DEVELOPING A METHOD FOR THE ENHANCEMENT OF THE BETA CAROTENE CONTENT OF SWEET POTATO (cv BRONDAL) USING PROTOPLAST TECHNOLOGY.

BY

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A thesis submitted in partial fulfilment of the requirements of the degree of Master of Science in Crop Science with options in Plant Breeding and Agronomy.

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The undersigned certify that they have read and recommend to the Department of Crop Science, the thesis entitled:

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ABSTRACT

Agronomically superior sweet potato (*Ipomoea batatas*) variety Brondal contains relatively low amounts of vitamin A as opposed to low yielding variety Nemagold, which is relatively rich in high amounts of the nutrient. The study focused on attempting to use protoplast technology, a tissue culture technique, to develop somatic hybrids with combined traits, such as high yield per unit area and high vitamin A content, from both varieties. For protoplast isolation, 10 enzymatic treatments were used, which were combinations of the enzyme cellulase with either macerozyme or hemicellulase. There were significant difference between treatments ($P < 0.05$) for protoplast yield and viability. The most effective enzyme concentration for sweet potato protoplast isolation was a combination of 2% hemicellulase and 2% cellulase to give the highest protoplast yield $LSD_{0.05}$ (3.446) and viability $LSD_{0.05}$ (4.242) at relatively low enzymatic concentrations. The isolated protoplasts were then tested for viability and the results of the analysis showed that an inverse relationship existed between protoplast yield and protoplast viability. There were significant differences between the ten-enzymatic treatments ($P < 0.05$) for protoplast viability. For protoplast fusion the $CaCl_2$ concentrations ranged from 0.1 M to 1 M. Statistical analysis revealed that there was a significant difference between treatments ($P < 0.05$). Mean separation reveal that all treatment means for protoplast fusion were significantly different from each other ($LSD_{0.05}$ = 2.591). CTAB 3% method was used to extract genomic DNA from both sweet potato varieties. In order to have a method for confirming successful fusions of the two lines, Random Amplified Polymorphic DNA method was used. Primers OPH5, OPH7, OPU5, OPU1 and OPH6 used in the experiment were able to detect polymorphisms in the two sweet potato genotypes. For every case, each given primer was able to produce bands in similar electrophoretic positions that matched in both sweet potato varieties (monomorphic bands). This revealed a level of similarity between the two genotypes. However, very few electrophoretic band positions produced by the same primer did not match (polymorphic) proving that the two sweet potato varieties had a degree of dissimilarity. The number of polymorphic bands produced per primer was between 2 and 3. Different fragment lengths were produced in the two varieties using the same primer, revealing that some of the banding patterns were similar in both sweet potato varieties. Visual assessment of the DNA was estimated from the intensity of UV-induced fluorescence emitted after ethidium bromide staining. However, quantitative analysis of electrophoretic banding patterns using sensitive such as the spectrophotometric method which is based on optical density (OD) was not done.

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Finally I would like to thank friends and family who are always there for me during life's ups and downs. Thank you God for the above people.

DEDICATION

I dedicate this project to my parents Mr. F and Mrs. B.B. Masekesa for their loving support both emotionally and financially throughout the whole Masters program. Thank you mum for instilling in me enough values and determination to meet the world out there. And to my dad for telling me that l should shine like the star that l am. I love you guys.

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ABBREVIATIONS USED IN THE TEXT

$CaCl_2$	Calcium Chloride
DNA	Deoxyribo Nucleic Acid
DTC	Development Technology Centre
2,4D	2, 4 Dichlorophenoxyacetic acid
EDTA	Ethylene diaminetetraacetate acid
GGPP	Geranlygeranyl diphosphate
Kbp	Kilo base pairs
KH_2PO_4	Potassium Hydrogen phosphate
KNO_3	Potassium Nitrate
LSD	Least significant difference
$MgSO_4.7H_2O$	Magnesium Sulphate (seven parts water)
MVA	Melavanic Acid
µL	micro litres
mg/L	Milligrams per litre
mM	millimolar
OD	Optical Density
PCR	Polymerase chain reaction
pH	Potential hydrogen ions
P-value	Probability value
RAPD	Random Amplified Polymorphic
RCBD	Randomised block design
Rpms	Rounds per milli second
Sec	Second
S.E.D	Standard Error of Differences
NaCl	Sodium Chloride
TBE	Tris-borate-EDTA
UV	Ultra-Violet light
VAD	Vitamin A deficiency
WHO	World health Organisation

CHAPTER ONE:

1.1 INTRODUCTION

The genetic manipulation of plants at various cellular levels is beginning to revolutionise conventional plant breeding. A new set of techniques for the isolation, modification and introduction of novel genes into plants can overcome the limitations of sexual gene transfer and produce new combinations of genetic material, some of which were unlikely to ever arise in nature (Evans, 1993). These manipulations can increase the efficiency, productivity and perhaps most importantly the profitability of plant breeding (Burns, 1993). For instance, this year alone over 100 million hectares of genetically modified (GM) crops has been grown the world over (James, 2006).

Protoplast technology is a tissue culture technique that can be used as a plant breeding tool to combine traits from different varieties at a cellular level. There are basically two methods of achieving fusion of protoplasts. The first method involves isolating individual plant cells from parent tissue using enzymes that degrade the cell wall and pectin to release protoplast cells. Liberated protoplast cells from the different varieties are then combined using a fusing agent such as calcium chloride (Bottino, 1992). In the second method, meristematic tissue from the two varieties to be combined are placed along side each other on regeneration media. Meristem cells only have a thin primary cell wall, such that as the two adjacent meristems begin to callus, the force of this growth forces cell contents of adjacent cells to mix thus achieving fusion (Pierik, 1987).

Enzymatic isolation of protoplasts and subsequent fusion is currently the most preferred method of protoplast fusion as each step of the procedure can be checked to determine whether it has been successful (Bottino, 1992).This is in contrast with the second method were fusion can only be confirmed when regeneratants are analyzed. The second method also has the disadvantage that extensive investigations have to be conducted to determine the precise type and levels of hormone supplementations that are required to induce callus production from meristems in two different varieties at the same time (Pierik, 1987).

Protoplast technology as a whole has the advantage that when the two nuclei of different varieties fuse, the chromosomal number is increased thus heightening the ploidy level. Increased polyploidy leads to an upsurge in general plant vigor and ultimately yield in the hybrid plants produced immediately after fusion (Musvosvi, personal communication).

After protoplast technology has been conducted and fused protoplasts have been regenerated, genetic finger printing procedures which detects nucleotide sequence differences such as Random Amplified Polymorphic DNA (RAPDs) can then be used to distinguish between the recombinant plants from the parental genotypes (Williams, Feldman and Tingey, 1993) such as Nemagold and Brondal.

Sweet potato is a classic case scenario where traditional breeding is difficult to employ and alternative technology such as protoplast technology can be used for improvement of traits. Under Zimbabwean conditions of high altitude and high diurnal temperatures, sweet potato does not produce viable seed, the ideal conditions being cooler diurnal temperatures. In

addition, sweet potato has a hexaploid genome (6n) and having such a large genome makes unbalanced segregation of chromosomes into respective gamates likely (Reginana, 2006).

Brondal (white fleshed), is a sweet potato variety in Zimbabwe that is high yielding (30 tonnes/ha) but has a relatively low beta-carotene (pro-vitamin A) content compared to Nemagold (orange fleshed), which has relatively high vitamin A but has low yield characteristics (Development Technology Centre, 2006). Given that genes in the nucleus encode quantitative traits like yield while pigments such as beta-carotene are coded for by genes in the chloroplast (Zhang, 2007); it might be possible using protoplast technology, to generate a somatic hybrid that exhibits agronomic traits similar to Brondal as well as showing vitamin A levels similar to that of Nemagold.

Dietary micronutrient deficiencies, such as a lack of vitamin A, are a major source of morbidity (increased susceptibility to disease) and mortality worldwide. Children in particular are affected, having their immune systems and normal development compromised causing disease and eventually death. An effective way to alleviate this plight would be to provide the public with nutrient-dense staple foods such as sweet potato especially biofortified varieties (those varieties producing and accumulating provitamin A i.e. orange fleshed varieties) in the case of vitamin A deficiencies (Welch and Graham, 2004).

In light of the vitamin A deficiency (VAD) faced by the majority of Zimbabweans (Bakai, 2006), a good way to alleviate problems of vitamin A deficiency at household and national level would be to provide and promote to the nation the production and consumption of high vitamin A containing foods such as sweet potato. Since Brondal is one of the most

widely distributed sweet potato varieties in the country, for instance 400,000 households growing it in 2007 according to a recent ASSf report (Robertson, personal communications), enhancing the vitamin A content of one the most consumed sweet potato varieties would aid in the alleviation of vitamin A deficiencies countrywide.

Protoplast work has not been conducted in the department of Crop science for close to 20 years (Robertson, personal communication). Due to financial constraints, new enzymes could not be purchased for the experiment hence the enzymes that had been kept in storage for 20 years were used. This necessitated enzyme viability tests at various levels and combinations. Some of the objectives of the study were to develop in the Crop Science laboratory standard protocols for protoplast isolation, protoplast viability assay and protoplast fusion for future departmental use.

1.2 REASARCH OBJECTIVES

1.2.1 MAIN REASARCH OBJECTIVE

To isolate protoplast cells from Brondal and Nemagold (sweet potato varieties), fuse the respective protoplast cells and regenerate the fused product into fully functional plants.

1.2.2 SPECIFIC REASARCH OBJECTIVES

1) To isolate protoplast cells from Brondal and Nemagold sweet potato using a range of enzymatic concentrations.

