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# PROTOPLAST ISOLATION AND PLANT REGENERATION IN BAMBARA GROUNDNUT: A PLATFORM FOR TRANSIENT GENE EXPRESSION

**by**

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**Thesis submitted in fulfilment of the requirements for the**

**Master of Technology: Agriculture**

**In the Faculty of Applied Sciences**

**at the**

**CAPE PENINSULA UNIVERSITY OF TECHNOLOGY**

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**Cape Town**
**2015**

## DECLARATION

I, Taiwo Betty Ayeleso, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

**Signed** **Date**

## ABSTRACT

Bambara groundnut (*Vigna subterranea*), a dicotyledonous plant is a legume which has a potential to contribute to food security and nutrition. Protoplasts are naked plant cells lacking cell walls. Viable protoplasts are potentially totipotent. Therefore, when given the correct stimuli, each protoplast is capable, theoretically, of regenerating a new wall and undergoing repeated mitotic division to produce daughter cells from which fertile plants may be regenerated through the tissue culture process. Protoplast systems are valuable and versatile cell based systems that are useful in observing cellular processes and activities.

In this study, the isolation of protoplast from the leaves of Bambara groundnut plant was extensively optimised. The factors affecting protoplast isolation considered in this study were ages of plant material, mannitol concentration, combinations and concentrations of enzymes and duration of incubation. Effects of ages of Bambara groundnut plant (4, 6, 8, 10 weeks), molarities of mannitol (0.4 M, 0.5 M. 0.6 M and 0.7 M), concentration and combination of enzymes (1%, 2% and 4% cellulase, 0.5% and 1% macerozyme and, 0.5% and 1% pectinase) at different incubation duration (4, 18, 24, 42 hours) were investigated. Overall, it can be deduced from this study that the optimal protoplast yield ($4.6 \pm 0.14 \times 10^5 ml^{-1}$/gFW) and viability (86.5 ± 2.12%) were achieved by digesting the leaves of four week old Bambara groundnut plant with 2% cellulase and 0.5 % macerozyme with 0.5M mannitol for 18 hours.

Freshly isolated protoplasts were then cultured at different densities of $1 \times 10^4$ - $2 \times 10^6$ protoplasts/ml using MS in three different culture (Liquid, agar and agarose bead) methods. First cell division was observed only in liquid medium. With several attempts, no division was achieved in the agar and agarose bead methods, division also did not progress in the liquid medium and hence, plant regeneration from Bambara groundnut protoplasts could not be achieved in this study. Consequently, a further study is underway to compare the proteomic profiles of freshly isolated protoplasts and cultured protoplasts in order to gain insights into the expression of proteins that could perhaps be contributing to the difficulty in regenerating Bambara groundnut plant through protoplast technology. The present study is novel because it is the first study to optimise the various factors that could affect protoplast isolation from the leaves of Bambara groundnut and thus developed an efficient protocol for protoplasts isolation from leaves of Bambara groundnut for cell manipulation studies.

**ACKNOWLEDGEMENTS**

I thank God for his divine mercy, strength, protection and journey mercies while running around for the completion of this research.

I appreciate my supervisor, Dr Richard Mundembe for his technical assistance, input and understanding. My thanks also go to my co-supervisor, Prof Francis Lewu for his technical support and encouragement.

I also want to thank CPUT for the university research funding to undertake this study.

My special appreciation goes to Mrs Linda O'Riley of the Department of Botany and Plant Biotechnology in the University of Johannesburg for her technical input, support and hospitality.

My special thank goes to my husband, Dr Ademola O. Ayeleso for his love, support, understanding and constant encouragement when I was too down to carry on, you are indeed a priceless gift from God. Also, to my jewels; Abisayo and Abisola for their understanding when mummy had to be in the laboratory even at odd hours, I love u both very much.

I am very grateful to my parents; Mr C.A. and Mrs S.O. Oluwagbemi for their unconditional love, always believing in me and making sure I am on the path to achieving my dreams.

My thank goes to Bonolo Mosime for her technical assistance in tissue culture experiments.

I also appreciate Prof Oziniel Ruzvidzo and Dr. Sandile Fuku of the Department of Biological sciences, North West University, Mafikeng campus for their immense assistance. Thank you so much.

## DEDICATION

This thesis is dedicated to God, who made the completion of this project possible.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| BAP | 6- Benzylaminopurine |
| $CaCl_2$ | Calcium chloride |
| CCoAOMT | Caffeoyl-CoA O-methyltransferase |
| DNA | Deoxyribonucleic acid |
| GUS | β –glucuronidase |
| HCL | Hydrochloric acid |
| PEG | Polyethylene glycol |
| KCl | Potassium chloride |
| pH | Potential of hydrogen |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| MES | 2-(*N*-morpholino) ethanesulfonic acid |
| MS | Murashige & Skoog |
| NAA | Naphtalene acetic acid |
| SD | Standard deviation |
| RNA | Ribonucleic acid |
| RPM | Rotation per minute |

| | |
|---|---|
| MALDI-TOF/MS | Matrix-assisted laser desorption ionization time of flight/mass spectrophotometry |
| 2,4-D | 2, 4 dichlorophenoxy acetic acid |
| 2-D PAGE | Two-dimensional polyacrylamide gel electrophoresis |

# CHAPTER ONE

## 1.0 INTRODUCTION

Bambara groundnut is an indigenous African crop that belongs to the family Fabacaea. It is a seed crop of African origin and it is cultivated mainly by farmers as a famine culture crop because of its agronomic values (Anchirinah *et al.,* 2001; Olaleye *et al.,* 2013). Bambara groundnut is the third most important legume after groundnut (*Arachis hypogea*) and cowpea (*Vigna unguiculata*) in Africa (Sellscope, 1962; Omoikhoje, 2008). It is a highly proteinous and nutritious crop with the potential to provide a balanced diet in areas where animal protein is scarce and / or expensive (Hillocks *et al.,* 2012). Its chemical composition can be compared to that of soy bean, a well-known nutritional crop (Murevanhema and Jideani, 2013). Bambara groundnut can be eaten fresh or cooked while still immature. At maturity, they become very hard and therefore require boiling before further preparation. Most recently, milk extracted from this nut has been used to produce a probiotic beverage (Murevanhema and Jideani, 2013). Bambara groundnut is popular in Africa because of its resistance to drought and its ability to produce reasonable yield when grown on poor soil compared to other species (Anchirma *et al.*, 2001). The plant is also useful in crop rotations as a source of residue nitrogen for the subsequent crop through nitrogen fixation (Ncube *et al*., 2007).

However, there are still challenges associated with Bambara groundnut production and utilization which include the presence of anti-nutritional factors such as tannins and oxalate which lower seed quality and protein availability (Odumodu, 1992) and susceptibility of the crop to pests and diseases (Gwekwerere, 1995). Bambara groundnut is also a very hard seed, which takes a long time to cook and has a poor milling characteristics, as it does not dehull easily (Barimalaa and Anoghalu, 1997). These factors make it less competitive than the other improved crop species; hence there are efforts to contribute towards improvement of the crop by various approaches, including traditional breeding and tissue culture (Basu *et al*., 2007; Okpuzor *et al*., 2010). Bambara groundnut has been successfully regenerated from embryonic axes, cotyledons and epicotyl and hypocotyl cuttings (Mongomake *et al*., 2009). As evident from available literature, no successful isolation has been reported in Bambara groundnut.

Protoplasts are useful materials in the production of novel genotypes of plants through somatic hybridization. Protoplasts were first isolated in tomato seedling roots by Cocking (1960) and since then isolation of protoplasts has been recorded in many crops following a variety of methods. Plant protoplasts provide a unique single cell system to understudy several aspects of modern biotechnology. Protoplasts are naked cells, the cell surface is fully exposed and

accessible and hence it provides the starting point for many techniques aimed at the genetic modification of plant cells and whole plant (Ling *et al*., 2010) towards crop improvement. Protoplast technology includes the isolation, culture and fusion of plant protoplast resulting in the production of whole plant (Pati *et al.,* 2008). Development of protoplast systems has become indispensable tools in genetic engineering and crop breeding and has increased the versatility of plants (Rao and Prakash, 1995).

In view of the many applications of plant protoplasts and the lack of studies on application of protoplast technology in Bambara groundnut, this study attempted to optimize a protocol that can be used to generate a large number of viable protoplasts from the leaves of Bambara groundnut. This study also attempted to optimise the conditions for cell division and plant regeneration from the isolated protoplasts. The study further proceeded to characterise the difference in protein profiling of both freshly isolated protoplasts and dividing protoplasts in culture. This in our view could provide useful insights into the presence of individual proteins that are involved in cell differentiation during protoplast culture and possibly help to explain the reason (s) why regeneration of Bambara groundnut plant could not be achieved in the present study.

### 1.1 Aims of the study

Protoplasts technology has been useful in both genetic and biochemical studies but there are no reports to date describing the isolation and culture of protoplasts of Bambara groundnut. This study was conducted to investigate the optimum conditions for Bambara groundnut protoplast isolation, culture and regeneration.

The specific aims were;

- To develop and optimise an efficient and reproducible protocol for Bambara groundnut protoplast production.
- To optimise an efficient protocol for protoplast culture towards regeneration of Bambara groundnut plants.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Overview of Bambara groundnut

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) belongs to the family *Fabaceae* and subfamily *Faboidea* (Bamshaiye *et al.,* 2011). It is a leguminous dicotyledonous plant that resembles groundnut in both cultivation and habit. Although, it is cultivated occasionally in Asia and elsewhere, it is grown rarely outside the continent of Africa (Hillocks *et al.,* 2012). It is a seed crop of African origin and it is cultivated mainly by farmers as a famine culture crop because of its agronomic values (Anchirinah *et al.,* 2001; Olaleye *et al.,* 2013). Bambara groundnut is the third most important legume after groundnut (*Arachis hypogea*) and cowpea (*Vigna unguiculata*) in Africa (Sellscope, 1962; Omoikhoje, 2008). The Bambara nuts are referred to as Jugo beans (South Africa), izindlubu (Zulu, South Africa), ntoyo ciBemba (Republic of Zambia), Gurjiya or Kwaruru (Hausa, Nigeria), Okpa (Ibo, Nigeria), Epa-Roro (Yoruba, Nigeria), Nyimo beans (Zimbabwe) (Bamshaiye *et al*., 2011), ìNduhu-mvendaî in Venda, South Africa (Swanevelder, 1998) and Izindlubu in isiZulu, South Africa (Mahbudhi et al, 2013).

#### 2.1.1 Habit and production of Bambara groundnut

Bambara groundnut is cultivated mainly on the flat but sometimes, on mounds or ridges which may be beneficial in wetter areas, as the crop does not tolerate water-logging (Hillocks *et al*., 2012). Bambara groundnut is propagated through the seeds which are round shaped and can be in colours ranging from red, cream/white and brown as shown in figure 2.1. It can be grown on poor soils with little rainfall and also produce better yields in better conditions (Mkandawire, 2007). It grows best under bright sunshine, high temperature and at least 4 months free of frost but frequent rain (Okonkwo and Opara, 2010). It usually takes 7 to 15 days under favourable temperature (28.5°C to 32.5°C) for Bambara groundnut to germinate; but under lower temperatures, it takes up to 31 days with some seeds remaining dormant indefinitely (Linnemann and Azam-Ali, 1993). It has a well-developed taproot with profuse geotropic lateral roots and the fibrous lateral roots form nodules for nitrogen fixation (Mazahib *et al*., 2013). Thus Bambara groundnut like any other legume provides farmers with an important alternative to diversify their farming systems and improve soil fertility via symbiotic $N_2$-fixation (Ncube *et al*., 2007).

Bambara groundnut is advantageous over cowpea and groundnut due to its adaptation to poor soils and tolerance to drought (Hillocks *et al*., 2012). The mechanisms which enable Bambara groundnut to produce at least some yield during severe drought are poorly understood, but may be linked with its relatively high root: shoot biomass ratio and small leaf area, which restricts transpirational loss of water (Collinson *et al.,* 1996). The stems are branched and hairy, with short internodes while the leaves are trifoliate and are borne on long slender petioles and the flowers are spread out close to ground level on hairy peduncles with each producing one to three flowers (Mazahib *et al*., 2013). It is suitable for intercropping with other crops and does not take up large areas of land that could be used for other crops considered more important or lucrative (Hillocks *et al*., 2012).

Figure 2.1: Bambara groundnut seeds

Figure 2.2: Bambara groundnut plant adapted from Fleissner, 2006

### 2.1.2 Nutritional importance and utilization of Bambara Groundnut

Bambara groundnut is a proteinous legume with reported approximate chemical composition of water 14.7%, ash 3.24%, crude protein 22.2%, fat 6.6%, cellulose 4.4% and carbohydrates 63.56 % (Ferrao *et al.,* 1987). Its chemical composition can be compared to that of soybean a well-known nutritional crop (Murevanhema and Jideani, 2013) and other common legumes as shown in Tables 2.1 and 2.2. It is an important source of protein in the diets of a large percentage of the population in Africa especially the poor who cannot meet the expense of costly animal protein (Bamshaiye *et al*., 2011). Legumes are good sources of essential amino acids and fats. Bambara groundnut is high in protein quotient, especially in methionine and this makes it more proteinous than any other bean (Olaleye *et al*., 2013). The essential amino acid content of Bambara groundnut is comparable to that of soybean with values 6.82g/16gN lysine, 1.85g/16gN methoinine, 1.24g/16gN cysteine and 6.24g/16gN lysine, 1.14g/16gN methionine, 1.80g/16gN cysteine respectively (Fetuga *et al.,* 1975; Omoikhoje, 2008). It also contains high concentration of soluble fibre than any other beans (Olaleye *et al*., 2013).

