P7	N160P12			
ZZL	N70P2	N86P6	N111P43	N271P3	N46P10	N166P5	N237P4	N149P2	
ZZM	N229P11	N238P13	N192P15	N214P6					
ZZN	N303P1	N106P5	N122P7	N169PA lin					
ZZO	N96P3	N104P2	N129P6	N145P5					
ZZP	N256P12	N77P4	N83P2	N155P4	N209P8				
ZZQ	N241P7	N297P7	N139P3	N166P6	N231P3				
ZZR	N272P4	N311P8	N108P19	N263P6					
ZZS	N345P4	N58P4	N15P2	N279P2					
ZZT	N56P2	N88P4	N106P9	N108P14					
ZZU	N336P14	N51P8	N129P2	N293P3	N329P11	N281P15	N292P2		

Appendix 4.1. Plants represented by letters A – ZZU in Fig.4.4