2) To determine sweet potato protoplast viability under various enzymatic combinations and concentrations.

3) To find the optimum $CaCl_2$ concentration that will induce a high number of protoplast fusions between two sweet potato varieties.

4) To attempt to induce cell-wall synthesis, cell division and then plantlets and regenerate fused protoplast products on suitable media.

5) To conduct random amplified polymorphic DNA (RAPDs) tests to distinguish between Brondal and Nemagold genomic DNA.

1.3 RESEARCH HYPOTHESES

1) H_1: There is a difference in the ability of available enzymes to remove the cell walls of sweet potato cells.

2) H_1: There is a difference in protoplast viability of cell isolated under various enzymatic concentrations and combinations.

3) H_1: There is a difference in the number of fusion products between the various $CaCl_2$ concentrations used.

4) H_1: There is a difference in the regenerative capacities of different media on sweet potato protoplasts.

5) H_1: There is a difference in the banding patterns produced when random amplified polymorphic DNA (RAPDs) tests are conducted to distinguish between Brondal and Nemagold genomic DNA.

CHAPTER TWO:

LITREATURE REVIEW

2.1 SWEET POTATO STATUS IN ZIMBABWE

Sweet potato (*Ipomoea batatas*) is a perennial crop grown as an annual crop in Zimbabwe. It belongs to the Convolvulaceae family and it is a vine type of plant with roots that become swollen and this then becomes the edible part. The crop is short-seasoned and grows best in a warm, humid climate(Tagwirei, 2006).

According to the consumer council of Zimbabwe report (2005) sweet potato is fast becoming a substitute to bread at breakfast, the latter being unaffordable to most families. Currently Brondal is one of the most widely consumed and cultivated sweet potato varieties in Zimbabwe, becoming popular in both the rural and urban settings (Tagwirei, 2006). The variety is partially drought tolerant and does well on marginal and degraded soil with little labour and few or no inputs from outside the farm (Tagwirei, 2006).

Despite excellent agronomic traits such as early maturity and high yielding, Brondal has a low Vitamin A content as evidenced by its white flesh. This is in sharp contrast with sweet potato varieties like Nemagold, Aristo and Jewel, which are agronomically weak but have high vitamin A content.

2.2 BIOCHEMICAL PATHWAY OF BETA-CAROTENE

Beta carotene, a pigment manufactured in the chloroplast and found exclusively in plants is the precursor to vitamin A(Omaye, Krinsky, Kagan, Mayne, Liebler and Bidlack, 1997) and gives Nemagold sweet potato tubers a characteristic orange colour. Upon ingestion by animals, a molecule of beta carotene is converted to roughly two molecules of vitamin A (Omaye, Krinsky, Kagan, Mayne; Liebler and Bidlack, 1997).

The biochemical pathway of all caroteniods is confined to the chloroplast (Omaye, Krinsky, Kagan, Mayne; Liebler and Bidlack, 1997). Beta-carotene is derived from isoprene units. The first committed step in the production of beta-carotene is the manufacture of Mevalonic Acid (MVA). Mevalonic Acid is produced from geranylgeranyl diphosphate (GGDP). Mevalonic acid is converted to phytoene by a membrane bound enzyme phytoene synthase. Phytoene is in turn changed to lycopene by the enzyme phytoene desaturase. Lycopene cyclase will then convert lycopene to beta-carotene. Beta-carotene absorbs visilble light in the 450 nm range such that it is yellow-orange in color (Omaye, Krinsky, Kagan, Mayne; Liebler and Bidlack, 1997). The presence or absence of beta-carotene is the major difference between yellow and white-fleshed sweet potatoes and mutation observations suggest that it is controlled by a single gene.

The major difference between white and orange fleshed sweet potato varieties is that orange fleshed varieties have the ability to produce the pigment beta carotene in the tubers while the white fleshed varieties have lost this capacity due to a mutation in chloroplast membranes. The enzyme phytone synthase is membrane bound and any mutations in the membrane will alter its function abilities and consequently causes a break in the biochemical pathway of beta-carotene.

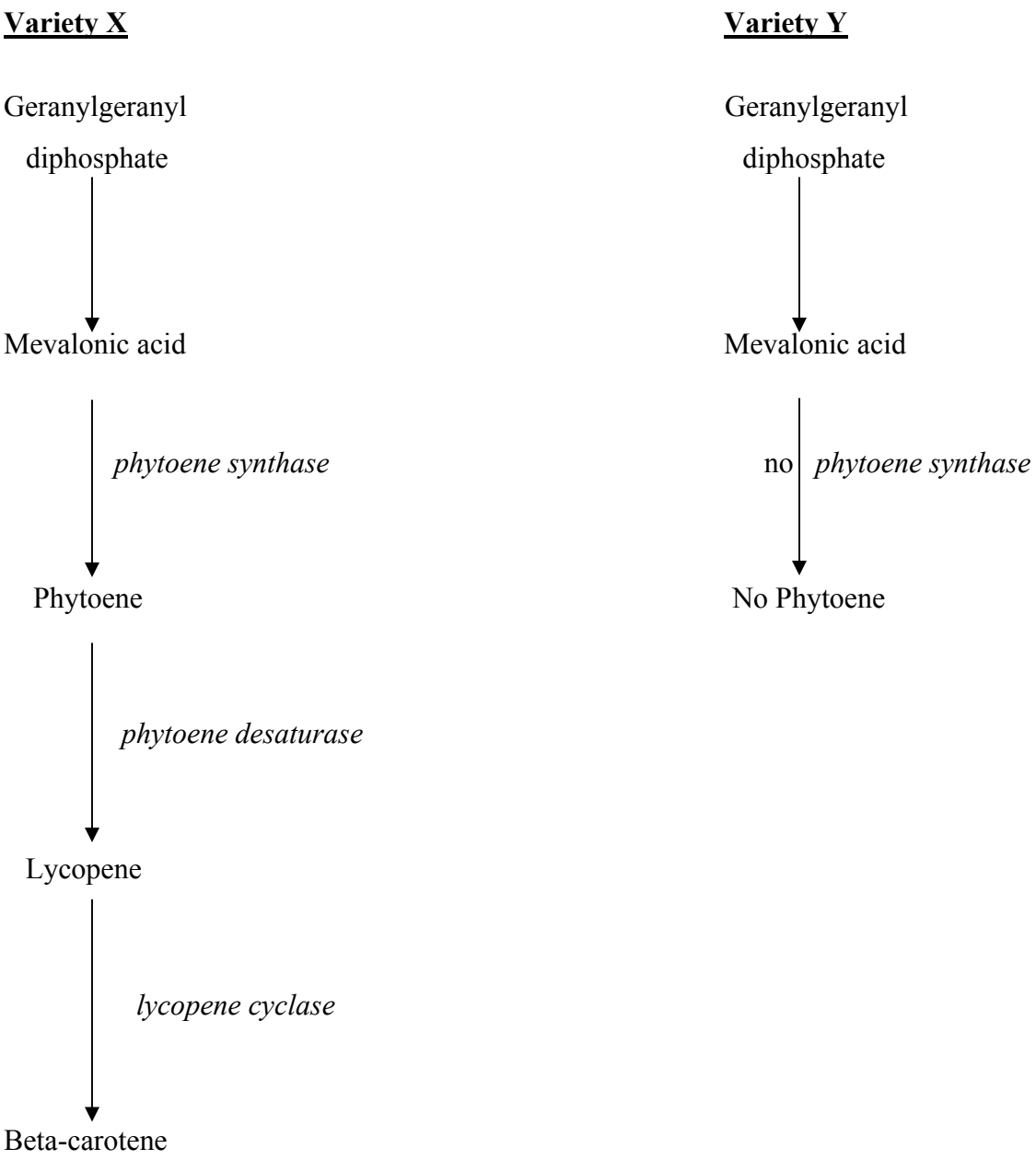

Figure 2.1 Biochemical pathway of for beta-carotene for two theoretical varieties and the enzymes involved were variety X has the ability to manufacture the pigment beta-carotene and Variety Y does not (adapted from **Beta-Carotene: Friend or Foe? Fundamental and Applied Toxicology, 2006.)**

Using protoplast technology it might be possible to create somatic hybrids that exhibit yield potentials similar to Brondal and vitamin A levels similar to Nemagold. When two different

protoplasts are fused and their cytoplasms mix but their nuclei remain separated within the fused cell then the product is called a heterokayon (Ngadze, personal communications). After meiosis, the resulting cells will have either of the parental nuclei or a combined nucleus and a cytoplasm that is composed of a mixture of organelles from both parents (Kanchanapoom, Jantara and Rahchard, 2001).

Therefore enhancing the vitamin A content of Brondal to levels similar to Nemagold might be achieved by creating somatic hybrids cells that have a mixture of Brondal and Nemagold chloroplasts. Chloroplasts contain about 3000 proteins of which 95% of them are encoded for by nuclear genes (Zhang, 2007). As a result, enzymatic and photosynthetic complexities within chloroplasts contain subunits encoded by two separate genomes. This necessities a close coordination of gene expression that involves two-way signalling between the nucleus and the chloroplast. While plastid development is largely under nuclear control, developmentally arrested or damaged plastids can, as is the case with membrane mutation, regulate nuclear gene expression via retrograde signalling pathways. Integration of signals is essential for co-coordinating gene expression to achieve appropriate physiological responses (Koussevitzky *et al.,* 2007). In the created somatic hybrids, the chloroplasts that originated from Nemagold it is postulated that they will still be able to proceed with beta-carotene production even under Brondal nuclear control.