The carbohydrate content of Bambara groundnut is made up of starch and non-starch polysaccharides with lesser amount of reducing and non-reducing sugar (Bamshaiye *et al*., 2011). According to a study by Okonkwo and Opara (2010), the fatty acid components found in Bambara nut oil were Caprylic acid (17.0%), Capric (8.0%), Lauric acid (9.0%), Palmitic acid (10.0%), Palmitoleic acid (21.0%), Oleic (18.0%) and Linoleic acid (12.0%). They further stated that Bambara nut can serve as a reliable food as it can provide these essential fatty acids in the diet, thereby possessing the ability to sustain the people in Africa continent. These fatty acids are basically used in the production of hormone like substance that regulates the wide range of functions in the body (Okonkwo and Opara, 2010). Bambara groundnut can be useful in the fortification of other foods for instance, Mbatha *et al*. (2009) fortified maize blended meal with Bambara groundnut and the results showed significant improvement in the mineral and essential amino acid contents i.e. the minerals especially zinc, calcium, magnesium, iron and iodine increased to 30% with addition of Bambara groundnut.

The distinctive properties and composition of Bambara groundnut makes it a balanced food due to the presence of all the major nutrients that promotes good health for people living in Africa (Okonkwo and Opara, 2010). In addition to the relatively high protein content in Bambara groundnut, the total nutritional composition reflects an excellent balance unusual in single plant products (Gabriel *et al.,* 2013). Bambara groundnut seeds may be consumed in various forms for food i.e. fresh seeds may be eaten raw, boiled, grilled and dry seeds can be made into a powdery form to make cakes (Adebowale and Lawal, 2002; Olaleye *et al*., 2013).

Table 2.1: Nutritional content of Bambara groundnut and other common legumes

| Nutritional content | Bambara groundnut | Soya | Phaseolus bean | Cowpea | Faba bean | Chickpea |
|---|---|---|---|---|---|---|
| Calories | 390 | 416 | 343 | 333 | 341 | 364 |
| Protein (%) | 21.8 | 36.5 | 23.8 | 23.6 | 26.1 | 19.3 |
| Carbohydrate (%) | 61.9 | 30.2 | 59.6 | 60 | 58.3 | 60.6 |
| Fat (%) | 6.6 | 19.9 | 2.1 | 0.8 | 5.7 | 6.0 |

Source: de Kock (undated report)

Table 2.2: Mineral content [mg 100 $g^{-1}$] in raw Bambara groundnut and its fermented flour

| Mineral content | Raw nut | Fermented flour |
|---|---|---|
| Calcium | 16.3 | 20.7 |
| Potassium | 74.1 | 80.6 |
| Magnessium | 66.7 | 69.3 |
| Sodium | 26.1 | 26.0 |
| Iron | 0.38 | 0.43 |
| Selenium | 0.12 | 0.14 |
| Phosphorus | 164.7 | 110.2 |

Source: Ijarotimi and Esho (2009)

The seeds become very hard at maturity and therefore require boiling before any specific preparation is to be done. Brough *et al*. (2003) prepared vegetable milk by soaking shelled Bambara nuts in water overnight, homogenising and removing insoluble material. The beany taste could be removed by dry-frying the beans after soaking and before homogenisation, milk from Bambara groundnut was preferred in taste and colour to those produced from cowpea, pigeonpea and soybean.

Fermentation as reported by Ijarotimi and Esho (2009) improved the mineral composition but had little effect on the amino acid content and decreased the anti-nutritional factors; oxalate, tannic acid, phytic acid and trypsin. Fermentation of Bambara groundnut results in the production of a condiment with characteristics similar to that produced by the fermentation of locust bean (Barimalaa *et al*., 1994). Nwanna *et al*. (2005) reported that fungal fermentation of Bambara groundnut improved upon the nutrients potential of the seed as a feedstuff resource.

The realization that Bambara groundnut has a potential to contribute to food security and nutrition is relatively recent, and there are efforts to contribute towards improvement of the crop by various approaches, including traditional breeding and tissue culture (Basu *et al*., 2007; Okpuzor *et al*., 2010). In spite of its high nutrition, Bambara groundnut remains one the crops most neglected by science (Basu *et al.,* 2007), the yields are low because its production and improvement has been neglected for many years by researchers (Bamshaiye *et al*., 2011).

### 2.1.3 Challenges to the utilization of Bambara groundnut

The challenges to Bambara groundnut production and utilization include unpredictable low yield and the presence of anti- nutritional factors (Mongomake *et al*., 2009). Anti-nutritional factors are a range of secondary defence metabolites synthesized by plants as part of their protection against attack by herbivorous, insects and pathogens or as means to survive in adverse growing conditions (Khokhar and Apenten, 2003). The anti-nutritional factors mainly occur in pulses, grain legumes and foods and feed material prepared from grain legumes and pulses (Friedman, 2001). The presence of these anti-nutritional factors in Bambara groundnut such as tannins and oxalate lower seed quality and protein availability (Mongomake *et al*., 2009). They also interfere with normal growth, reproduction and health, when consumed regularly in amount existing in a normal component of diet (Bora, 2014).

Bambara groundnut is also a very hard seed, which takes a long time to cook and has a poor milling characteristics, as it does not dehull easily (Barimalaa and Anoghalu, 1997). The reduction in Bambara groundnut production, according to farmers is due to lack of adequate processing techniques to encourage utilization (Lord, 2010; Mazahib *et al*., 2013). These factors make it less competitive than the other improved crop species hence the need for crop improvement approaches.

### 2.1.4 Crop improvement attempts in Bambara groundnut

In spite of the all the limitations to the utilization of Bambara groundnut, reports of improvement strategies on the plant are quite limited. Until recently, only selection breeding was practiced in which existing landraces were evaluated and their seeds multiplied (Massawe 2003). Bambara groundnut like any other leguminous plant is a self-pollinating crop and hence, natural cross pollination is difficult due to the morphology of the flowers. Massawe, (2003) attempted artificial pollination of Bambara groundnut plant which resulted in a poor success rate as low as less than 2% harvested hybrid seeds. The development of improved varieties through conventional breeding is laborious as it involves a well-controlled and coordinated multi locational breeding programme through single genotypes or through mixes of similarly adapted genotypes (Aliyu *et al*. 2015).

Recent biotechnology approaches in crop improvement which includes protoplast fusion and genetic transformation by direct integration of genes into plants protoplast is an explorable opportunity for the improvement of Bambara groundnut. In comparison with conventional breeding methods, somatic hybridization may allow the exploitation of a wider gene pool, as sexual incompatibilities are bypassed. In order to overcome interspecific incompatibility,

protoplast technology is an appropriate system of creating a new plant with desired traits (Chamani, *et. al.*, 2012).

### 2.2 Plant protoplasts

The protoplast is the living material of a plant or bacterial cell, including the protoplasm and plasma membrane after the removal of the cell wall. The plant cell wall although playing a key role in processes involving plant structure and function is a major impediment in exploring direct DNA transfer to individual cells and the production of somatic hybrids by cell fusion (Veilleux *et al.*, 2004). Plant protoplasts (naked cells) provide a unique single cell system to form the basis for several aspects of modern biotechnology (Davey *et al*., 2005). Protoplast technology includes the isolation, culture and fusion of plant protoplast resulting into the production of whole plant (Pati *et al*., 2008). Protoplasts were first isolated by Cocking (1960), and since then isolation of protoplasts has been recorded in many crops with adapted modifications.

Protoplasts are usually produced by the treatment of mesophyll tissue with a mixture of cell wall degrading enzymes in solutions which contain osmotic stabilizers to sustain the structure and function of protoplasts (Chabane *et al*., 2007; Al-maarri *et al*., 2014). Development of protoplast systems has become indispensable tools in genetic engineering and crop breeding and has increased the versatility of plants (Rao and Prakash, 1995). Protoplast isolation from plants is now a common procedure. Viable protoplasts are potentially totipotent, therefore under optimal conditions each protoplast is not only capable of regenerating a new wall but can also undergo repeated mitotic division to produce daughter cells from which fertile plants may be regenerated through the tissue culture process (Davey *et al*., 2004). Protoplasts from different species and even from different tissues of the same species commonly differ in their nutritional requirements (Mac´kowska *et al*., 2014). Somatic hybridization through plant protoplast fusion allows combine parent genes in higher plants as well as overcoming barriers existing between plant species (Navrátilová, 2004). Protoplasts provide many convenient features to study cellular processes during transient overexpression or suppression of specific gene's products (Ul-Rehman *et al.*, 2011).

#### 2.2.1 Protoplast isolation

The two methods of isolation of protoplasts are mechanical and enzymatic methods. Although, mechanical methods which involves the slicing of plasmolysed tissues are now not often used for protoplast isolation, they are useful with large cells and when limited (small) numbers of protoplasts are required (Davey *et al*., 2005). The enzymatic method has been successfully

applied to several plants and it involves the use of enzymes to dissolve the cell walls thereby releasing protoplasts and this could be one step method (direct method) or two step method (sequential method) (Tomar and Dantu, 2010).

The two step method which is also referred to as the sequential method was first attempted by Takebe *et al.,* (1968), protoplasts are isolated from the tissue or callus by first incubating in one enzyme i.e. pectinase and then treated with another enzyme i.e cellulase (Tomar and Dantu, 2010) for the release of protoplasts. The one step enzymatic method of protoplast isolation was used by Power and Cocking (1970) for protoplast isolation. In this method, protoplasts are isolated directly by the use of the two enzymes simultaneously to reduce time. The mixture of a cellulase and a pectolytic enzyme like pectinase or macerozyme is usually suitable for majority of plant tissues.

Isolation of protoplasts by enzymatic method is restricted to parenchymal cells with unlignified cell walls due to fact that cells with lignified cell walls are resistant to enzymes. It is an advantageous method because protoplasts are collected at a high quantity while the cells are not damaged and the osmotic conditions may be influenced (Navrátilová, 2004). When large populations of protoplasts are required, which is the norm, efficient enzymatic digestion of source tissues is essential (Davey and Kumar, 1983; Eriksson, 1985; Davey *et al*., 2000, 2003).

### 2.2.2 Factors affecting protoplast isolation in plants

Several factors affect the release of protoplast in plants. These factors influence different species and genotypes differently. Hence, several studies have been conducted on the modification of these factors towards optimal yield and viability of isolated protoplasts in different plants.

#### 2.2.2.1 Source and age of plant material

The physiological state of the source tissue greatly affects the yield and viability of isolated protoplasts (Raikar*,* 2007). Almost any plant organ or tissue can be a source of protoplasts. However, Dovzhenko *et al.* (2003) reported a reproducible and rapid cotyledon-based protoplast system for *Arabidopsis thaliana,* which facilitated molecular studies with this model species. Similarly, Raikar *et al.* (2008) reported that protoplast yield from cotyledons 4 days after seed germination were substantially higher than when leaves were used as a tissue for isolation. Guard cells have also been reported to be a unique source of totipotent protoplasts. Pandey *et al.* (2002) reported both large-scale and small-scale procedures to isolate guard cell protoplasts of *A. thaliana* for use in physiological studies.

Among the most popular and the most convenient sources of protoplast isolation are leaf mesophyll tissue and callus or suspension cultures (Vasil and Vasil, 1980). Seedling leaves has been said to be a convenient source of totipotent protoplasts (Davey *et al.,* 2006). Leaf mesophyll cells of a wide range of plants have been used with success as a protoplast source. It was also reported that for plants grown in soil under natural conditions, young plants with expanding leaves before the differentiation of flowers generally give best results (Saker *et al.*, 1999). This is consistent with other studies that reported that age of plant material affects the yield and viability of isolated protoplast. Pongchawee *et al.,* (2006) reported that it was more difficult to isolate protoplast from older leaves because there was more lignin substances accumulation in cell wall of older cells.

#### 2.2.2.2 Pretreatment of source tissue

Dark pretreatment and preplasmolysis of the donor tissue proved beneficial for the yield and viability of the protoplasts. Plasmolysis prior to enzymatic digestion of source tissues in some salts and/or sugar alcohol solutions reduces cytoplasmic damage and spontaneous fusion of protoplasts from adjacent cells. Plasmolysis of leaves for 90 minutes in 13% sorbitol solution greatly increases the number of protoplast obtained from leaves of Apricot (Ortin-Parraga and Burgos, 2003). Mechanical treatments prior to enzyme application also greatly influence the release of protoplasts. Some procedures also routinely remove the epidermal cell layer mechanically by either peeling, brushing off the epidermis carefully and delicately or thinly cutting the tissue into cross sections. These methods require some care and practice to avoid excessive damage of the inner cell layer.

#### 2.2.2.3 Enzyme treatment and length of incubation

Plant cell walls contain celluloses, hemicelluloses, pectins, some other polymers and proteins. The complex physical and chemical structures of the plant cell wall restrict the activity of enzymes against it. Hence, enzymes mixtures of cellulases, hemicellulases, and pectinases are routinely used to break cell wall towards protoplast isolation. The treatment of a plant tissue with a mixture of pectinase and cellulase would simultaneously separate cells and degrade their cell walls (Power and Cocking, 1970). The type and concentration of enzymes are important were important for isolation of protoplasts (Shao *et al.*, 2008). Length of incubation in enzyme solution also greatly influences the release of protoplasts from plant tissues, usually the increasing time of contact with lytic enzymes may increase the chances of lysis of already formed protoplasts thereby decreasing the total protoplast yield (Kumari and Panda 1992). Pongchawee *et al*. (2006) reported that time of incubation significantly affects the yield and viability of protoplast in *Anubia nana* engler, they observed that yield and viability decreased when the length of incubation was extended beyond 4 hours. Yoo *et al.,* (2007)

reported that prolonged incubation of leaves for 16-18 hours is stressful for protoplasts but might be important for the dedifferentiation and regeneration processes.