2.3 APPLICATION OF PROTOPLAST TECHNOLOGY IN THE IMPROVEMENT OF CROPS.

Conventional plant breeding is based on a highly regulated system of hybridisation where crosses are limited to genetically related plant species. Higher plants are subject to incompatibility barriers, which restrict the gene pool. Traditional plant breeding is time consuming because it requires extensive backcrossing with the superior variety in order to eliminate most of the genome of the undesirable parent while retaining the useful genes. Protoplast technology is a non-conventional genetic procedure involving fusion between isolated somatic protoplasts and subsequent development of their product (heterokaryon) into a somatic hybrid with combines traits from parents (Bottino, 1992).

A protoplast is a plant cell that has had its cell wall either enzymatically or mechanically removed and is sometimes referred to as a naked cell. The cell can now be subjected to cytoplasmic additions or subtractions as well as insertion or deletions of certain traits at molecular (genomic DNA) level. Protoplast technology is not only useful in plant breeding but also in various fundamental studies such as membrane transport, cell compartmentalisation, cell division and the cytoskeleton in relation to the cell cycle (Bottino, 1992).

Contributions made by this technology to crop improvement is mainly in the form of somatic hybrids, cybrids, direct DNA transfer, electroporation and microinjection (Bottino, 1992). Protoplast technology has been used to produce germplasm previously unavailable to the plant breeder (Calbiochem, 1996). The unique gene combinations arising from segregation of mixed organelles coupled with cytoplasmic and nuclear gene recombinations as well as somaclonal

variation, ensures that new plant varieties are derived from somatic hybridisation (Bottino, 1992).

2.3.1 Protoplast Technology

2.3.1.1 Protoplast Isolation

Protoplasts must be isolated from disease free, active and aseptically growing plant tissues (Ngadze, personal communication) since this has implications on the regenerative capacity of the protoplasts. Experiments previously conducted show that it is best to isolate protoplasts from etiolated plantlets as this would lead to the production of more protoplasts after enzyme additions. Etiolation induces the cell wall to stretch thus exposing more surface area upon which the hydrolic enzymes can work on (Kanchanapoom, Jantara and Rahchard, 2001).

Prior to protoplast isolation, the plant tissue from which the protoplasts are to be isolated are soaked and incubated in a hyper-tonic solution of sugars such as mannitol or sorbitol solutions referred to as osmoticum which causes the cells in the tissue to plasmolyse and that makes it easier to digest away the cell walls enzymatically. In the absence of a cell wall that controls cell shape, the cell becomes susceptible to the surrounding osmotic pressure and can either burst or shrivel depending on whether the surrounding environment is hypotonic or hypertonic. Osmotic stabilizers such as sorbitol and inositol ensure that water does not flow either into the cell or out such that the cell shape is maintained and so protoplast isolation is conducted in an osmoticum or an osmotic stabilizer to maintain the outer plasma-membrane's structural integrity (Kanchanapoom, Jantara and Rahchard, 2001).

The levels of enzymes to be used for isolating protoplasts are species and tissue dependent as the chemical composition of the cell walls of cells of these tissues and species vary considerably. For instance hardwoods which would have undergone suberisation require higher enzymatic concentrations compared to herbaceous plants such as sweet potato. Meristem cells require relatively lower enzyme levels since they only have a primary cell wall (Burgess, 1985) compared to for example tissue such as xylem which would have been lignified and thus would requires more stringent hydrolytic enzymes.

Cellulase is an enzyme that hydrolyses cellulose, the main constituents of cell walls. Pectinases breakdown the pectin that acts as the glue holding plant cells together in plant tissue. Hemicellulases destroys the crosslinking bonds of both substances. Plant cells are surrounded by a rigid, semi-permeable cell wall which defines the shape of the cell and also confers integrity and rigidity to the tissue. The cell wall serves as a barrier to the movement of particulate material between the cell and its outer environment (Burgess, 1985). Chemically the cell wall is composed of mainly polysaccharides with inclusions of some proteins and lipids. The three main polysaccharides are cellulose, pectin and hemicelluloses. Cellulose is an unbranched polymer of beta (1-4) D glycopyranosyl units associated with microfibril bundles. These microfibrils are cross-linked by hemicellulose, which is in itself a polymer of beta (1-4) D xylopynosyl units. This cross-linked structure is then embedded in a pectin matrix, which is primarily composed of alpha (1-4) polygalacturonic acid backbone, which can be randomly acetylated and methylated (Capitana and McCann, 2000).

Somatic hybridization is the combination of nuclear and cytoplasmic genetic information by fusing somatic cells. Somatic hybrids are not genetically modified organisms and therefore not regulated by lengthy genetic engineering directives in the European Union or in Zimbabwe (Welters, 2007). Protoplasts are individual plant cells without cell walls and thus by fusing the protoplasts it is possible to combine traits from related plant varieties which are unable to be crossed sexually (Pierik, 1987).

The commercial preparations of cellulase are typically mixtures of enzymes containing high cellulase activity with some hemicellulase activity as well. These enzyme mixtures are capable of degrading cellulose, mannans, xylans, galactomannans, pectin and other polysaccharides. Macerozyme R10, is a pectinase isolated from *Rhizopus* species which catalyses the hydrolysis of 1-4 alpha D galactosidiuronic links in pectin and other galacturonans (Capitana and McCann, 2000). This enzyme is robust and might be expected to survive in the laboratory's cold room or fridge for a long time. However with power cuts interrupting the continuity of the cold storage it is of interest to discover if they are still effective. Enzymes being proteins in nature gradually become denatured if not stored at the appropriate temperature (Lehninger, 1989).

The first stage in the isolation of protoplasts occurs with pectinases such as macerozyme R10 that dissolves the middle lamella followed by the breakdown of the cell wall structure by cellulose and hemicellulase. The enzymes used are not pure but crude extracts from bacteria and fungi, which explains the different trade names given to the same enzymes or products and their different reactivities.

2.3.1.2 PROTOPLAST VIABILITY

Gahan (1985) defines protoplast viability as the capacity of an isolated protoplast to continue to grow in culture, replace its lost cell wall and to form suspension cells, calli or plantlets. Viability tests are conducted to determine whether the isolated protoplasts are still physiologically intact and undamaged by the process of enzymatic digestion. High protoplast viability is very important because it has implications on the regenerative capacity of the isolated protoplasts. There are basically four categories of protoplast viability tests but the most commonly used are the plasmalema-based tests which include the dye exclusion methods. These tests indicate the viability of protoplasts by virtue of its intactness and primarily normal physiological functions. Basically the methods depend on the ability of the plasma membrane to block the entry of dye molecules and an example is Evans Blue test for viability. After 5 minutes from its application at 1%, protoplasts with damaged cell membranes will stain blue while those, which would still be viable and have a functional membrane will actively exclude the blue stain from the cell (Ishii, 1988).

2.3.1.3 Protoplast Fusion

Usually the aggregation of two or more protoplasts is not enough to bring about fusion since protoplasts membranes bear a strong inherent negative charge. These negative charges are a result of hydrogen phosphate groups $[PHO_4]^-$ adorning the plant cell membrane. Animal cells also have this inherent negative charge, which is due to sialic acid residues. As a result, protoplasts that have just been isolated tend to naturally repel each other thereby reducing the frequency of fusion. For significant protoplast fusion purposes fusing agents called fusagens have to be used (Navratilova, Greplova, Vyvadilova and Klima, 2007).

Spontaneous protoplast fusion is of no value in breeding but rather induced fusion has to be instigated between chosen plant varieties. In animals inactivated Sendai virus is used to induce fusion while in plants the inducing agents bring the protoplast cells into close proximity, which causes them to adhere to each other until their membranes associate and their cell contents mix. Some of the fusagens that are used include polyethylene glycol, nitric acid and treatment with a high calcium chloride concentration at high pH as well as electrophoresis (Senbursch, 2005).

Nitric acid was one of the first and most successful methods for fusing plant protoplasts especially protoplasts isolated from root tips of oats and maize seedlings. However it has since been relegated to lower levels in terms of preferences as it has a low fusion frequency especially where highly vacuolated mesophyll protoplasts are concerned (Senbursch, 2005). Calcium chloride at a high concentration plus high pH induces protoplast fusion by neutralizing the negative charge on the surface of protoplast membranes. Both the Ca^{2+} and H^{+} ions upon release into the mixture containing the protoplasts serve to neutralise the negative charge imposed by the hydrogen phosphate groups on membrane surfaces. Ca^{2+} is a bivalent cation while phosphate groups bear a single negative charge and thus not only neutralize the charge but also serves to combine two neighbouring protoplasts by bonding a hydrogen phosphate group in one protoplast and then ionically bonding with another hydrogen phosphate group on another protoplast. This ensures that the protoplasts are in close proximity and may eventually fuse. This method is considered better than that of using nitric acid as a fusagen because fusion percentage is significantly higher reaching in some instances 59% (Bhojwani and Razdan, 1983). Particular care has to be taken where high pH is used since too high a pH level results in toxicity in some crop species (Senbursch, 2005).

Polyethylene glycol (PEG) has been used since 1974 and is used universally because it has a high frequency of protoplast fusion with reproducible results and involves low cytotoxicity in most crop species. Exceptions include sweet potato where protoplasts treated with PEG failed to regenerate into plantlets (Sihachakr and Ducreux, 1989). The mode of action of this chemical is that it causes protoplast cell agglutination. With electrofusion protoplasts are literally forced to fuse using electrical currents. Protoplasts are placed into a small culture cell containing electrodes and a potential difference is applied inducing the protoplasts to move lining up between the electrodes. At this point a short, square wave of electric shock is applied and the protoplasts will fuse (Kanchanapoom, Jantara and Rahchard, 2001).

A cybrid is a hybrid cell that contains a nucleus of one variety and the cytoplasm of another (Ngadze, personal communication). A synkaryon is a fusion product that results from the fusion of diploid cells, which then forms a tetraploid cell but has only one nucleus due to nuclear fusion giving the cell a form of polyploidy. Heterokaryons are formed when two somatic protoplasts are fused and the nuclei remain as separate entities (Kanchanapoom, Jantara and Rahchard, 2001).

2.3.1.4 Protoplast Culture

Protoplast culture is based on the theory of totipotency by Steward (1994), who altered hormonal balances in order to bring single carrot cells to go through developmental changes and finish up as plantlets. These plants did indeed set seed and complete the growth and development cycle showing that a single cell's DNA had the complete programme for producing a complete plant (Robertson, personal communication). In addition to the hormones

however, very specific media requirements have to be met as the protoplasts cells have to undergo a number of processes such as cell wall regeneration and cell division before achieving functional status. Protoplast culture media is very specific and depends on the plant type to be cultured. For instance the types of sugars found on the cell walls of one plant species might not necessarily be the same as those found in anther (Pierik, 1987).

Once cell wall regeneration has been achieved on the protoplast then cell divisions can occur 2-7 days after the cell wall has been reconstructed. When microcalli are formed, the calli can then be transferred to a new media with altered hormone levels that encourages and induces the calli to differentiate into plantlets that can be acclimated in compost to become the true plant. Protoplast culture is best conducted in liquid media, which is constantly agitated to oxygenate the protoplasts. The liquid media ensures that all the submerged protoplasts are protected from the external environment since they would be in a vulnerable state (Pierik, 1987).

Protoplasts can also be regenerated using a culture system known as a nurse culture system. With a nurse culture system an embryogenic callus of the same species obtained from meristem culture is partially submerge by liquid media with hormonal supplements. The solid media upon which the callus rests is then separated from the liquid phase by a sterile filter paper to prevent the regenerating protoplasts from physically mixing with the callus but allowing them to exchange exudates. The protoplasts are then inoculated into the liquid phase of the system and cultured. The principle behind this system is that the calli act as feeder cells that provide the protoplast cells with some unstable components that cannot be catered for in conditioned culture media (Pierik, 1987).

It is important that regeneration occurs at very high frequencies so that tests for somaclonal variation and positional effects can be conducted. Care must be taken when isolating protoplast cells for culture. Tissue from pith or cortex must be avoided, as they will form calli that is friable in nature, which are likely to produce dividing and expanding cells that never differentiate. Somaclonal variation can also occur in cells that have been incubated for too long. The prehistory to the plant, its hormonal status, can make a difference to the epigenetic control of the genome (Robertson, personal communication).

2.4 Molecular Biology as a Diagnostics tool

Traditionally breeders have had to wait to grow out the seed that would have been bred to check for its agronomic standing before being certain whether the traits of interest are indeed in the hybrid. This has lead to some breeding programmes taking an average of eight to ten years as the trait has to be checked for consistency over the years (Godwin, 1992). With the use of molecular technology it is possible to check whether the traits being bred are present in the hybrid cell and also if the trait is carried and expressed in the right form at cellular level without having to wait to grow out the hybrids (Dudley, 1994).

A wide array of markers exist which include morphological, biochemical, protein and DNA markers. Genetic relationships between plants have been estimated over the years using morphological, heterosis and pedigree data, but these all have inherent disadvantages that make then unreliable (Dudley, 1994). For plant breeding purposes as well as germplasm studies,

DNA markers are mostly favored because of increased accuracy and informativeness of nucleotide sequence-based determination of polymorphisms (Godwin, 1992). In addition DNA-based markers are preferred the most because they produce an almost unlimited number of polymorphisms and at the same time enabling the investigator to study the genome directly.

The two best used DNA markers are Restriction fragment length polymorphism and Random amplified polymorphic DNAs. Restriction fragment length polymorphism analysis basically involves digesting the subject genome with restriction enzymes, fractionating the fragment electrophoratically, and then preferentially visualizing fragments containing particular homologous sequences by hybridizing them to a specific DNA probe (De Verna and Alpert, 1990). Random amplified polymorphic DNAs detect nucleotide sequence polymorphisms in a DNA amplification-based manner using only a single primer of arbitrary sequence (Tingey *et al.,* 1992).

2.4.1 RAPDs are used in germplasm evaluations

Genetic markers have been used since the early 1930s in genetic experiments and as a means of indirect selection. Many characters of interest in plant breeding are of very low heritability and in some cases may be difficult or expensive to measure. They therefore pose a problem when it comes to selection in large segregating populations. A marker gene, which is easy to assay for, is highly heritable and closely linked to the gene of interest may be used to select indirectly for a trait. Genetic markers can be used to gain estimates of genetic variability. Tissue culture regenerants are a typical population where some measure of genetic variation is present due to somaclonal variation (Godwin, 1992).

Coinheritance or genetic linkage is a familiar concept in genetic studies where it was shown that combinations of genes tended to be transmitted together because they were close to each other on the same chromosome (Godwin, 1992). Markers must be polymorphic, which means it must exist in different forms so that the chromosome carrying the mutant gene can be distinguished from the one carrying the normal gene by the phenotype of the gene that it carries.

A big advantage of RAPDs is that they can be performed at any plant developmental stage meaning that 1gram of plant DNA is enough to allow assay for a number of RAPD loci and hence their characters, thus increasing the speed of selection. Random Amplified Polymorphic DNA (RAPDs) can also be used for germplasm protection against piracy. A company can collect genetic fingerprints of its varieties and use them as a reference point against rival company products (Godwin, 1992).

However, Random Amplified Polymorphic DNA (RAPDs) have proved to be very expensive due to the equipment required compared to morphological markers. The ploidy level does not influence the number of fragments per primer and this attribute of independence of RAPDs bands from genome size is due to mismatch between the primer and the template as well as to primer competition (Tingey *et al.,* 1992).

2.4.2 Events that may have lead to polymorphisms detectable by RAPDs.

Many events can theoretically result in a polymorphism that can be detected by RAPD analysis. These range from (1) insertion of a piece of DNA between the two annealing sites which may render the original fragment too large to amplify resulting in its loss; (2) the

deletion of a DNA fragment carrying one of the two primer annealing sites also results in the loss of a fragment (Tingey *et al.,*1992); (3) a nucleotide substitution may affect the annealing of one or two of the primers at a given site because of changes in homology, which can lead to a presence or absence of polymorphism or to a change in fragment size; or (4) insertion or deletion of a small piece of DNA can lead to a change in the size of the amplified fragment. In our case it can be suspected that there has been a single mutation and that this will show up under RAPD investigation as a single polymorphism difference, or (5) DNA rearrangements could also result in presence or absence of a polymorphism (Weising *et al.,* 1995).

The resultant individual plants from fused protoplasts can then have their genomic DNA extracted and subjected to the RAPDs procedure as the parents were. The electrophoretic banding pattern produced by both the parents and regenarents will be compared with each other to determine whether fusion between the parents would have taken place.

CHAPTER THREE:

MATERIALS AND METHODS

3.1.1. Samples used

Plant material used was taken from aseptically growing *invitro* Brondal sweet potato. Leaf material used was obtained from one month old seedlings. A week prior to protoplast isolation the sweet potato seedlings were left in the dark to etiolate them. All plant materials were provided by Agribiotech (Pvt) Ltd Zimbabwe.

3.1.2 Chemicals and reagents used

All the chemicals and reagents were bought from Fermatas. The three enzymes types used in the experiments were bought from Sigma group of companies in 1989. The experiments were carried out in two laboratories namely the Crop Physiology laboratory in the department of Crop Science and the Molecular Biology laboratory in the department of Biochemistry, Faculty of Science.

3.2 TISSUE CULTURE

3.2.1 Protoplast Isolation Procedure

The protocol for protoplast isolation by Menon and Tyagi (1999), was followed which recommended that 1% cellulase and 0, 25% macerozyme enzyme levels be used. The protocol was modified in light of the fact that the enzymes to be used in the experiments were 20 years old and their viability after numerous power cuts during storage was suspected to be low. This lead to the development of ten enzymatic treatments where cellulase ranged from 0, 5 to 4 g

while macerozyme was ranged from 0, 5 g to 2 g. Hemicellulase was also found in storage and included in the experiments.

Aseptically growing leaves were taken from their containers and immersed in 0.75 M sterile mannitol solution at pH 5, 6 for an hour. The enzyme solution contained per 100 ml; 27,2 mg/L of KH_2PO_4, 101 m/L KNO_3, 1480 mg/L $CaCl_2$, 246 mg/L of $MgSO_4.7H_2O$ and 0.75 M mannitol. The pH of the solution was adjusted to 5.6 using concentrated NaOH then autoclaved at 21 Kpsi for 15 min. The solution was allowed to cool before the enzymes were added. This made up the enzyme solution. After the leaf material was added to the enzyme solution the mixture was then incubated in the dark for 16 hours.

The experimental treatments were laid out in a completely randomised design (CRD) with ten treatments. Treatment means were separated using 5% least Significant Difference ($LSD_{0,05}$). The treatment number and corresponding enzyme combinations and concentrations are given in the Table 3.1 below.

TABLE 3.1. Enzyme combination and concentrations used as treatments for protoplast isolation and viability.