#### 2.2.2.4 Nature and molarity of osmoticum

Protoplasts are very fragile, so the enzymes must be included in an isolation mixture that contains an osmotic stabilizer, a calcium salt, phosphate and a buffer (Gamborg *et al.*, 1976). Plant cells have an internal pressure (turgor pressure) which is contained by the rigid cell wall. Generally, protoplast burst in hypertonic solution and collapse in hypotonic solution (Ohshima and Toyama 1989) hence, standardizing the kind and concentration of osmoticum to be used in isolation buffer is crucial to harvesting a high yield of viable protoplasts. Examples of osmoticum that has been used include glucose, sucrose, sugar alcohols like sorbitol, mannitol. Of all these sugars, mannitol is the most commonly used osmoticum (Tomar and Dantu, 2010)

#### 2.2.2.5 Protoplast purification and harvest methods

Protoplasts are usually separated from plant debris and undigested tissues by centrifugation and filtering through meshes with pore sizes suitable for the size of the protoplasts. Repeated centrifugation and washing can be done usually 3 -10 minutes at 750-1000rpm (Ondrej, 1985) to achieve cleaner and purified protoplasts. Floatation of protoplasts on sugar or sugar alcohols gradient can also be used. Sucrose and Percoll are usually used for purification and washing because of their high buoyant densities.

#### 2.2.2.6 Others factors affecting protoplast isolation in plants

Other factors that can affect the release of protoplast include the pH of enzyme- buffer solution (Sinha *et al*., 2003). This can ultimately affect the activities of enzymes; several studies confirmed that a pH range of 5.6-5.8 is suitable for optimal enzyme activities in isolation buffer. Others are condition of growth of explants which is mainly influenced by environmental and seasonal conditions, shaking/agitation (Davey *et al*., 2005), light, sterilization procedures for greenhouse grown plants and temperature. Kovas and Subik (1970) observed that unfavourably high temperature may cause agglutination of certain organelles in isolated protoplasts and stability of protoplast membrane.

### 2.2.3 Protoplast culture and regeneration

Efficient system for plant regeneration from protoplasts is a key requirement for protoplast-based biotechnologies (Hou and Jia, 2004). Plants regeneration from protoplasts was first achieved by Nagata and Takebe (1971) in tobacco (*Nicotiana tabacum* L). The high regeneration tendency of protoplasts is a pre-requisite for protoplast utilization in crop

improvement programmes. In order to get a high regeneration frequency, optimization of the conditions for protoplast isolation, culture density, culture media composition, growth regulator concentrations, dark and light periods in the initial culture phase and the developing phase of calli capable of transfer to an induction medium is essential (Kaur *et al.*, 2006). The two methods that are often used for plants regeneration from cell cultures are somatic embryogenesis and organogenesis which are influenced by plant hormones and other factors that are supplied to the culture medium. Somatic embryogenesis is the process by which somatic cells, under induction conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes (Quiróz-Figueroa *et al*., 2006) while organogenesis involves the formation of adventitious organs from undifferentiated cell mass by the process of differentiation.

In a study conducted by Hou and Jia (2004), an efficient and reproducible system for plant regeneration from protoplasts isolated from embryogenic calli of *A. melilotoides* was done. They were able to regenerate plantlets from protoplast-derived calli simultaneously through somatic embryogenesis and organogenesis. Also, plant regeneration from 'Rangelander' alfalfa protoplasts using a protocol defined for alfalfa cell suspension-derived embryogenesis was reported (Monteiro *et al.*, 2003). Masani *et al.* (2014) studied the transformation of oil palm protoplasts by DNA microinjection and they were able to regenerate transgenic microcalli expressing green fluorescent protein as a visible marker to determine the efficiency of transformation.

#### 2.2.3.1 Physiology and biochemistry of cell wall regeneration

Plant cells are contained in rigid complex walls consisting of polysaccharides, proteins, and/or lignin, which varies in composition and amount depending on the cell types (Zhong and Ye, 2007). The plant cell wall is characterised by a dynamic nature critical not only for cell division, enlargement, and differentiation, but also for intercellular communication, responses to environmental cue including biotic and abiotic stresses (Carpita and Mccann, 2000) Protoplasts are in a transient state and regardless of their direct culture environment will set off the synthesis of a new cell wall a few hours after isolation and eventually revert to a single walled cell (Tomar and Dantu, 2010). The ability of protoplasts to undergo divisions and thus regenerate into plants is reliant on several processes involved in cytodifferentiation. A very important step in gaining totipotency in protoplast culture is biosynthesis of the cell wall, as confirmed by previous studies (Meyer and Abel, 1975; Nagata and Takebe, 1970).

Cellulose and callose are 2 polysaccharides which are mainly significant in the process of cell wall regeneration. Cellulose (β-1, 4-glucan) is the most prevalent polymer in plant walls,

constituting 30–90% of structural polysaccharides (Lee *et al*., 2011). Cell wall regeneration around protoplasts varies in different species ranging from as soon as 10 min following transfer of cells to regeneration medium to 5 days (Amstel and Kengen 1996; Kwon *et al*., 2005; Yang *et al*., 2008). Tan *et al*. (2011) also noticed the initiation of wall synthesis at multiple sites indicating a novel mechanism for wall regeneration in protoplasts as compared to wall synthesis during cytokinesis.

During wall regeneration in rice suspension cells, Sharma and co-workers (2011) observed that the removal of the cell wall stimulates cell wall synthesis from multiple sites of a protoplast simultaneously and leads to chromatin reorganization. They also observed that the genes associated with cell wall-related functions, stress response and transcription were up-regulated. On the contrary, transcription factors and genes regulating cell cycle and nucleic acid metabolism were down-regulated. Protoplasts of numerous legume crops are considered recalcitrant to *in vitro* culture, mainly due to their limited ability to divide. Examples of such crops include yellow lupin (*Lupinus luteus* L.) and grass pea (*Lathyrus sativus* L.) where regeneration into plants using protoplast culture is still a challenge (Wiszniewska and Piwowarczyk, 2014). The structure of the cell wall and its dynamic changes may be precise indices of developmental competences and ability of the cell (Malinowski & Filipecki 2002). The reconstruction and alteration of the cell wall in the rate of resynthesis as well as in the composition, physical and chemical properties of the regenerated wall affect the mitotic activity of the cells (Wang *et al.,* 1991; Suzuki *et al.,* 1998). Where regeneration experiments are not going well, it is important to investigate the source of the problems. Transciptomic and proteomics approaches may be used to investigate the proteins that are down-regulated or up-regulated during the process.

#### 2.2.3.2 Protein and gene expressed during regeneration

A number of studies have worked on the proteomics analysis of cultured protoplasts to gain an insight into proteins involved in the process of cell wall regeneration. This proteomic approach usually makes use of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and matrix-assisted laser desorption ionization-time-of-flight/mass spectrometry (MALDI-TOF/MS) technologies. One of the steps involved in cell wall dynamics is the assembly and rearrangement of cell wall structures through the actions of extracellular proteins (Kwon *et al*., 2005). Majority of identified proteins that may be involved in protoplast profileration are part of four main cellular processes; energy metabolism, defence or stress response, secondary metabolism, and protein synthesis and folding (de Jong *et al.,* 2007). In cultured protoplasts of

Arabidopsis suspension cultured cells, several proteins encoded by AtXH11 and AtXYL1 were found to be expressed (Kwon *et al*., 2005).

Nishitani (1997) suggested that AtXTH11 may be involved in both wall assembly and restructuring during the cell wall regeneration and expansion process. Also several proteins that are associated with stress including a chitinase (At2g43610) and a β-glucanase (At1g66280) were identified. These defense proteins may be induced by various stresses that may be produced during protoplast preparations (Kwon *et al*., 2005). In the proteome analysis of cultured *Medicago trunculata* protoplasts, Caffeoyl-CoA O-methyltransferase (CCoAOMT), an enzyme believed to be involved in the biosynthesis of lignin was up regulated in the first 4 days of culture after which the expression was decreased. This upregulation suggests that this enzyme may be involved in primary cell wall formation (de Jong *et al*, 2007).

Arabinogalactan proteins (AGPs) are a family of highly glycosylated, hydroxyproline-rich glycoproteins implicated in various aspects of plant growth and development which include the initiation of differentiation processes. The response of cultured protoplasts may be linked to the presence of AGPs in the cell wall. It has been shown that AGPs play a role in the proper binding of components in the new wall (Rumyantseva, 2005) and also involved in changing the course of the *in vitro* development of plant cells and tissues (Wiśniewska and Majewska-Sawka, 2007; Shibaya and Sugawara, 2009). It is therefore, important to investigate the roles of these proteins in cell-wall regeneration and cell differentiation especially in cultures of recalcitrant species.

Even though, evidence indicates that some AGPs are involved in cell to cell communication and cell to matrix interactions during plant development, cell proliferation and somatic embryogenesis (Nothnagel, 1997), Gao and Showalter (1999) suggested that some types of AGPs could be markers of cells entering the apoptosis pathway. In the cultures of grass pea and lupin, which are known recalcitrant species, cells containing AGPs were predominantly degenerating. AGPs seem to play a certain role in triggering apoptosis (Rumyantseva, 2005).

### 2.2.4 Factors affecting protoplast culture and plant regeneration

There are a number of factors that affect cell differentiation, callus formation and plant regeneration in cultured protoplasts. There are several reports on the optimization and modification of these factors towards optimal plating efficiency and a successful protoplast to plant system in different plant species.

#### 2.2.4.1 Plant species and genotype

Genotype is one of the major factors that affect regeneration in different plant species (Mamman *et al.,* 2013). Protoplast differentiation and plant regeneration has been relatively easy to achieve in plant species like *Nicotiana tabacum* (*Nicotiana*, *Solanaceae*) even from different source tissues. Conversely in the *Gramineae* (grasses family), regeneration of protoplasts into a plant has been very difficult and challenging even after several attempts with numerous genotypes of many species. A number of leguminous plant species are also known to be recalcitrant to protoplast culture (Wiszniewska and Piwowarczyk, 2014). Dhir *et al.* (1992) reported significant differences in the plating efficiency of cultured protoplasts in fourteen different genotypes of Soy bean (Glycine max L. Merr.). It is a well-documented fact that even very small genetic difference leads to varying protoplast responses to culture conditions (Tomar and Dantu, 2010)

#### 2.2.4.2 Culture methods

Several culture methods have been used in the culture of isolated protoplasts from different plant species. These methods have also been confirmed to be influential on protoplast differentiation. Generally some of the methods that have been used are liquid medium/suspension, semi solidified medium (e.g agar and agarose) and the thin layer culture methods. Some of the methods are also combined as in the agarose bead method where protoplasts are embedded in agarose and the gel blocks are transferred into a liquid medium (Dhir *et al.,* 1992). Even though the liquid medium makes it easier to gradually change the osmolarity of the medium for protoplast growth, the protoplast tend to agglutinate and some even degenerate which adversely affect the dividing protoplasts. Phansiri *et al.* (2001) reported that the use of agarose prevented the agglutination of protoplast which favours cell division and callus formation in *Broussonetic papyrifera*. The agarose bead method also facilitates the removal of used medium and frequent addition of fresh liquid medium without damaging the protoplasts (Tan *et al*., 1987). In the protoplast culture of *C. wenditii* the culture methods influenced significantly the plating efficiency and survival rate of cultured protoplast, even though protoplasts were able to divide in liquid and the agarose bead culture, no colony formation was observed when liquid medium was used. Other culture methods that have been exploited are hanging drop method (Kao *et al.,* 1970), multi drop array technique (Potrykus *et al.,* 1979) and the micro-culture technique which was used successfully by Durand *et al.* (1973) to culture protoplasts of *Petunia*.

#### 2.2.4.3 Culture media

Protoplasts from different species and from different tissues of the same species may vary in their nutritional requirements (Mac´kowska *et al*., 2014). Thus, the optimum medium for long-term culture must be determined empirically (Davey *et al.,* 2005). Nutritional requirements for the growth of protoplast differ from that of other cells in culture. There is loss of metabolites from protoplast during culture due to the absence of cell wall (Tomar and Dantu, 2010), hence, to make up for these losses of metabolites, a culture medium supplemented with increased metabolite is necessary for a successful protoplast culture (Kao and Michyaluk, 1975). Generally the culture media should contain macro and micro elements, vitamins, growth regulators and sugar (Nitch and Nitch, 1969). Carbon source is very important in any culture medium for callus embryogenesis. Media are usually based on the MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968) and the more complex KM8P medium containing coconut milk (Kao and Michayluk, 1975).

#### 2.2.4.4 Plating density

The plating density of protoplasts in the culture medium needs to be optimized and standardized as it affects the survival of the protoplasts and plating efficiency. It is very important for optimal wall regeneration and concomitant daughter cell formation (Davey *et al.*, 2005). Kiełkowska and Adamus (2012) also reported that the optimal plating density of protoplasts has an influence on the division ability of the cells and the formation of microcalli. A very high plating density rapidly depletes nutrients as protoplasts compete with one and another, while at very low density, loss of metabolites from protoplast is more (Tomar and Dantu, 2010) and consequently, protoplasts fail to undergo sustained division. Generally, the optimum plating density is in the range $5\times10^4$–$1\times10^6$ protoplasts per ml (Davey *et al.*, 2005). Tahami *et al. (*2014) reported that the plating density of protoplast influences the initiation of cell division in *Lilium ledebourii*.