	Enzyme combinations and concentrations In grams per 100 ml
Treatment 1	0.5 g Cellulase and 1 g Macerozyme
Treatment 2	1 g Cellulase and 1 g Macerozyme
Treatment 3	1 g Cellulase and 2 g Macerozyme
Treatment 4	2 g Cellulase and 2 g Macerozyme
Treatment 5	2 g Cellulase and 2 g Hemicellulase
Treatment 6	2 g Cellulase and 3 g Hemicellulase
Treatment 7	3 g Cellulase and 3 g Hemicellulase
Treatment 8	3 g Cellulase and 4 g Hemicellulase
Treatment 9	4 g Cellulase and 4 g Hemicellulase
Treatment 10	4 g Cellulase and 5 g Hemicellulase

3.2.2 Protoplast Viability

After 16 hours of incubation in the dark, the harvested protoplasts were exposed to light and 0, 1 microlitres of the suspension was taken and placed on a slide and mounted on a microscope. The viability of the protoplasts was tested using 0.1% Evans Blue stain. The stain was applied to the slide containing the harvested protoplasts and left to react for 5min. Protoplasts with damaged cell membranes allowed Evans Blue stain to seep into the cell, giving the cell a blue tint. The protoplast cells were counted using a Fuchs-Rosenthal haemocytometer with an Improved Neubauer counting chamber. The counting chamber had a total ruled area of $9mm^2$

and a depth of 0,1mm^3. The volume between the counting chamber and the coverslip was 0,1mm^3.

The experimental treatments were laid out into a completely randomised design (CRD) with ten treatments. Treatment means were separated using 5% least Significant Difference ($LSD_{0,05}$). The treatments correspond to enzyme combinations and concentrations are given in the Table 3.1 above.

3.2.3 Protoplast Fusion procedure

The protoplast suspension was then exposed to light for an hour to completely denature the enzymes and stop any further reactions. From each of the respectively harvested sweet potato protoplast varieties, 1 ml was taken and placed in a container containing $CaCl_2$ fusion mix at pH 11.4. The $CaCl_2$ fusion mixture was made by mixing $CaCl_2$ and 0.75 M mannitol together and the pH of the solution was adjusted to 11.4 using concentrated NaOH. This fusion mixture was then autoclaved at 21 Kpsi for 15 min to sterilise the mixture. It was allowed to cool before the isolated protoplasts from each sweet potato variety were added. The fusion mixture was then incubated in the dark for 24 hours at 25^{o}C.

The experimental treatments for protoplast fusion were laid out in a completely randomised design (CRD) with ten treatments. Treatment means were separated using 5% least Significant Difference ($LSD_{0,05}$). The treatment number and corresponding calcium chloride combinations and concentrations are given in the Table 3.2 below.

TABLE 3.2 Protoplast fusion treatment number and calcium chloride concentrations.

	Calcium Chloride concentrations Molar concentrations
Treatment 1	0.1 M
Treatment 2	0.2 M
Treatment 3	0.3 M
Treatment 4	0.4 M
Treatment 5	0.5 M
Treatment 6	0.6 M
Treatment 7	0.7 M
Treatment 8	0.8 M
Treatment 9	0.9 M
Treatment 10	1 M

3.2.4 Protoplast regeneration media

Three different media regimes were tested for their ability to regenerate the fused protoplast products. These media, all based on Murashige and Skoog (1964) had their hormonal levels varied with artificial plant growth hormones namely BAP and 2, 4 D. The hormonal levels were selected on the basis of the findings of Ngadze (personal communication) and Kangara (personal communication) who recommend that 1 mg BAP for sweet potato meristems and a ratio of 2:1 for 2,4 D to BAP for sweet potato explants respectively be used for sweet potato regeneration.

Table 3.3 shows the various hormonal levels tested out against the three different states of media. After four months of trying these media attention was turned to nurse cell culture. Nurse cell cultures were set up with the same variations of hormones as the conditioned media. These regeneration media were tested out for their ability to induce cell divisions in the fused protoplasts and also for microcalli induction.

Table 3.3: Shows the three different media forms that were tested for protoplast regeneration and the hormonal treatments used.

Solid media (MS)			Liquid media (MS)			Nurse culture system (MS)		
BAP	2.4D	Regeneration	BAP	2.4D	Regeneration	BAP	2.4D	Regeneration
0,6	0		0,6	0		0,6	0	
1,0	0		1,0	0		1,0	0	
0,25	2,0		0,25	2,0		0,25	2,0	
0,5	4,0		0,5	4,0		0,5	4,0	
0,4	1,0		0,4	1,0		0,4	1,0	
0,8	2,0		0,8	2,0		0,8	2,0	

3.3 Molecular Biology tools

3.3.1 Genomic DNA Extraction using 3% CTAB method.

Approximately 100 mg sterile sweet potato (cv, Brondal) leaf material was ground to a fine paste and then placed in a sterile 1.5 µL eppendorf tube. To this 1 ml of 3% CTAB DNA extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1,4 M NaCl, 3% CTAB) prewarmed to 65^{o}C was added. The resultant mixture was vortexed briefly to homogenise the contents. The eppendorf tube was then incubated for 5min at 65^{o}C. After 5 min had elapsed, an equal volume of chloroform was added to the eppendorf tube and shaken vigorously for 30 sec.

The eppendorf tubes were then centrifuged for 10 mins at 13 000 revolutions per minute (rpms). The supernatant (upper phase) was then transferred to a new tube. The above step was repeated until the supernatant was clear. To the clear supernatant in the new tube 1% CTAB solution (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1,5% CTAB) was added and mixed by inversion and the mixture was incubated for an hour at room temperature. Centrifugation followed for 10 mins at 13 000 revolutions per minute. The supernatant was discarded and the resultant pellet was re-suspended in 1 M NaCl and to this 400µL of isopropanol was added and mixed until the pellet had dissolved. Again the eppendorf tube was centrifuged for 10 mins at 13 000 revolutions per minute. The resulting supernatant was discarded and the remaining pellet was washed using 70% ethanol. The pellet was then allowed to dry in the air over night at room temperature. The following morning the DNA pellet was re-suspended in 50µL of TE buffer (10 mM Tris-HCl, 0, 1 mM EDTA).

To determine the purity of the extracted genomic DNA, samples were electrophoresed on a 0.8 % agarose gel against a 1.5 Kbp molecular weight marker.

3.3.2 RAPDs Procedure

For the PCR reaction the following table shows the PCR reagents in microlitres for RAPD reactions assuming a 10% pippeting error. Ten different primers were used to detect polymorphisms in both Brondal and Nemagold.

Water	5X Buffer	dNTPs + MgCl2	Primer	Template DNA	Taq enzyme
10	4	10	2.5	5.4	1.0

3.3.3 Thermal cycling conditions

A total of 39 cycles were performed. In the first cycle the temperature settings were 91^{o}C for denaturation for 60 sec, 42^{o}C for annealing for 15 sec followed by elongation at 72^{o}C for 70 sec. The subsequent 38 cycles had denatration time set at 15 sec, annealing for 15 sec and elongation at 70 sec with temperatures similar to those of the first cycle for each of the three steps. The thermocycler used was model Geneamp PCR system 9700. The qualities of the isolated genomic DNA will be determined by electrophorasing the DNA on 0, 8% agarose gel (Fermatas) and stained with ethidium Bromide (Fermatas).

CHAPTER FOUR

RESULTS

Table 4.1 gives the results obtained for protoplast yield and viability against the ten enzyme treatments used. For both protoplast isolation and viability the treatments used were varying the enzyme combinations and concentrations of cellulase, macerozyme and hemicellulase.

Table. 4.1 Effect of varying enzyme concentration on protoplast isolation and protoplast viability per 0.1 μL of solution.

Treatment Number	Enzyme combination and concentrations	Protoplast Yield (Count data)	Protoplast Viability (As a percentage)	
1	0.5 g Cellulase and 1g Macerozyme	20 ^{a}	88 ^{e}	
2	1 g Cellulase and 1g Macerozyme	28 ^{b}	86 ^{e}	
3	1 g Cellulase and 2 g Macerozyme	34 ^{c}	81 ^{d}	83 e
4	2 g Cellulase and 2 g Macerozyme	42 ^{d}	79 ^{d}	
5	2 g Cellulase and 2 g Hemicellulase	73 ^{e}	61 ^{c}	
6	2 g Cellulase and 3 g Hemicellulase	62 ^{e}	53 ^{c}	
7	3 g Cellulase and 3 g Hemicellulase	76 ^{e}	44 ^{b}	
8	3 g Cellulase and 4 g Hemicellulase	80 ^{e}	41 ^{ab}	
9	4 g Cellulase and 4 g Hemicellulase	84 ^{f}	44 ^{b}	
10	4 g Cellulase and 5 g Hemicellulase	85 ^{f}	37 ^{a}	
P-value		0.000	0.000	
SED		1.66	2.03	
$LSD_{0.05}$		3.446	4.242	

Means followed by the same superscript are not significantly different from each other at $P < 0.05$.

4.1 Protoplast isolation

There was a significant difference between treatments ($P < 0.05$) for protoplast yield therefore suggesting at least one of the means was significantly different from the others. For this reason

the LSDs for mean separation were conducted to determine which particular means differed from the rest. The $LSD_{0.05}$ (3.446) shows that the first four means were significantly different from each other. Treatments 5, 6, 7 and 8 were not significantly different from each other and similarly treatment means for treatments 9 and 10 were not significantly different from each other. From the generated information from protoplast isolation data it can be noted that in general increasing the concentration of enzymes will result in increased protoplast yield. The levels were higher than recommended by established protocols in anticipation that the enzymes may have lost potency in storage.

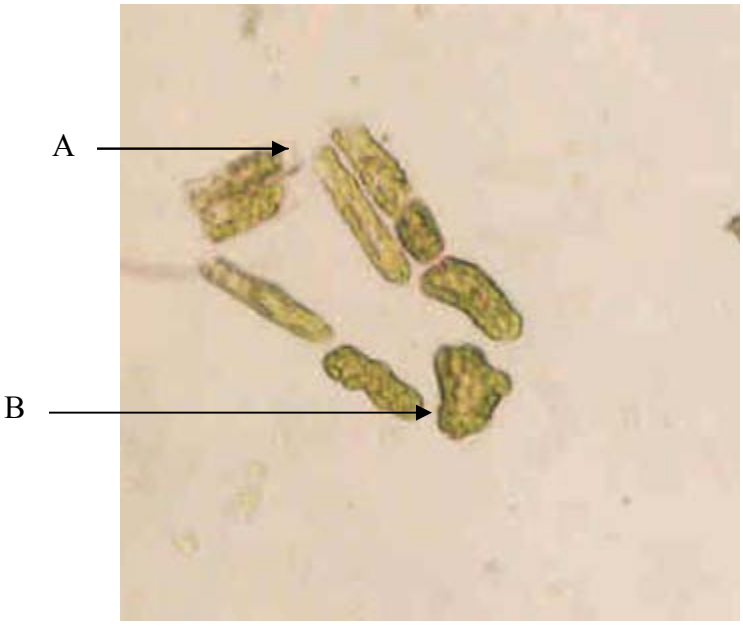

Figure 4.1: Sweet potato cells isolated using cellulase and macerozyme enzymes. **A** is part of a cell wall and **B** shows a protoplast cell. Magnification was at 10X x 40X using a standard light microscope. Type of camera used was Nikon 104 HFX labophat Fx35A.

Figure 4.1 shows the result of subjecting 1 month old sweet potato (Brondal) seedling leaves to 0,5 g cellulase and 1 g macerozyme enzyme suspension following the protocol of protoplast isolation by Menon and Tyagi (1999). Various cell entities were released from the parent tissue and these included plant cells and protoplast cells. Plant cells with their cell walls still intact had defined longitudinal shapes whereas protoplast cells which would have lost their cell walls were spherical in shape.

4.2 Protoplast viability

There was a significant difference between the ten enzymatic treatments ($P < 0.05$) for protoplast viability. The LSDs for separating treatment means ($LSD_{0.05} = 4.242$) showed that the means for treatment 2, 3 and 4 were not significantly different from each other, treatments 5 and 6 were not significantly different from each other and that treatments 9 and 10 were not significantly different from each other. The rest of the treatment means were significantly different from all the others.

4.3 Protoplast Fusion `

Table 4.2: The effect of increasing the molarity of calcium chloride on the number of fused protoplasts.

Calcium chloride Concentration (Molarity)	Fused protoplast Numbers (per 0.1 micro litres)
0. 1	9 [a]
0. 2	14 [b]
0. 3	18 [c]
0. 4	25 [d]
0. 5	30 [e]
0. 6	33 [f]
0. 7	42 [g]
0. 8	48 [h]
0. 9	54 [i]
1	56 [j]
SED	1.23
$LSD_{0.05}$	2.591
P-values	0.000

Means followed by the same letter are not significantly different from each other at $P < 0.05$.

Statistical analysis revealed that there was a significant difference between treatments ($P < 0.05$). LSDs revealed that all the ten treatment means were significantly ($LSD_{0.05} = 2.591$) different form each other.

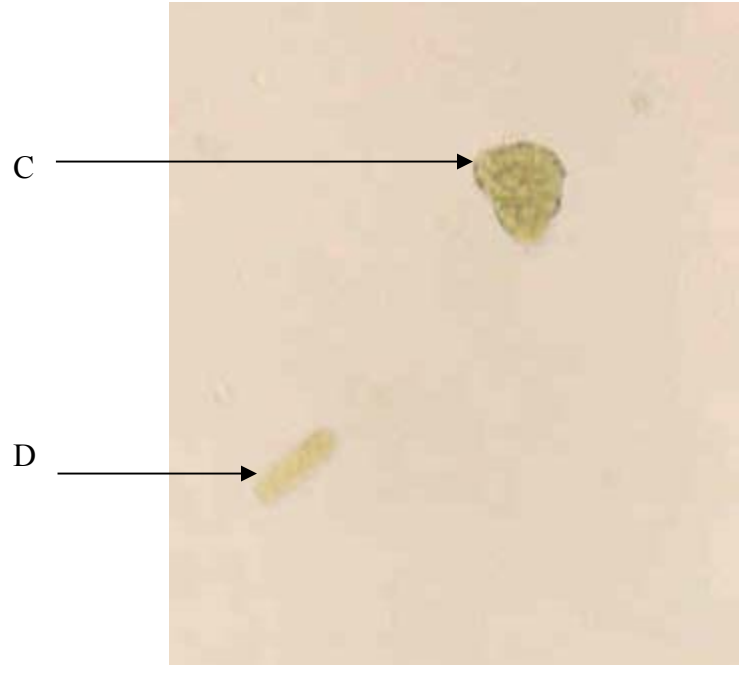

Figure 4.2: Sweet potato cells. C is a fusion product while D is a single plant cell. Note the differences in size between the two. Magnification was at 10X x 40X using a standard light microscope. Type of camera used was Nikon 104 HFX labophat Fx35A.

Figure 4.2 shows two individual cell entities. Cell labelled D was a single Brondal cell that still had its cell wall intact. Cell entity labelled C was a result of treating isolated protoplast cells with 0.1 M Calcium chloride concentration. Cell C was considerably larger compared to cell D a single cell, hence the former had to be a fusion product.

4.4 Protoplast Culture

The table below on protoplast culture shows that regeneration occurred in both nurse culture systems and liquid phases despite the different hormonal supplements.

Table 4.3: The three different media forms that were tested for protoplast regeneration and the hormonal treatments used.

Solid media (MS)			Liquid media (MS)			Nurse culture system (MS)		
BAP	2.4D	Regeneration	BAP	2.4D	Regeneration	BAP	2.4D	Regeneration
0.6	0	X	0.6	0	X	0.6	0	X
1.0	0	X	1.0	0	X	1.0	0	√
0.25	2.0	X	0.25	2.0	X	0.25	2.0	X
0.5	4.0	X	0.5	4.0	√	0.5	4.0	X
0.4	1.0	X	0.4	1.0	X	0.4	1.0	X
0.8	2.0	X	0.8	2.0	X	0.8	2.0	X

√ represents were regeneration occurred and X represents were regeneration did not occur.

From the above table 4.3, it was observed that production of microcalli occurred only in the liquid phases and not the solid phase of Murashigie and Skoog media. It was also noted that regeneration to the microcalli stage occurred in the liquid states of media despite the

differences in hormonal supplements. For instance in nurse culture systems regeneration occurred in media containing 1,0 mg/L BAP and 0 mg/L 2.4 D while the same result was obtained in liquid phase media with hormonal supplements 0,5 mg/L BAP and 4,0 mg/L 2.4 D.

4.5 DNA extraction

The results of running the genomic DNA of both sweet potato varieties on an 0, 8% agarose gel is in the figure 4.8 below. A molecular weight marker size 1.5 Kbp was used as reference point. Both genomic sizes appeared above the 1.5 Kbp band of the marker indicating that their size range was above 1.5 Kbp.

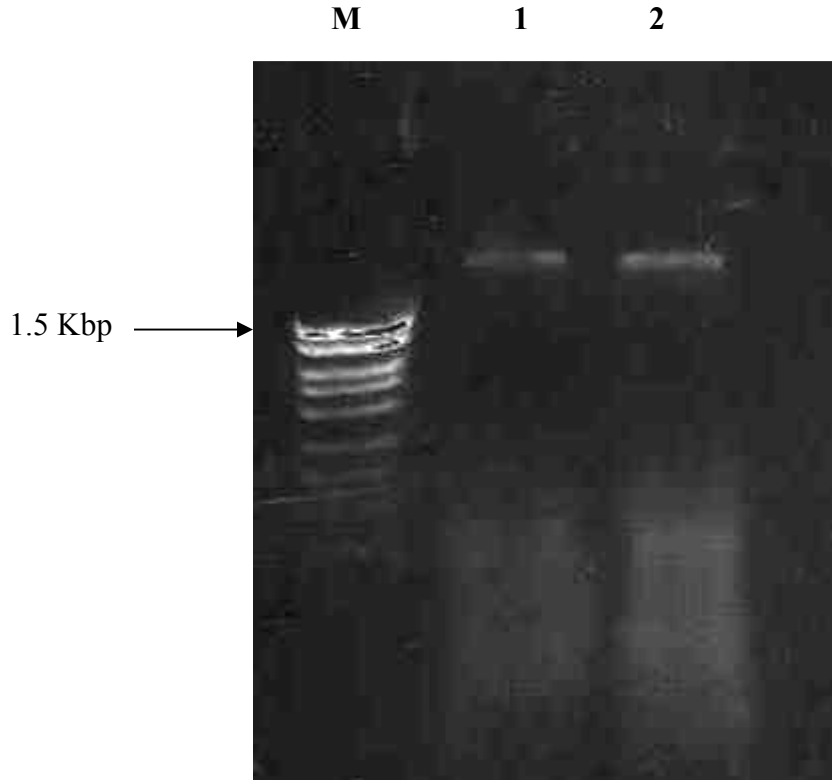

Figure 4.3: Genomic DNA from sweet potato varieties isolated using SDS extraction method. M is molecular weight marker 1.5 Kbp in size (Fermatas), Lane 1 is Brondal and Lane 2 is

Nemagold. The qualities of the isolated genomic DNA were determined by electrophorasing the DNA on 0, 8% agarose gel (Fermatas) and stained with ethidium Bromide (Fermatas).