#### 2.2.4.5 Type, combination and concentration of plant growth regulators

The addition of plant growth hormones to culture media can also influence the profileration of cultured protoplasts. Callus formation and shoot regeneration from cultured protoplasts require proper balance of plant growth hormones in the culture medium that must be modified for each species of plant. Usually protoplast culture medium is supplemented with a combination of auxin and cytokinin. The type of plant hormones and the balance between auxin and cytokinin are important factors controlling organ differentiation (Jomori *et al*., 1995). Growth of protoplast is reliant on the interaction between naturally occurring endogenous growth substance and growth regulator added to the medium (Pongchawee *et al*., 2006). Al Maarri *et al.* (2014)

reported that protoplasts of potato grown on hormone free medium did not undergo any division but turned brown and died. The nutritional and hormone requirements of cultured protoplasts are constantly changing depending on the stage in the regeneration process (Tomar and Dantu, 2008).

### 2.2.5 Application of protoplast technology

#### 2.2.5.1 Somatic hybridization

Protoplast fusion is a physical phenomenon during which two or more protoplasts come in contact and adhere with one another either spontaneously or in presence of fusion inducing agents (Verma *et al.,* 2008). A number of recent papers have described the generation of unique plants through somatic hybridisation by protoplast fusion, together with aspects of the procedures involved in this technology, including the selection of hybrid cells and plants (Davey *et al*., 2000, 2004). In addition to isolation and fusion of protoplasts, the process of somatic hybridisation also involves the selection of somatic hybrids and culture of somatic hybrids to generate a complete desired plant (Tomar and Dantu, 2010). Protoplast fusion has become a significant means of gene manipulation because it overcomes the barriers of sexual incompatibility imposed by conventional mating systems. Protoplast fusion technique has a great potential for genetic analysis and crop improvement approaches (Muralidhar and Panda, 2000).

Fusion of protoplast can be spontaneous or induced. Spontaneous induction hardly occurs between protoplasts because isolated protoplasts carry negative charge may makes them very likely to repel one and another (Verma *et al.,* 2008). Induced Fusion can be through chemical inducing agents commonly known as fusogens. Common fusogens include Sodium nitrate, PEG and Calcium ions. Keller and Melchers (1973) developed a method to effectively induce fusion of tobacco protoplasts at a high temperature ($37^{o}C$) in media containing high concentration of $Ca^{+}$ ions at a highly alkaline condition (pH 10.5). Another method of fusion which is more reproducible and less cytotoxic is the electrofusion. Electrofusion involves the use of mild electrical stimulation to fuse protoplasts. Two glass capillary microelectrodes are placed in contact with the protoplasts. Application of high intensity electric impulse ($100kv\ m^{-3}$) for some microseconds results in the electric breakdown of membrane and subsequent fusion.

Somatic hybridisation can lead to the production of hybrids or cybrids. Hybrids usually contain the somatic chromosome of both the parental species or sometimes preserve only the genetic material of one parent organism while cybrids are produced by fusion of one specie with another, having enucleate protoplast or loss of chromosomes of one parent by repeated

mitotic division. The different level of fusion and recombination involved in somatic hybridization of isolated protoplasts helps in the production of a new, desired or even better plant species.

#### 2.2.5.2 Genetic transformation

Macromolecules (DNA, RNA and proteins) can be delivered into protoplasts by various techniques such as PEG–calcium fusion, electroporation and microinjection. Transient gene expression assays using protoplasts are versatile tools for gene expression studies (Abel and Theologies, 1996). Efficient systems for transient gene expression using protoplast have been developed for a wide range of plant species which include but not limited to *Arabidopsis thaliana* (Hoffman *et al.*, 1994), maize (Sheen, 2001) and rice (Bart *et al.*, 2006). Guo *et al.* (2000) electrofused protoplasts of cell suspensions of Bonnaza navel orange (*C. sinensis*) with mesophyll protoplasts from seedless Red Blush grapefruit (*C. paradisi*) to generate tetraploid and diploid plants. Treatment of protoplast-plasmid mixtures with PEG and/or electroporation is the approach normally exploited to induce DNA uptake into protoplasts. Garcia *et al.* (1998) isolated protoplasts from stems and leaves, used PEG to induce DNA uptake and detected transient gene expression by a spectrophotometric GUS assay. Dhir and co-workers (1998) electroporated DNA into petiole protoplasts and also demonstrated expression of the GUS gene.

#### 2.2.5.3 Proteomic studies

A proteome approach provides new insights to analyze the complex functions of model plants and crop species at different levels (Xi *et al*., 2006). Proteomics is a powerful means that is used in revealing the protein complement of subcellular organelles and to get new insights into intracellular protein sorting and biochemical pathways (Xi *et al*., 2006). It allows for separation and identification of hundreds proteins which has been very useful in investigating protein expression (Wang *et al*., 2010). Expression of proteins differ depending on the particular species, variety, stage of growth, organ and tissue in particular environment and the expression profile is closely related to the function of proteins (Xi *et al.*, 2006). Fontes *et al*., (2010) reported that protoplasts and vacuoles from grapevine could be useful as models for both basic research and biotechnological approaches which include proteomics, solute uptake and compartmentation, toxicological assessments and breeding programs.

A known method for proteomics is 2-DE, a technique that was started by O' Farrell more than twenty years ago (O' Farrell, 1975; Xi *et al*., 2006). 2-DE gel image analysis is very important in the field of proteomics and can be used to identify and characterise many forms of a particular protein encoded by a single gene (Xi *et al*., 2006). In a study conducted by Wang *et*

*al*. (2010), useful information on the performance of growth, development and resistance of a citrus diploid cybrid plant produced by protoplast fusion between Satsuma mandarin and pummelo were provided through the proteome of leaves. The differentially expressed proteins in the study suggested an alteration in photosynthesis, stress resistance and metabolism in the cybrid plant. Proteomic analysis of *Nicotiana tabacum* protoplast culture medium for 24 hours showed relations among regulatory elements of exocytotic processes (Ul-Rehman *et al.*, 2011).

The proteome study of the proliferation of cultured *Medicago truncatula* protoplasts has been carried out to investigate molecular changes involved in protoplast proliferation (de Jong *et al*., 2006). The proteins that were identified in the study were part of four main cellular processes that may be involved in protoplast proliferation: energy metabolism, defence or stress response, secondary metabolism and protein synthesis and folding. Kwon *et al*. (2005) did a proteomic study on apoplastic proteins involved in regeneration of cell wall in *Arabidopsis thaliana* protoplast suspension-cultured cells and different sets of proteins with differing status of their post-translational modifications which include phosphorylation and glycosylation were identified. The study also showed a set of enzymes that were specifically involved in cell wall expansion and construction in suspension-cultured cells.

### 2.2.6 Reports of protoplasts isolation in some legumes

Protoplasts isolation and regeneration have been reported in different plant species including legumes. Vieira *et al.* (1990) was able to achieve whole plant regeneration from protoplasts isolated from seedling cotyledons of forage legume; *Stylosanthes guianensis, S. macrocephala and S. scabra.* Plants regeneration from protoplasts isolated from alfalfa (Atanassov and Brown, 1984; Monteiro *et al.,* 2003), *Lotus cornilatus* (Ahuja *et al.,* 1983; Pati *et al.,* 2005), *Onobrychis viciifolia* (Pupilli *et al.*, 1989), soy bean (Dhir *et al*. 1992) and *Astragalus melilotoides* (Hou and Jia, 2004) have also been achieved. In other way, protoplasts of numerous legume crops such as the grass pea and yellow lupin are considered recalcitrant to *in vitro* culture because of their limited ability to achieve sustained division. In recent researches, attempts are being made to enhance the regeneration ability of protoplasts of legumes through the selection of the optimal donor explants and optimization of the isolation and culture conditions (Sinha *et al*., 2003; Wiszniewska and Pindel, 2009).

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 Seeds Germination and Growth

Bambara groundnuts were germinated both in the greenhouse and in the laboratory to determine the best germination and growth method for optimal protoplast isolation.

#### 3.1.1 Green house germination

Three (3) seeds were planted in each pot of potting mix composed of fine composted pine bark, coarse sand and coco peat. The pots were placed in a glasshouse maintained at a temperature of 28.5 ± 2$^{o}$C and watered every 48 hours, the seeds germinated within 10-12 days of planting.

#### 3.1.2 *In-vitro* germination

The germination was done after surface sterilization of the seeds with 70% ethanol for 2 minutes and Jik® containing 4% Na-hypochlorite for 20 minutes. They were rinsed three times with sterile distilled water and incubated in darkness at 25°C after plating on plain agar (0.8%) in sterile plastic containers. After 5 days, the germinated seedlings were transferred into a culture bottle containing Murashige and Skoog medium (MS medium) (Murashige & Skoog, 1962) in agar for growth at 25°C in the light. The pH of the culture medium was adjusted to 5.7 ± 0.1 before the addition of agar and sterilised by autoclaving at 121ºC for 15 minutes.

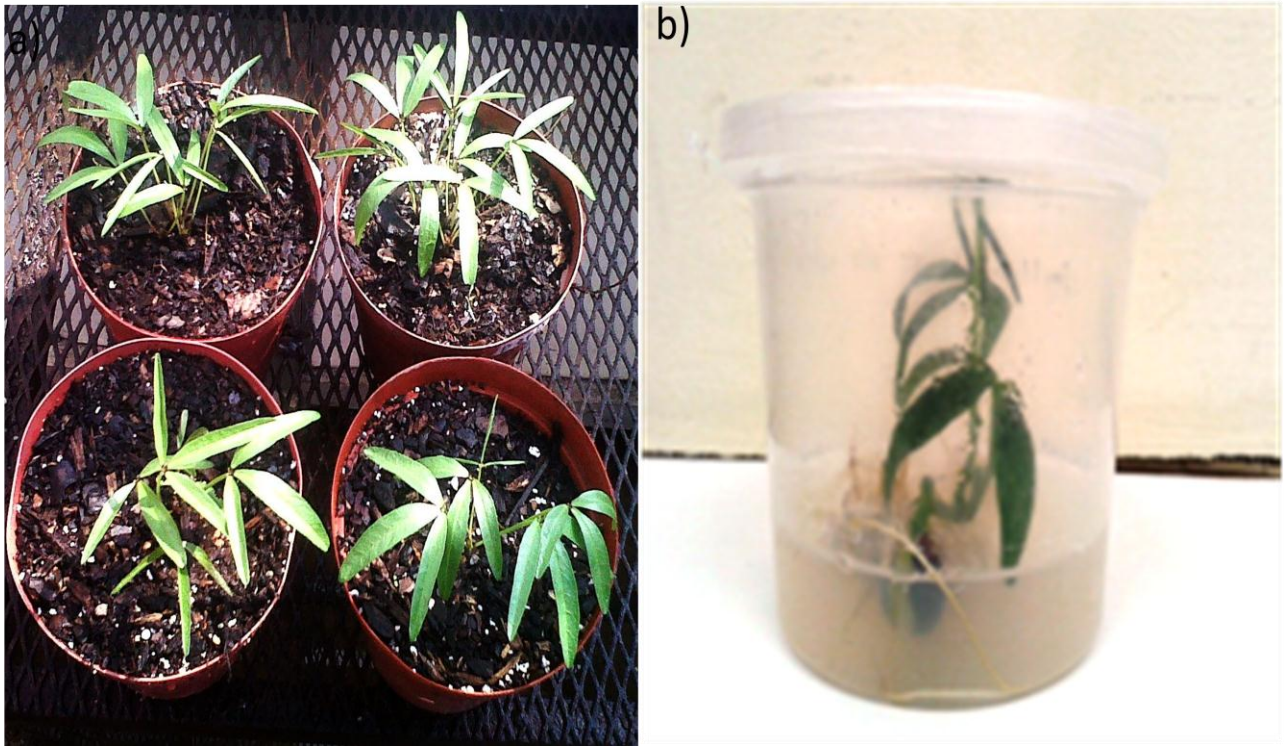


Figure 3.1: Bambara groundnut plant growing in (a) potting soil and (b) on agar

### 3.2 Protoplast preparation

Both mechanical and enzymatic methods of isolation were carried out;

#### 3.2.1 Mechanical isolation

The leaves of the laboratory and green-house germinated Bambara groundnut plant were ground in isolation buffer with minimal force in a mortal. Samples from the resulting solution were checked for released protoplasts.

#### 3.2.2 Enzymatic Isolation

This was carried out using twelve different concentrations of enzyme combinations containing digestive enzymes; cellulase 'Onozuka' R10 (Yakult Honsha Co Ltd, Japan), pectinase (Sigma Aldrich) and macerozyme R-10 (Yakult Honsha Co. Ltd, Japan) as shown in Table 3.1. Other factors that can influence the release of protoplast which include age of plant leaves, period of incubation, molarity of osmoticum were also varied and their effects investigated.

Using the method of Sheen (2001) with some modifications, leaves at the different ages of the plant (4, 6, 8 and 10 weeks) were incubated in the buffer-enzyme solution. The buffer solution consists of osmoticum (mannitol) of varying osmolarity (0.4, 0.5, 0.6 and 0.7 M), 20 mM Calcium chloride ($CaCl_2$), 40 mM Potassium chloride (KCl) and 10 mM monohydrate buffer (MES) PH 5.7. Green-house grown leaves were surface sterilized in 70% ethanol for 30 seconds, and Jik® containing 1.5% hypochlorite for 10 minutes. They were rinsed three times with autoclaved distilled water to remove any trace of sterilizing agents. Leaves epidermis were scraped off gently with a blade, they were thereafter sliced thinly in a petri dish with brand new 22 inches blades (figure 3.2) used blade was being replaced with a new one after cutting 2-3 leaves, this is to ensure smooth cutting of the leave tissue to avoid damage to the mesophyll cells. The enzymes were added to the buffer solution and placed in a water bath at $55^{o}C$ for 10 minutes to enhance enzyme solubility (Yoo *et al.,* 2007). The enzyme-buffer solution was sterilized with a 0.22 µm syringe filter unit. In each petri dish, 10 ml of the filter sterilized buffer-enzyme solution was added to the thinly cut sections of the leaves. The petri dishes were covered with parafilm and incubated at room temperature in the dark for protoplast release. Incubation was done at different time intervals (4, 18, 24 and 42 hours) to determine the optimal time for the release of protoplasts. Isolation was carried out in a sterile environment provided by a laminar flow unit, all equipment and material used were autoclaved and sterilized as necessary to prevent contamination. The experiment was carried out twice (each in triplicates).