The genomic DNA samples of Brondal and Nemagold were preliminary analysed by agarose gel electrophoresis in TBE buffer to test if the samples were significantly degraded. The appearance of distinct electrophoretic bands and no smears in figure 4.3 points to relatively good quality DNA. Visual assessment of the DNA quality was estimated from the intensity of UV-induced fluorescence emitted after ethidium bromide staining. However, quantitative analysis of extracted DNA using sensitive methods such as the spectrophotometric method which is based on optical density (OD) was not done.

4.6 Degree of polymorphisms as revealed by RAPDs.

Figure 4.4 below shows a typical example of a RAPD banding pattern obtained with 5 different primers. Further proof of the quality of the DNA extracted was confirmed by the ability of the used primers to amplify certain portions of the DNA under described PCR conditions. All 5 primers used in the experiment were able to detect polymorphisms in the two sweet potato genotypes. For every case, each given primer was able to produce electrophoretic position of the bands that matched in the two sweet potato used, although this was not always the case. For instance, in lanes 9 and 10 the primer produced 1 matching electrophoretic band (monomorphic bands) in both genotypes. This revealed a level of similarity between the two genotypes. However, also in lanes 9 and 10 each had an electrophoretic band position that did not match the other proving that the two sweet potato varieties had a degree of dissimilarity. Some of the RAPD bands produced were polymorphic. The number of polymorphic bands produced per primer was between 2 and 3. Different fragment lengths were produced in the

two varieties using the same primer, revealing that some of the banding patterns were similar in both sweet potato varieties.

Figure 4.4 below shows the various banding patterns produced after RAPDs were conducted on Brondal and Nemagold sweet potato varieties. Five different primers OPH5, OPH7, OPU5, OPU1 and OPH6 were used to detect polymorphisms in the two genotypes. Labels A and B indicate similar electrophoretic bands produced in the two sweet potato varieties using primer OPH6.

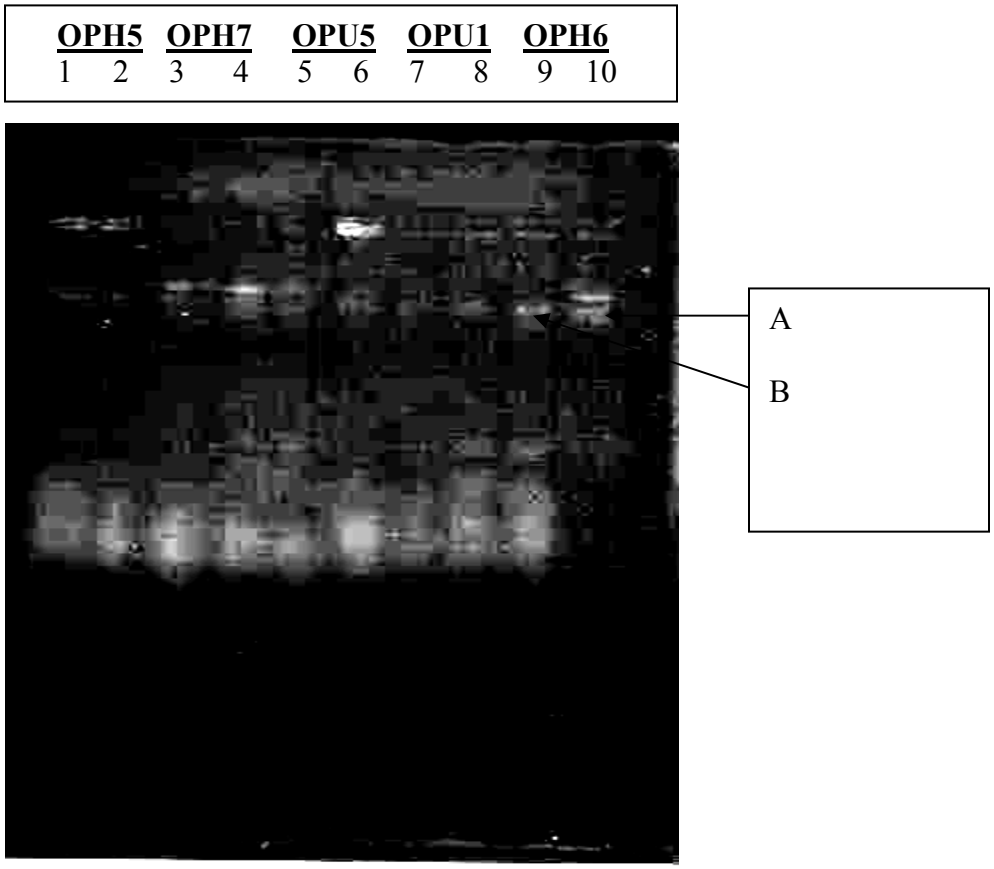

Figure 4.4 An Ethidium bromide stained agarose gel displaying RAPD banding patterns obtained with primers OPH5, OPH7, OPU5, OPU1 and OPH6 respectively in two sweet potato

varieties Brondal and Nemagold. Lanes 1,3,5,7 and 9 contained Brondal genomic DNA and lanes 2,4,6,8 and 10 contained Nemagold genomic DNA. The position of each electrophoretic band was compared between both genotypes under the same primer. A and B represent matching electrophoretic bands in two genotypes with the same primer.

CHAPTER FIVE:

DISCUSSION

For purposes of protoplast technology, protoplast isolation is of major importance since the yield obtained will determine the possible number of protoplasts that fuse and consequently the number of somatic hybrid plants. In the investigation on protoplast isolation from sweet potato variety Brondal, the results showed that using enzymes such as cellulase, hemicellulase or macerozyme all contributed to a significant protoplast yield ($p < 0.001$). These results are consistent with studies done by Sihachackr and Ducreuex (1989) who reported that for any unit increase in enzyme concentration, they would be a corresponding increase in protoplast yield.

Increasing the levels of cellulase, such as in the case from 1 g to 2 g in treatment 3 and 4 respectively or from 3 g to 4 g in treatments 9 and 10 lead to a significant ($P< 0.05$) increase in the release of protoplast cells. This increase is due to the fact that cellulase as a hydrolytic enzyme catalyses the breakdown of the beta (1-4) D glycopyranosyl bonds in cellulose, which is the main constituent of plant cell walls. It follows therefore that as more of the enzyme is added, the more protoplast cell are liberated from plant tissue (Capitana and McCann, 2000). Increasing the concentration of the enzyme means conversion is also increased resulting in more protoplasts being yielded. It was gratifying to the laboratory that the enzyme proved to be still viable after such long and erratic storage thus achieving the first objective.

Whenever the level of hemicellulase increased such as from 2 g to 3 g, there was a significant ($P<0.05$) increase in the number of protoplasts isolated. This increase in protoplast yield can be

attributed to the fact that as more hemicellulase is added, more of the beta (1-4) D xylopyranosyl bonds in the cell wall chemical structure are hydrolysed. These bonds hold together, in a cross linked structure, the cellulose microfribrils that are the main component of plant cell walls and in essence hemicellulases breakdown the structure that confers the cell wall with structural integrity (Capitana and McCann, 2000). Again it was pleasing that this enzyme too was viable.

Macerozyme R10, levels when increased showed a significant ($P<0.05$) increase in protoplast yield as evidenced in the case of treatments 2 and 3 were a doubling of the macerozyme concentration led to an increase in protoplasts from 28 to 34 per unit microlitre of solution. Macerozyme R10 is a pectinase that breaks down the pectin that acts as glue holding the cell walls together (Capitana and McCann, 2000). As more of the enzyme is added more protoplasts are released because the bonds that were previously holding the cells together would have been hydrolysed. The third relevant enzyme was thus proved to be effective showing that these three enzymes are still robust.

The combined effect of 2 g cellulase and 2 g hemicellulase gave a relatively higher protoplast yield at significantly ($P<0.05$) lower enzyme levels or combination as revealed. This result shows that substituting hemicellulase for macerozyme will result in a greater protoplast yield. This is because cellulase and hemicellulase attack the cell wall components that makeup the core structure that holds cells together. These findings are in contrast with the work of Menon and Tyagi (1999) of India who found that the best concentration to use for sweet potato protoplast isolation was 1 g cellulase and 0,25 g macerozyme. This contrast can also be due to

the fact that the macerozyme enzyme could have lost some of its viability over the long period of time that it was stored compared to the other two enzymes (Robertson, personal communication).

In 1903 Michaelis studied and founded the theory of enzyme action. He found that when an enzyme is added to any substract an enzyme-substract complex is formed and this is a necessary stage in enzyme catalysis. In the case of hydrolysing enzymes such as cellulase, macerozyme and hemicellulase, in the enzyme-substract complex that's were hydrolysis occurs and once catalysis has been completed the complex then breaks down to yield the protoplast cells. The saturation effect experienced after 2 g cellulase and 2 g hemicellulase were any further increases in enzyme concentration did not lead to any further significant increases in protoplast yield is explained by the fact that at very high enzyme levels all available enzymes will form the enzyme-substract complex meaning that the substract becomes the limiting factor to protoplast production. All enzymes are saturated with the substract and reaction reaches equilibrium.

The stability of the 3 studied enzymes is not known but inference made on the basis that they are protein in nature (Lehninger, 1989), suggests that the enzymes cellulase and hemicellulase are more tolerant to fluctuations in temperature that might occur in storage than macerozyme.