Table 3.1: Component of enzyme mixtures used for enzymatic isolation

| Enzyme mixtures | Cellulase Onzuka R-10 % w/v | Macerozyme R-10 % w/v | Pectinase % w/v |
|---|---|---|---|
| 1 | 1.0 | 0.5 | |
| 2 | 2.0 | 0.5 | |
| 3 | 4.0 | 0.5 | |
| 4 | 1.0 | 1.0 | |
| 5 | 2.0 | 1.0 | |
| 6 | 4.0 | 1.0 | |
| 7 | 1.0 | | 0.5 |
| 8 | 2.0 | | 0.5 |
| 9 | 4.0 | | 0.5 |
| 10 | 1.0 | | 1.0 |
| 11 | 2.0 | | 1.0 |
| 12 | 4.0 | | 1.0 |

Figure 3.2: Scraped leaves' epidermis of Bambara groundnut leaves

### 3.2.3 Protoplast Purification

After incubation, the petri dishes were agitated gently and briefly to release isolated protoplasts, the protoplast containing enzyme-buffer solution was diluted with equal volume of washing solution (125 mM $CaCl_2$, 154 mM NaCl, 5 mM KCl and 2 mM MES PH 5.7) after which the released protoplasts were collected by sieving through 8 layers of cheese cloth, the protoplast containing solution was centrifuged at 800 rpm for 5 minutes and the pellet was resuspended in the washing solution. The resuspended pellet was overlayed on different sucrose concentrations (10%, 15%, 20%, 30%, 40% and 60%) to make a gradient (figure 3.3). Protoplasts were collected at the interface of different sucrose concentrations to locate and retrieve purified protoplasts. A sample of the collected protoplast was viewed and photographed under the microscope (×40).

### 3.2.4 Protoplast quantification and viability test.

Protoplasts were quantified by the use of haemocytometer (Gleddie, 1995). Viability of the protoplasts was examined by Trypan blue staining assay (Chamani *et al.*, 2012). Equal volume of 0.04% trypan blue in buffer solution was mixed with 100µl protoplast suspension, about 10µl of trypan blue/cell mix was pipetted on to a Neubauer Hemocytometer (Reichert, USA) with cover slips. The protoplast suspension was allowed to fill the entire chamber. The filled heamocytometer slide was viewed under the light microscope at 100x magnification. Trypan blue was excluded by living protoplasts whereas dead protoplasts and cell debris were stained a deep blue color. The number of unstained cells (viable protoplasts) was counted.

The concentration of protoplasts per ml per gram of leaves was determined by the formula; **Average number of cells in one large square /weight of leaves material used × dilution factor × $10^{4.}$**

Percentage viability was also determined as; **No of Viable Cells Counted / Total Cells Counted (viable and dead) × 100.**

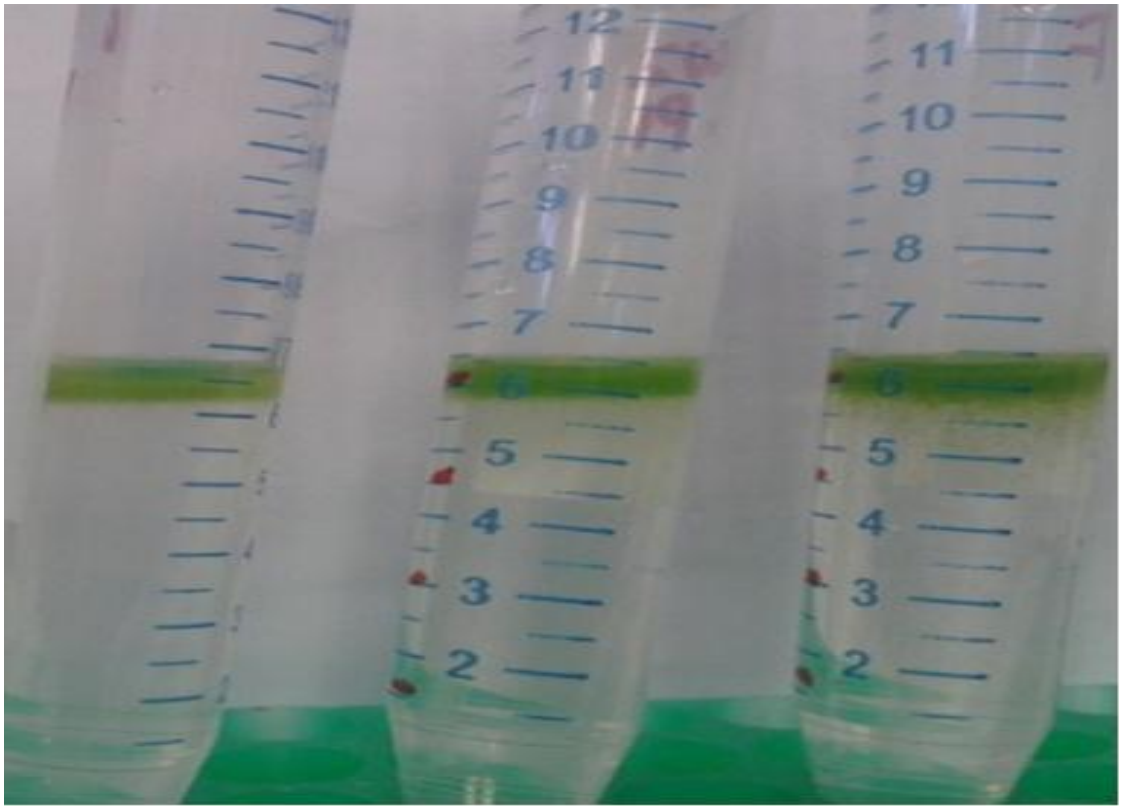


Figure 3.3: Protoplasts purification by layering on sucrose gradients

### 3.3 Protoplast culture

Protoplast culture was carried out following three different culture methods as described by Tahami *et al* (2014) with modifications. Freshly isolated and purified protoplasts were plated at different densities quantified by the use of a haemocytometer. The different culture methods evaluated were liquid MS medium, agar plating and agarose bead.

#### 3.3.1 Liquid Culture

Protoplasts were cultured in modified liquid MS medium containing 1% sucrose and 9% mannitol using the method of Tahami *et al*. (2014). Protoplasts were resuspended in 4 ml of media in small Petridishes (5.5 cm diameter).The petridishes were sealed with parafilm and covered with tin foil for incubation.

#### 3.3.2 Agar plating

Protoplasts were plated on MS medium containing 1% sucrose, 9% mannitol solidified with 0.8% agar in petri dishes.

#### 3.3.3 Agarose bead plating

The agarose bead method was carried using the method of Gandhi and Khurana (2001). Protoplasts were resuspended in liquid MS-medium supplemented with 1.2% low melting point agarose (Seakem® LE). The protoplast-agarose mixture was pipetted and dropped on to the petridishes. After solidification, the droplets were covered with 3ml of modified liquid MS medium. The petri dishes were sealed and incubated at room temperature in the dark and subsequently monitored daily for cell division. There were frequent addition of fresh liquid medium and removal of used medium, without damaging the protoplasts.

#### 3.3.4 Preparation of medium

Full (8.8g/L) and half (4.4g/L) strength MS medium containing 1% sucrose and 9% mannitol was prepared by dissolution of the required mass of reagent in appropriate volume of distilled water. For agar plating and agarose bead method, 0.8g/L of agar and 1.2% agarose was added to the medium respectively, the mixture was then gently heated with continuous stirring until complete dissolution. The culture media were adjusted to pH 5.7±0.1 by a pH meter with 0.1M NaOH or HCl as necessary and was sterilised by autoclaving.

#### 3.3.5 Stock preparation of hormones

Stock solutions (1mg/ml) of auxins (NAA and 2, 4-D) and cytokinin (BAP) used were prepared by dissolving in appropriate solvents (Table 3.2) before making up with distilled water to the final volume. They were filter-sterilised with a 0.22 µm filter unit and store at -20$^{o}$C.

### 3.3.6 Optimization of the culture medium

In all the three culture methods evaluated, full (8.8 g/L) and half (4.4 g/L) strength MS medium were used. The medium was supplemented with 1% sucrose, 9% mannitol and different concentration and combination of hormones (NAA, 2, 4-D and BAP). Yeast extract and casein hydolysate were also added to investigate their effect on protoplast division.

### 3.3.7 Optimization of plating density

Protoplasts were plated at densities ($1\times10^4$, $2\times10^4$, $1\times10^5$, $2\times10^5$, $1\times10^6$, and $2\times10^6$) to investigate the effect of plating density on protoplast differentiation and also determine the optimal density for the culture of bambara groundnut leaves protoplast. Plating densities were estimated by the use of a Nebauer haemocytometer.

### 3.3.8 Optimization of the culture conditions

Protoplast cultures were maintained in a growth chamber under a photoperiod cycle of 16/8h as light/dark provided by cool white fluorescent lights (13.5 µmol.m-2.$s^{-1}$) and also consistently in the dark to investigate the effect of light and dark periods on protoplast proliferation. Both conditions were maintained at 27±2°C.

Table 3.2: Solvents used for the dissolution of hormonal supplements

| Hormones | Solvents |
|---|---|
| NAA | 0.1N NaOH |
| 2,4-D | 70% Ethanol |
| BAP | 0.1N NaOH |

Table 3.3: Different supplementations in MS culture media

| Media | NAA mgL-1 | BAP mgL-1 | 2,4-D mgL-1 | Yeast Extract mgL-1 | Caseine hydrolysate mgL-1 |
|---|---|---|---|---|---|
| 1 | 1 | 0.5 | - | - | - |
| 2 | 1 | 0.5 | 0.2 | - | - |
| 3 | | 0.5 | 0.2 | - | - |
| 4 | 1 | 0.5 | | 200 | 250 |
| 5 | 1 | 0.5 | 0.2 | 200 | 250 |
| 6 | | 0.5 | 0.2 | 200 | 250 |

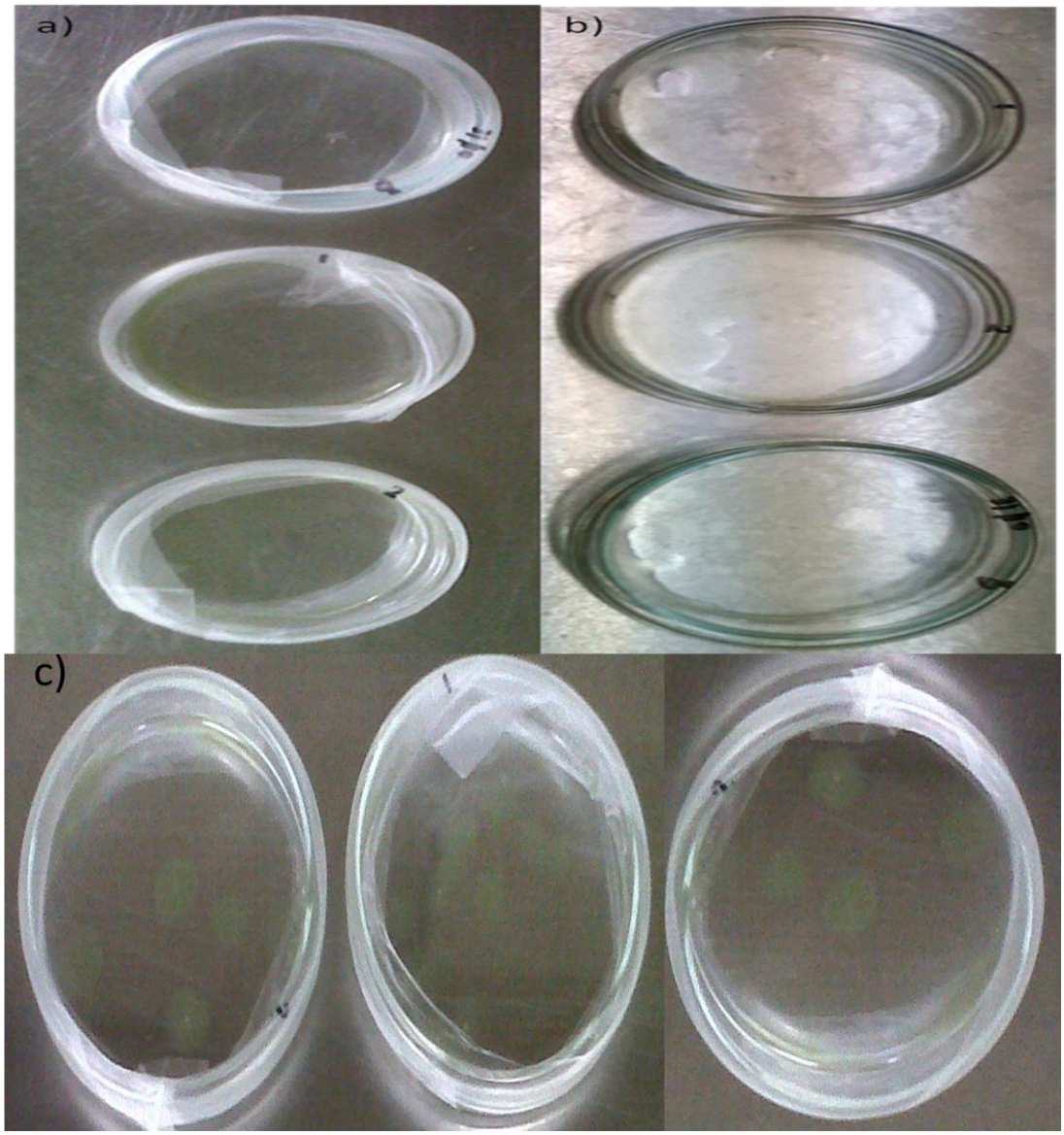


Figure 3.4: Bambara groundnut protoplast culture (a) on MS agar (b) in liquid MS medium (c) in agarose beads

### 3.4 Proteomics analysis

Protein profiling of freshly isolated protoplasts and cultured protoplasts in liquid medium were compared. Protoplasts were isolated as described earlier, for cultured protoplasts, isolated protoplasts were cultured at a density of $1x10^6$ in a liquid medium containing 1.0 $mgl^{-1}$ NAA 0.5 $mgl^{-1}$ BAP and 0.2 $mg^{-1}$ 2, 4-D. After 5 days in culture, the protoplasts containing medium was centrifuged at 800 rpm for 7 minutes to harvest protoplasts cells. They were subsequently washed twice by centrifuging in washing buffer at 800 rpm for 5 minutes. Both samples (freshly isolated and cultured protoplasts) were collected and transferred into a 2 ml eppendorf tubes.