Protoplast viability plays a vital role in protoplast technology as unviable or damaged protoplasts could fail to regenerate into complete plants. An increase in protoplast yield resulted in decreased protoplast viability (Ishii, 1988). The enzymes cellulase, hemicellulase

and macerozyme are basically hydrolytic enzymes that catalyse the breakage of bonds in complex sugars, polysaccharides. Polysaccharides are abundant in both the structure of the cell wall as well as that of the cell membrane (Lehninger, 1989). For this reason whenever the hydrolytic enzymes are in solution with plant cells they do not discriminate between complex sugars in the cell wall and those in the cell membrane. Once hydrolysis of the cell wall is complete then the released cell becomes vulnerable to continued enzymatic attack on its membrane surface. Consequently the more the protoplasts are released the greater is the chance that some of those protoplasts will be damaged as well. This is consistent with the work of Ishii (1988) of Japan who postulated that the hydrolyzing enzymes cause cell fragmentation, which disassemble cortical microtubules in the plasma membrane of cells leading to decreased protoplast viability.

Ishii (1988) goes on further to suggest that protoplasts lose their viability during the isolation process due to oxygen toxicity. He explains that superoxide radicals (O^{2-}) are generated when plant tissue is treated with any pectinase enzyme. This may also account for the greater lose in viability in protoplasts isolated using macerozyme, a pectinase, compared to those isolated using hemicellulase. The addition of antioxidants or altering the isolation suspension's pH could be a viable option to decrease lose of protoplast viability as antioxidants neutralise the radical activity of superoxide (Lehninger, 1989).

Protoplast fusion is essential in the proposed breeding process since it allows for the creation of various genomic as well as chloroplast recombinations within the fusing protoplasts. Calcium chloride concentrations as low as 0,1M showed significant ($P<0.05$) increases in

protoplast fusions and is in line with the recommended concentration of calcium chloride in most fusion protocols (Kanchanapoom *et al.*, 2001). The highest number of fusion products was with 1M $CaCl_2$ concentration, which gave a fusion percentage of 52%. Bhojwani and Razdan (1983) cited that in their experiments protoplast fusion ranged from 20% - 62%, which is in keeping with the results that were obtained in this study.

The fusion of the protoplast cells of Brondal and Nemagold in all treatments is due to the fact that when the protoplasts are placed into a $CaCl_2$ solution the Ca^{2+} ion will be in solution as well. This double cation will serve to neutralise the negative charge that is inherent on membrane surfaces of plant cells that is as a result of the hydrogen phosphate groups that bear a negative charge. The Ca^{2+} ion becomes a fusagen by binding together the hydrogen phosphoric groups $[PHO_4]^-$ on two different protoplasts through mutual attraction between the negative hydrogen phosphate group $[PHO_4]^-$ and the positive Ca^{2+} ion. Once the protoplasts are in close proximity to each other then it becomes inevitable that the protoplasts will have their membranes associating and subsequently fusion occurs (Kanchanapoom *et al.*, 2001).

Previous experiments have found that with increased $CaCl_2$ concentration beyond a certain level the number of fusing protoplasts began to drop subtly. Senbursch (2005) postulated that this could be due to the fact that as more Ca^{2+} ions became dissociated in solution then the Ca^{2+} ions began to repel each other. This repelling effect of the calcium chloride ions will result in bond breakage for those protoplasts that would not yet have fused. If a bond would have been formed by a single Ca^{2+} ion that bounds and links together two-adjustcent protoplasts then an

increase in charged ions between these protoplasts may well result in the protoplasts moving apart as they would be forced apart by the repulsive force (Senbursch, 2005).

Of the media tested out for protoplast regeneration the results showed that the most pertinent issue was not hormonal supplementation but rather the phase in which the protoplasts are regenerated. The results revealed that protoplast culture is best done in liquid media since cell wall resynthesis, cell division and microcalli development was possible in both the liquid culture as well as nurse culture system but not in the solid media phase with similar hormonal supplements. Jullien *et al.,* (1998) suggested that for protoplast regeneration a stepwise approach should be taken, were protoplasts are inoculated into liquid media first to induce cell division. After microcalli development, this should then be followed by inoculation onto solid media for microcalli support. With liquid cultures or nurse culture systems the aqueous phase offers the protoplasts protection against the external environment. Without the cell wall the protoplast cells become vulnerable and hypersensitive to any fluctuations that might occur within their immediate environment (Pierik, 1987).

The protoplasts in liquid media are completely submerged and thus constantly nourished and replenished. On the other hand those protoplasts that would have been cultured on solid media are prone to exposure from the elements such as temperature extremes that induces drying out of the cells. Another factor that could have contributed to protoplast regeneration in both the liquid culture and the nurse cell culture systems could be the osmotic adjustments that were made to ensure that the protoplasts did not burst upon inoculation upon an aqueous phase. The

solid media was not adjusted for osmolarity control and this could be one of the contributing factors to the failure of the solid media to regenerate the fused protoplasts.

Calli growth from fused protoplasts of Brondal and Nemagold was achieved in both liquid culture and nurse cell culture. This proves that conditioned media is adequate to induce calli growth and that any unstable chemical compounds that might have been produced by the feeder cells in nurse culture systems have no bearing on the regenerative capacity of protoplasts. Regeneration of plantlets remains to be achieved and this worldwide has so far proved difficult as no reproducible protocol has yet been published although sporadic success has been achieved (Prakash, 1994).

Looking at figure 4.3 of genomic DNA the results show that both DNA were relatively pure after extraction and relative to the marker used, the size shows that it is indeed genomic DNA that has been extracted. RAPDs were done to distinguish polymorphisms between the two varieties. Figure 4.4 shows the different banding patterns that are produced when RAPDs were conducted on the two sweet potato varieties. Most of the banding patterns were similar in both Brondal and Nemagold and only a few were different. Similar banding patterns proves that both sweet potato varieties were genotypically similar since both varieties were breed for commercial purposes.

The different banding patterns could be accounted for by the fact that Brondal was mainly bred for high dry matter production which translates to yield while Nemagold was chosen for it's vitamin A content. Not many different bands were produced between the two varieties and

many were similar showing that only slight variations exists between the two varieties. This is expected because both sweet potato varieties were bred for by the same company and also for commercial purposes, thus making it more likely that the same starting material was used to bred both sweet potato varieties (Robertson, personal communications).

CHAPTER SIX:

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

From the study conducted, it can be concluded that for maximum protoplast isolation from sweet potato (Brondal) leaves, an effective enzyme combination and concentration to use would be 2 g cellulase and 2 g hemicellulase and any further increase in enzyme concentration above these levels would not yield a significantly ($P<0,05$) greater protoplast yield and would therefore be a waste of resources. It should be noted that these concentrations refer to the enzymes held by the Crop Science laboratory and may not be the same for fresh enzymes.

For protoplast viability it has been shown that with increased enzyme concentration there is a corresponding increase in lose of protoplast viability. *Protoplast isolation is therefore a compromise between maximum yield and protoplast viability.* The recommended enzyme levels that would offer the best compromise here between high protoplast yield and high protoplast viability would again be 2 g Cellulase and 2 g hemicellulase.

Evidence from the conducted experiments on sweet potato protoplast fusion between Brondal and Nemagold protoplasts shows that by increasesing the $CaCl_2$ concentration will result in a corresponding increase in the percentage of fused protoplasts. However it is advised that the $CaCl_2$ level should not exceed 1 M since any further increase could possibly result in negative correlation that is decreasing levels of fusion and would therefore be a waste of resources.

For sweet potato protoplast culture, it can be concluded from the results obtained that for the initiation of cell wall synthesis, cell division and microcalli development stage, it appears best to use liquid cultures instead of solid cultures. Protoplasts require continuous protection from the external environment given their vulnerable state and that this is best offered by liquid cultures.

From the results obtained from the electrophoresis of the two genomic DNA, it has been shown that using the CTAB 3% method for DNA extraction results in relatively pure DNA (Nemagold and Brondal) and that only by conducting RAPDs is it possible to distinguish between the two sweet potato varieties because of the different polymorphisms that were produced. The results of conducting RAPDs on genomic DNA of both varieties showed that these primers will be able to distinguish whether or not the two genomes have been combined in the regenerated plants. The banding patterns produced in the parents will be compared with those produced in the regenerated plants.

6.2 RECOMMENDATIONS

- I would therefore recommend that for future experiments on protoplast isolation studies focus more on such parameters as effect of varying the environmental conditions around protoplast isolation such as light and temperature as these have a significant effect on enzyme activity. I would also like to recommend that the same experiments be conducted on all varieties of sweet potato found in Zimbabwe to see whether the same results will be consistent cross the board.

- In future studies on protoplasts viability, I recommend that other tests for protoplast viability be used not only the Evens Blue exclusion method as it only tests for damaged protoplast cell membranes and is hardly the best indicator of protoplast viability.

- Experiments on sweet potato protoplast culture, in future, should concentrate on ways to induce microcalli to differentiate into shoots and roots.

- Future experiments on varietal differences using molecular techniques, I recommend that chloroplasts from both varieties be extracted and their DNA sizes determined. Further more, I suggest that other plant molecular markers be used for evaluating the differences and similarities between any plant varieties.

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APPENDICES

APPENDIX 4.1

Analysis of variance for protoplast isolation

Source	DF	SS	MS	F	P
Treatment	9	17420.7	1935.6	139.93	0.000
Error	20	276.7	13.8		
Total	29	17697.4			

APPENDIX 4.2

Analysis of variance for protoplast viability

Source	DF	SS	MS	F	P
Treatment	9	11288.7	1254.3	61.09	0.000
Error	20	410.7	20.5		
Total	29	11699.4			

APPENDIX 4.3

Analysis of variance for protoplast fusion

Source	DF	SS	MS	F	P
Treatment	9	7847.2	871.91	115.74	0.000
Error	20	150.67	7.53		
Total	29	7997.87			