#### 3.4.1 Protein extraction

Protein was extracted sequentially using the ReadyPrep™ sequential extraction kit from Biorad. The kit consists of Reagent 1, reagent 2, reagent 3, and reducing agent TBP to be used for three sequential stages of extraction. The first stage of extraction was done with Reagent 1 (40 mM Tris) after reconstitution by adding 50ml of deionised water. 0.5 ml of reagent 1 was added to both samples, this was vortexed vigorously for 5 minutes after which they were centrifuged on a bench top centrifuge at 5000 rpm for 10 minutes. The supernatants were retrieved and protein quantification was carried out.

The second stage of extraction was done with 1% of reducing TBP in Reagent 2 (8M urea, 4% w/v CHAPS, 40mM Tris and 0.2 %( w/v) Biolyte 3/10 ampholyte). The resulting pellet from the first stage of extraction was washed twice in extraction reagent 1 to remove residual medium soluble protein. 0.25ml of extraction reagent 2 was added to the washed pellet, the mixture was vortexed for 5 minutes and centrifuged at top speed until there was a firm pellet and clear supernatant. The supernatant was recovered and protein quantification was carried out.

The third stage of extraction was done with a 1% dilution of reducing TBP in Reagent 3 (10 ml of 5 M urea, 2 M thiourea, 2 % w/v CHAPS, 2 %(w/v) SB 3-10,40 mM Tris and 0.2 %(w/v) Biolyte 3/10 ampholyte). The resulting pellet from the third stage of extraction was washed twice in extraction reagent 2 to remove residual medium soluble proteins. 0.25 ml of extraction reagent 3 was added to the washed pellet, the mixture was also vortexed for 5 minutes and centrifuged at top speed until a hard pellet and clear supernatant was formed. The supernatant was retrieved and protein quantification was carried out.

Protein quantification of all the samples was carried out using the nano-drop machine and Bradford assay. Prior to use in 2D- analysis, samples were also loaded on the SDS- PAGE gel for qualitative analysis of the protein samples. All the protein samples from both freshly

isolated protoplasts and cultured protoplasts at the three stages of extraction were aliquoted, labelled accordingly and stored in -80$^{o}$C until further use in 2-D experiments.

### 3.4.2 Protein Quantification by Bradford assay

The Bradford reagent was filtered through a filter paper before use. Protein standards of 0.1-1.0 mg/ml were prepared by making a serial dilution of 1mg/ml stock of BSA. 0.1ml of each known standard, blank and each sample was added to 3ml of Bradford reagent, vortexed and incubated for 10 minutes. The absorbances at 595nm were measured with a UV-visible spectrophotometer and a standard curve was generated by plotting the absorbance of each BSA standard as a function of its concentration. The concentrations of the samples were then extrapolated from the standard curve using the equation “y=mx+b” where y= absorbance at 595nm and x= protein concentration.

### 3.4.3 SDS-PAGE gel analysis of samples

SDS-PAGE gels were prepared according to the method of Laemmli (1970) using a 12% resolving gel (Table 3.4) and 5% stacking gel (Tables 3.5). Electrophoresis was conducted in the mini-PROTEAN electrophoresis system (BIORAD Hercules, USA). Protein bands were visualised by staining the gels with Commasie Brilliant Blue-250 using the Fairbank staining technique (Fairbank *et al*., 1971).

Table 3.4: 12% SDS-PAGE Running gel

| Reagent | Volume |
|---|---|
| 30% Acrylamide stock | 4 ml |
| 0.8% SDS | 1.25 ml |
| 3M Tris Hcl PH 8.8 | 1.25 ml |
| 10% APS | 50 µl |
| 100% TEMED | 20 µl |
| Sterile Distilled $H_2O$ | 3.43 ml |
| Total | 10 ml |

Table 3.5: 5% SDS-PAGE Stacking gel

| Reagent | Volume |
|---|---|
| 30% Acrylamide stock | 800 µl |
| 0.8% SDS | 625 µl |
| 3M Tris Hcl PH 8.8 | 625 µl |
| 10% APS | 25 µl |
| 100% TEMED | 20 µl |
| Sterile Distilled $H_2o$ | 2.905 ml |
| Total | 5 ml |

### 3.4.4 IEF First Dimension run

The first dimension run was done on a Protean® i12™ IEF System machine with 7cm Readystrip™ IPG strips pH 3-7. Prior to the run, passive rehydration with the sample was carried out in a rehydration tray for 18 hours. The rehydration volume for each sample was prepared by diluting 100 µg of the respective sample in 125 µl of readyprep® rehydration buffer. The rehydration volume was pipetted along the lane of the rehydration tray; the strips were carefully laid gel-side down on the rehydration volume and covered with 2.0 ml of mineral oil to prevent evaporation of the samples. After rehydration, the strips were sparingly rinsed with sterile distilled water and gently blotted dry with a filter paper. They were then transferred on to a focusing tray on the IEF machine and focused under conditions shown in Table 3.6. After the focusing, the first dimension strips were equilibrated with equilibration solutions 1 and 2 (Table 3.7) for 15 minutes each on a rocking shaker before proceeding to the second dimension run on SDS- PAGE.

Table 3.6: Focusing conditions for loaded IPG strips

| Step | Voltage(V) | Ramp | Time | Units |
|---|---|---|---|---|
| 1 | 250 | Rapid | 0;15 | |
| 2 | 4000 | Gradual | 1;00 | |
| 3 | 4000 | Rapid | 15,000 | |
| 4 | 500 | | Hold | |

Table 3.7: Equilibration buffers

| Equilibration Buffer 1 | Equilibration buffer 2 |
|---|---|
| 6M Urea | 6M Urea |
| 30% Glycerol | 30% Glycerol |
| 2% SDS | 2% SDS |
| 50Mm Tris- Hcl | 50Mm Tris- HCl |
| 2% DTT | 2.5% 40% Acrylamide/Bisacrylamide |

### 3.4.5 Second dimension SDS-PAGE analysis

After equilibration, the second dimension was run on 10% running gel, the strips were placed on the gels, sealed with overlay agarose and run at 240V until the bromophenol blue reached the end of gel.

### 3.4.6 Fairbank Comassie brilliant blue staining

The gels were stained with Comassie blue according to the method of Fairbank *et.al.* (1971). Three staining solutions containing decreasing concentrations of Commassie blue and isopropanol with 10% acetic acid (Table 3.8) were prepared and used sequentially to stain the gels. Each gel was placed in a plastic pouch containing 100 ml of CBB staining solution A and heated for 2 minutes in a microwave or until boiling point. They were then placed on a rotary shaker to cool for 5 minutes at room temperature with gentle shaking. Fairbank staining solutions B and C were sequentially added and the process of heating and cooling was repeated.

### 3.4.7 Destaining of Comaassie brilliant blue

The destaining solution D (Table 3.8) containing only 10% acetic acid was added to the gels, they were also heated and cooled as described for the staining procedure. The process was repeated until a clear background was obtained on the gels.

### 3.5 Statistical analysis

All data collected from the experiments were subjected to statistical analysis. Data are expressed as mean ± S.D. (standard deviation). One-way analysis of variance (ANOVA) was used to test for significant differences at $P<0.05$.

Table 3.8: Component of Fairbank staining and destaining solutions (Fairbank *et al.,* 1971)

| Solution | CBB-R250 (%) | Isopropanol (%) | Acetic acid (%) |
|---|---|---|---|
| A | 0.05 | 25 | 10 |
| B | 0.005 | 10 | 10 |
| C | 0.002 | - | 10 |
| D | - | - | 10 |

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 Protoplast isolation, quantification and viability

Figure 4.1a shows freshly isolated protoplasts from Bambara groundnut leaves. Freshly isolated protoplasts were bright, spherical in shape and well separated. Figure 4.1b shows viable and non-viable protoplasts isolated from Bambara groundnut leaves. Trypan blue, a staining dye used to monitor the viability of the protoplasts was excluded from the living protoplasts but densely stained dead cells and cell debris. Yield and viability of protoplasts were measured and compared statistically at the different conditions and factors involved in the isolation of protoplasts.

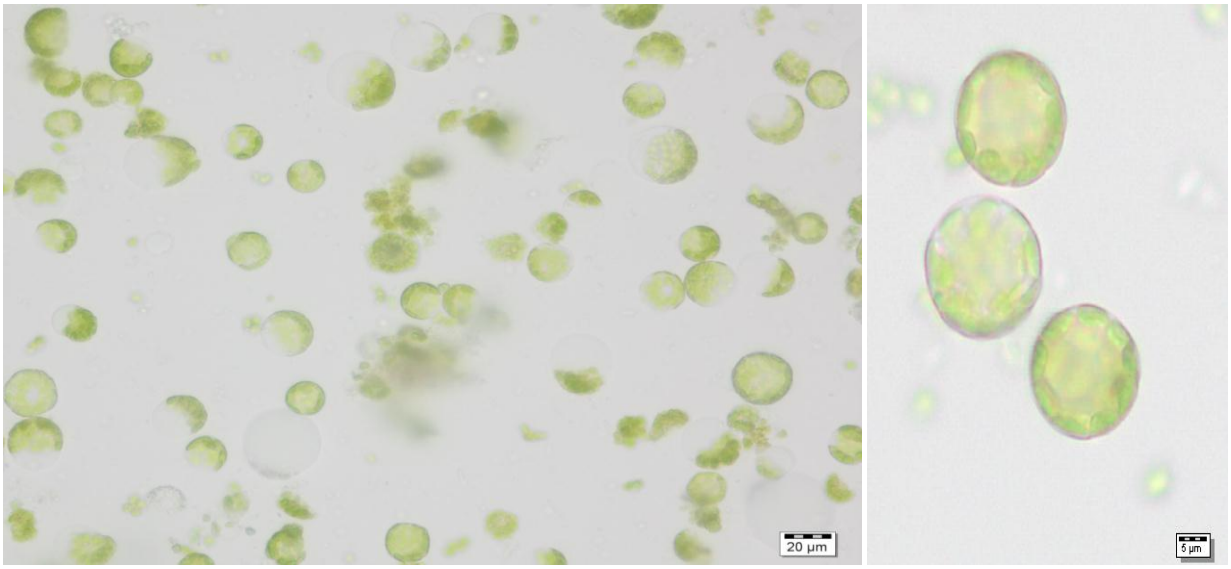


Figure 4.1a: Freshly isolated protoplasts from Bambara groundnut leaves as seen under the microscope (×40).

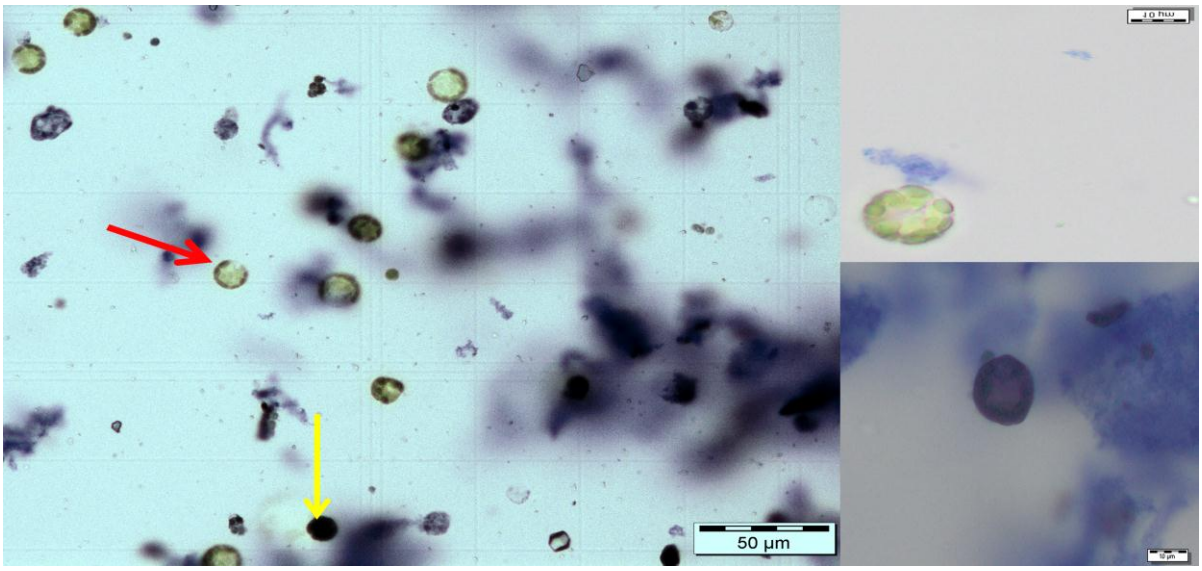


Figure 4.1b: Viable (red arrow) and non-viable protoplasts (yellow arrow) in 0.04% trypan blue solution on a heamocytometer as seen under the microscope (×40).

### 4.1.1 Effect of age of plant material on the yield and viability of isolated protoplasts

The effects of ages of plants on the yield and viability of protoplasts with 2% cellulase and 0.5% macerozyme in 0.5 M mannitol buffer solution for 18 hours in the dark are shown in figures 4.2a and 4.2b. The results showed that the yield of protoplasts when protoplasts were digested from leaves of four week old Bambara groundnut plant was significantly ($P<0.05$) higher than 6, 8 and 10 weeks. There was no significant difference in the protoplast viability in 4 weeks when compared with 6 weeks old leaves. The protoplast viability was significantly ($P<0.05$) higher in both 4 and 6 weeks when compared with 8 and 10 weeks. However, the results also showed that the viabiltity of protoplast in both 8 and 10 weeks were not significantly different from each other. Therefore, a four-week-old leaf was the appropriate age for protoplast isolation from Bambara groundnut plant.


Figure 4.2a: Effect of age of plant material (leaves) on the yield of protoplasts.


Figure 4.2b: Effect of age of plant material (leaves) on the viability of isolated protoplasts.

Bars having the same alphabets are not significantly different from each other while bars having different alphabets are significantly different from each other at $P<0.05$

### 4.1.2 Effect of incubation period on the yield and viability of isolated protoplasts

The effects of different incubation periods on the yield and viability of protoplasts isolated from the leaves of four weeks old Bambara groundnut plant with 2% cellulase and 0.5% macerozyme in 0.5 M mannitol buffer solution are shown in figures 4.3a and 4.3b. At 4 hours of incubation, there was a very low yield of protoplast when compared to 18, 24 and 42 hours of incubation. There were very minimal debris and broken cells at 4 hours due to a much reduced incubation period and hence, had higher viability of protoplasts which is not significantly different from 18 hours incubation. There was a significantly higher yield of protoplast at 18 hours although, not significant different when the incubation was extended to 24 hours and even up to 42 hours. Protoplast viability reduced at 24 hrs though not statistically significant and became significantly ($P<0.05$) reduced at 42 hours as most of the protoplast had broken or bursted thus leading to increased debris and chloroplasts. Therefore, in this study, 18 hours is the optimal age for protoplast isolation from Bambara groundnut leaves.

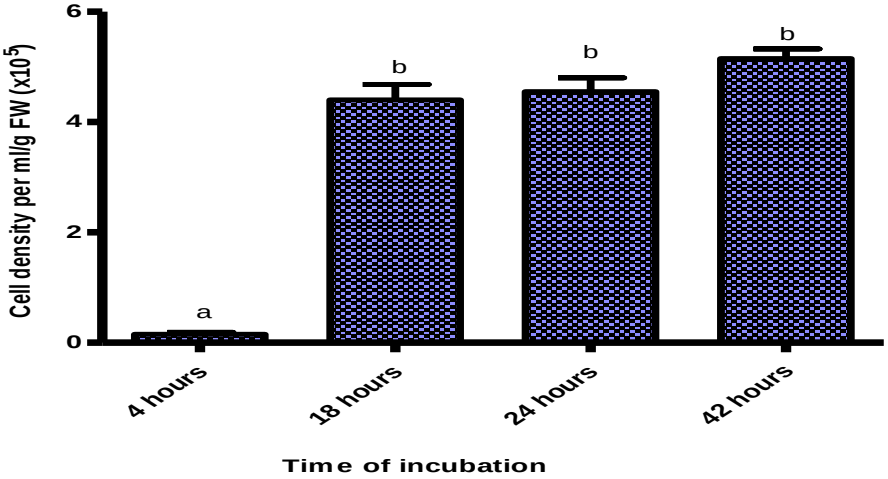


Figure 4.3a: Effect of incubation time on the yield of isolated protoplasts

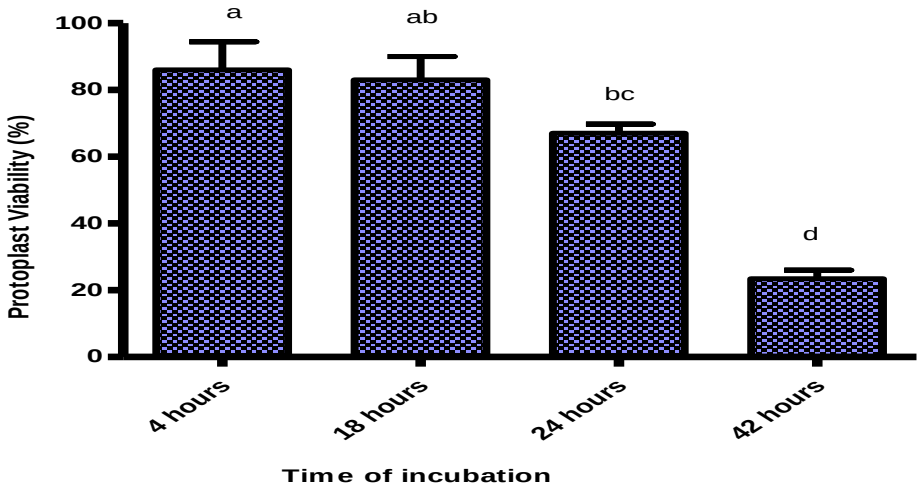


Figure 4.3b: Effect of incubation time on the viability of isolated protoplasts

Bars having the same alphabets are not significantly different from each other while bars having different alphabets are significantly different from each other at $P<0.05$

### 4.1.3 Effect of molarity of osmoticum (mannitol) on the yield and viability of isolated protoplasts

The effects of mannitol at different molarities on the yield and viability of protoplasts isolated from the leaves of four weeks old Bambara groundnut plant with 2% cellulase and 0.5% macerozyme are shown in figures 4.4a and 4.4b . At 0.4 M mannitol, the protoplast yield was significantly higher than 0.6 M and 0.7 M at $P<0.05$. However, no significant difference in protoplast yield was shown when 0.6 M was compared with 0.7 M. The protoplast yield and viability with 0.5M mannitol were significantly higher when compared with 0.4 M, 0.6 M and 0.7 M at $P<0.05$. The protoplast viability at 0.4 M was not significantly different from 0.6 M while both were significantly higher than 0.7 M at $P<0.05$. In this study, 0.5 M mannitol was the most suitable osmotic stabiliser for protoplast isolation from Bambara groundnut leaves.

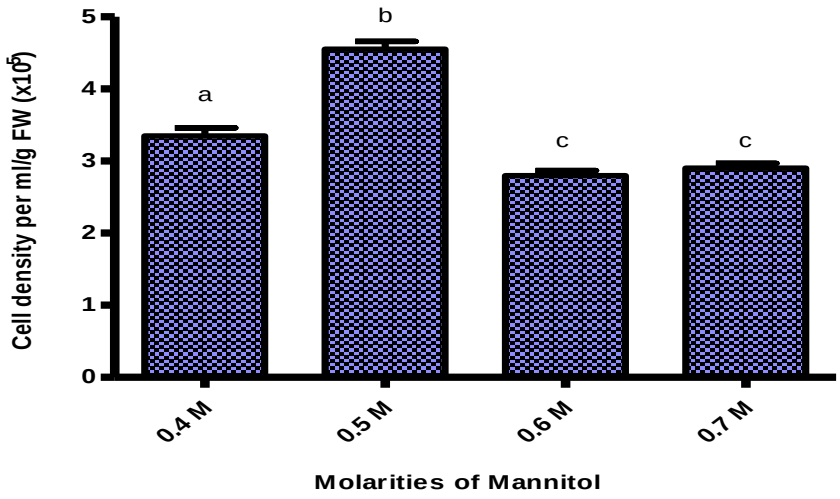


Figure 4.4a: Effect of molarity of mannitol on the yield of isolated protoplasts

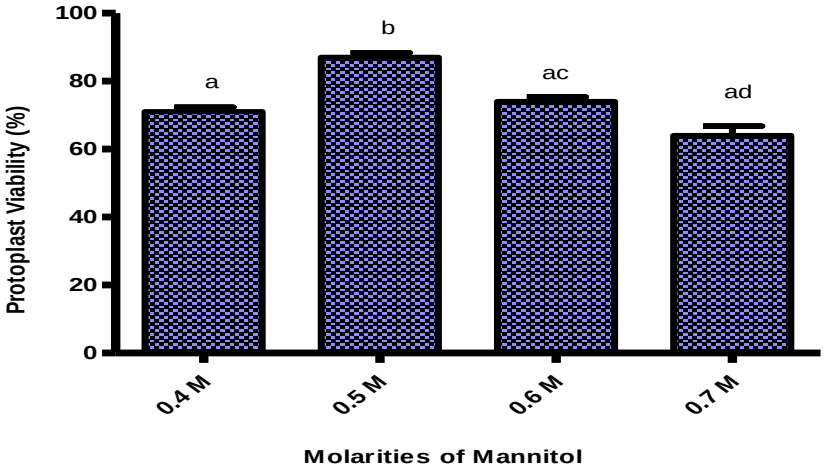


Figure 4.4b: Effect of molarity of mannitol on the viability of isolated protoplasts

Bars having the same alphabets are not significantly different from each other while bars having different alphabets are significantly different from each other at $P<0.05$

### 4.1.4 Effect of concentration and combination of enzymes on the yield and viability of isolated protoplasts

The effect of digestive enzymes on the yield and viability of isolated protoplasts greatly influenced by the length of incubation of source tissue in the enzyme-buffer solution. Hence, four weeks old leaves of Bambara groundnut plant were digested with different combination and concentration of enzymes (Table 3.1) in buffer containing 0.5 M mannitol at the different incubation periods.

#### 4.1.4.1 Effect of concentration and combination of enzymes at 4 hours of incubation

The yield and viability of protoplasts isolated with different combination and concentration of enzymes at 4 hours incubation are shown In figures 4.5a and 4.5b. The results showed that 4% cellulase and 0.5% pectinase which had the highest protoplast yield ($0.16 \pm 0.00 \times 10^5$ /ml/gFW) was not significantly different from protoplast yield at 2% cellulase and 0.5% macerozyme, 4% cellulase and 0.5% macerozyme, 2% cellulase and 1% macerozyme, 4% cellulase and 1% macerozyme, 1% cellulase and 1% pectinase and 2% cellulase and 1% pectinase, 4% cellulase and 1% pectinase. Furthermore, the results showed that protoplast yield at 4% cellulase and 0.5% pectinase was significantly ($P<0.05$) higher than protoplast yield at 1% cellulase and 0.5% macerozyme, 1% cellulase and 1% macerozyme, 1% cellulase and 0.5% pectinase and 2% cellulase and 0.5% pectinase. There was no statistical significant difference in the protoplast viability in all the enzyme concentrations and combinations used in this study at 4 hours incubation.

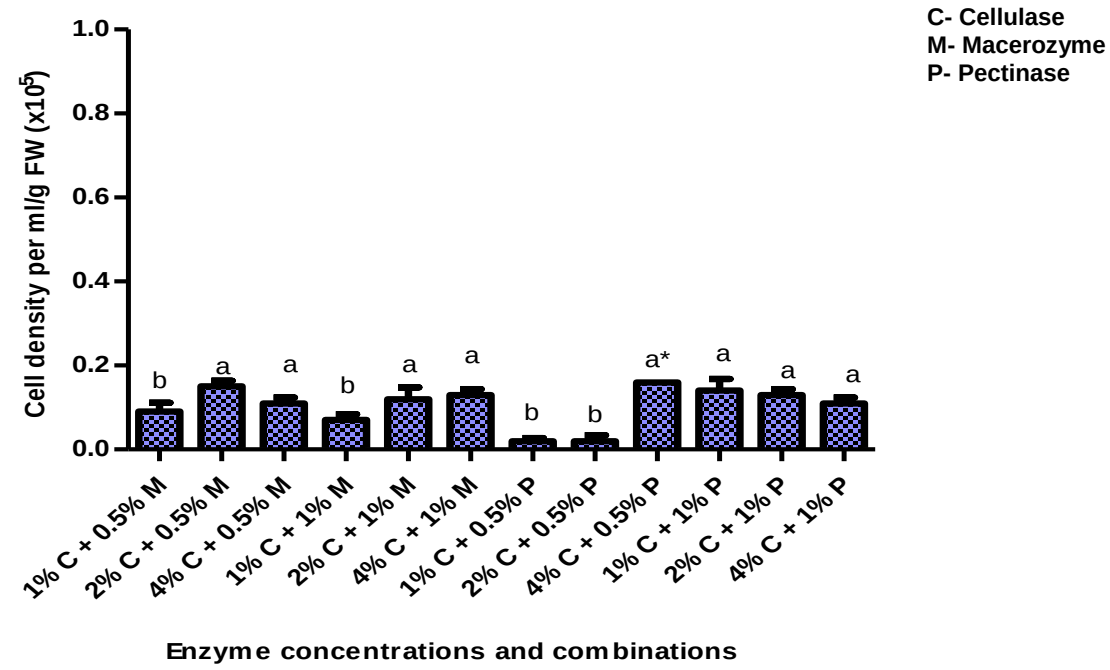


Figure 4.5a: Effect of concentration and combination of enzymes on the yield of isolated protoplasts at 4 hours of incubation

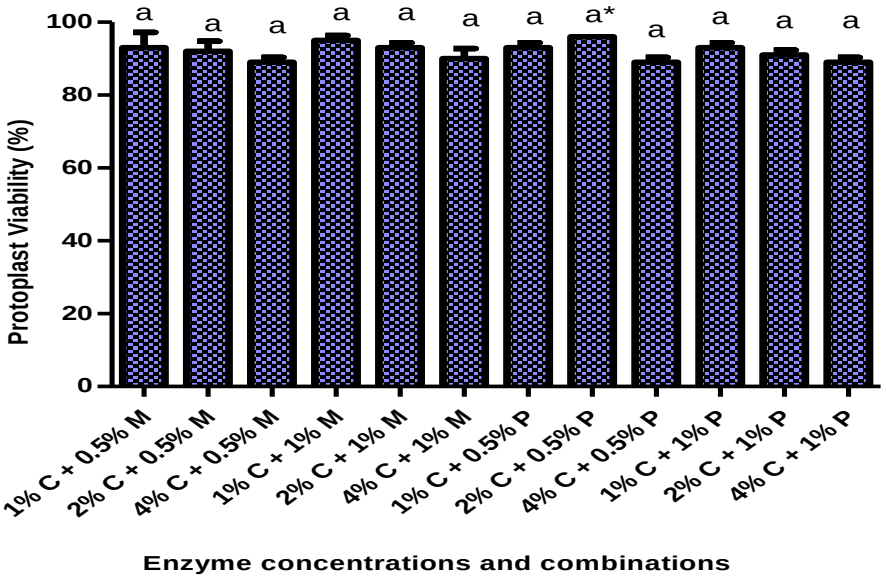


Figure 4.5b: Effect of concentration and combination of enzymes on the viability of isolated protoplasts at 4 hours of incubation

[a] is not significantly different from [a*] while [b] is significantly different from [a*] at $P<0.05$

#### 4.1.4.2 Effect of concentration and combination of enzymes at 18 hours of incubation

The yield and viability of protoplasts isolated with different combination and concentration of enzymes at 18 hours incubation are shown in figures 4.6a and 4.6b. The results showed that 2% cellulase and 0.5% macerozyme with the highest protoplast yield ($4.6 \pm 0.14 \times 10^5$/ml/gFW) was not significantly different from protoplast yield at 2% cellulase and 1% macerozyme, 4% cellulase and 1% macerozyme and, 4% cellulase and 0.5 % pectinase. Furthermore, the results also showed that protoplast yield at 2% cellulase and 0.5% maceroenzyme was significantly ($P<0.05$) higher than protoplast yield at 1% cellulase and 0.5% macerozyme, 4% cellulase and 0.5% macerozyme, 1% cellulase and 1% macerozyme, 1% cellulase and 0.5% pectinase, 2% cellulase and 0.5% pectinase, 1% cellulase and 1% pectinase, 2% cellulase and 1% pectinase, 4% cellulase and 1% pectinase. In addition, there was no significant difference in the protoplast viability at 2% cellulase and 0.5% maceronzyme which has the highest protoplast viability ($86.5 \pm 1.41\%$) at 18 hours incubation when compared with protoplast viability at 2% cellulase and 1% macerozyme and 2% cellulase and 0.5% pectinase. However, there was statistically significant difference ($P<0.05$) at 2% cellulase and 0.5% maceronzyme when comapared with the protoplast viability at 1% cellulase and 0.5% macerozyme, 4% cellulase and 0.5% macerozyme, 1% cellulase and 1% macerozyme, 4% cellulase and 1% macerozyme, 1% cellulase and 0.5% pectinase, 4% cellulase and 0.5% pectinase, 1% cellulase and 1% pectinase, 2% cellulase and 1% pectinase, 4% cellulase and 1% pectinase

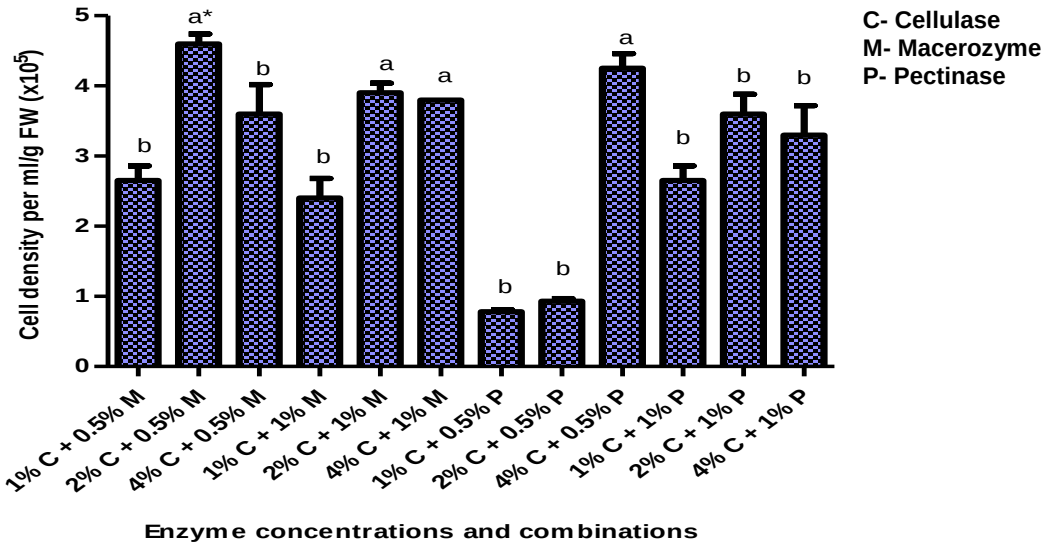


Figure 4.6a: Effect of concentration and combination of enzymes on the yield of isolated protoplasts at 18 hours of incubation

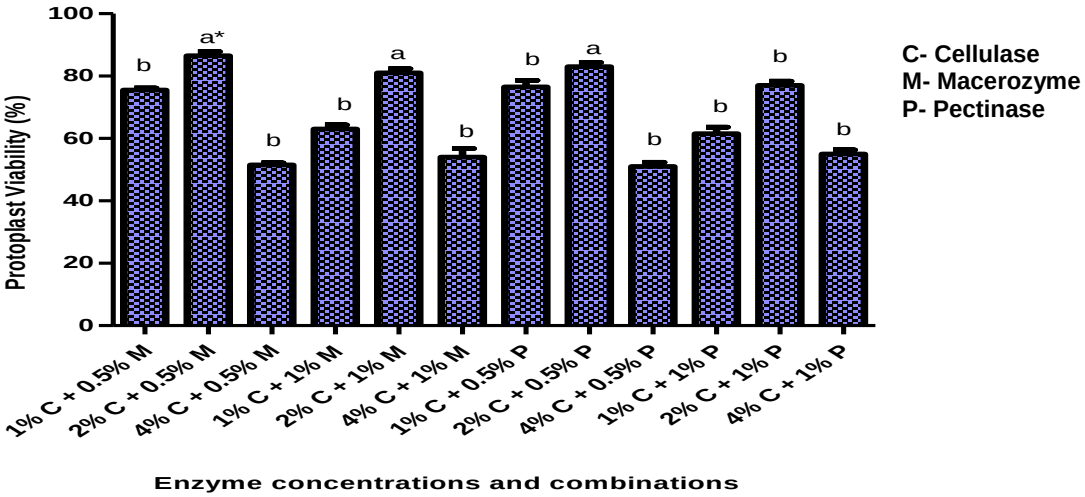


Figure 4.6b: Effect of concentration and combination of enzymes on the viability of isolated protoplasts at 18 hours of incubation

[a] is not significantly different from [a*] while [b] is significantly different from [a*] at $P<0.05$

#### 4.1.4.3 Effect of concentration and combination of enzyme at 24 hours of incubation

The yield and viability of protoplasts isolated with different combination and concentration of enzymes at 24 hours incubation are shown in figures 4.7a and 4.7b. The results showed that 2% cellulase and 0.5% macerozyme which has the highest protoplast yield ($4.80 \pm 0.14 \times 10^5$ /ml/gFW) was not significantly different from protoplast yield at 2% cellulase and 1% macerozyme and, 4% cellulase and 0.5% pectinase. Furthermore, the results showed that protoplast yield at 2% cellulase and 0.5% maceroenzyme was significantly ($P<0.05$) higher than protoplast yield at 1% cellulase and 0.5% macerozyme, 4% cellulase and 0.5% macerozyme and, 1% cellulase and 1% macerozyme, 4% cellulase and 1% macerozyme, 1% cellulase and 0.5% pectinase, 2% cellulase and 0.5% pectinase, 1% cellulase and 1% pectinase, 2% cellulase and 1% pectinase, 4% cellulase and 1% pectinase. From the results, although 2% cellulase and 0.5% macerozyme had the highest protoplast yield, the protoplast viability ($66.50 \pm 4.95\%$) was statistically significant ($P<0.05$) and lower than 2% cellulase and 0.5% pectinase which had the highest protoplast viability ($79.00 \pm 1.41\%$) but with poor protoplast yield ($1.30 \pm 0.14 \times 10^5$/ml/gFW) at 24 hours incubation.

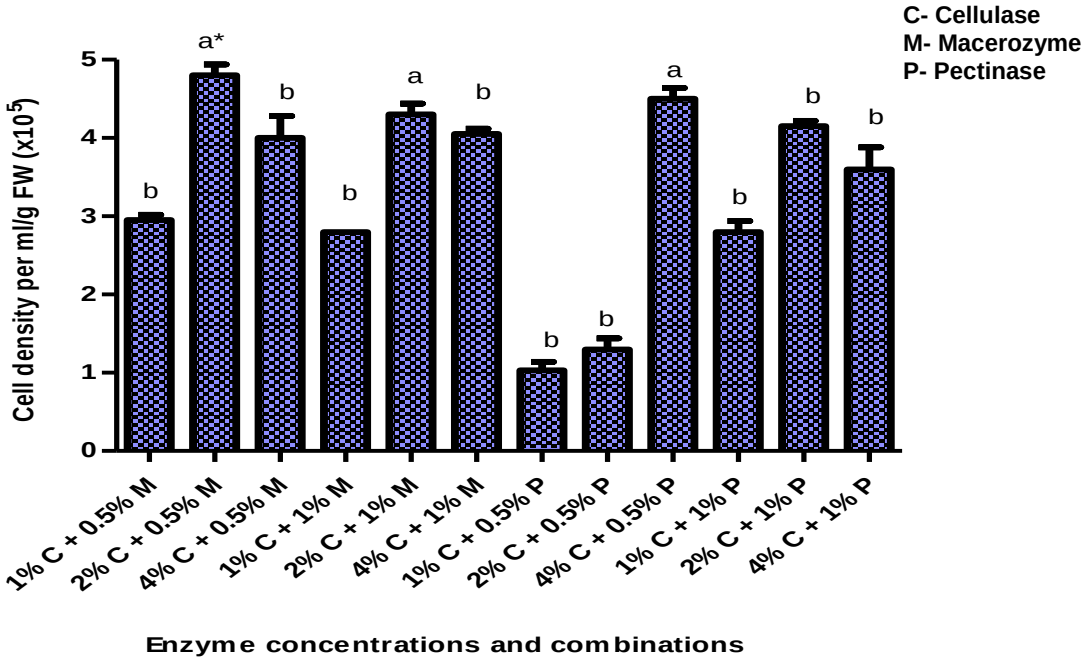


Figure 4.7a: Effect of concentration and combination of enzymes on the yield of isolated protoplasts at 24 hours of incubation

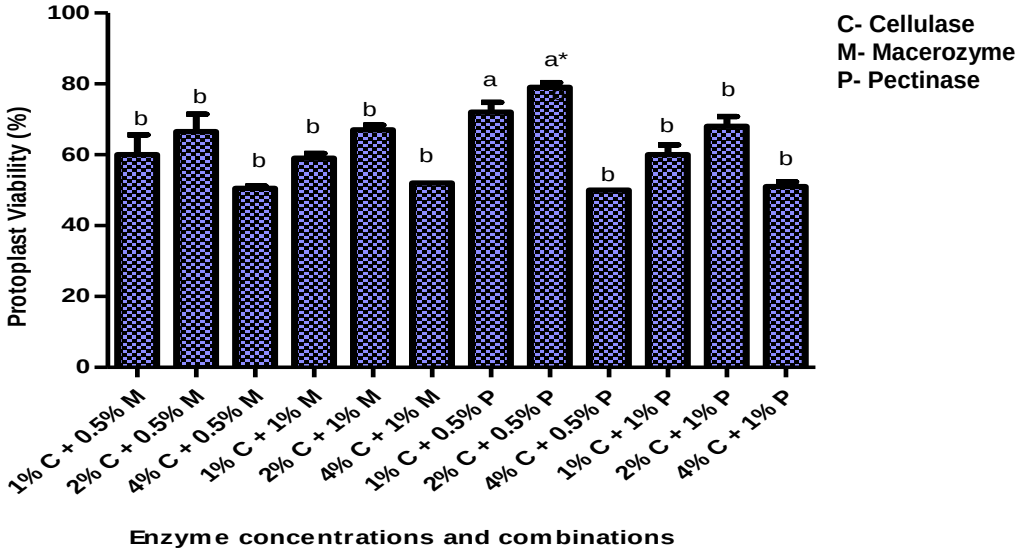


Figure 4.7b: Effect of concentration and combination of enzymes on the viability of isolated protoplasts at 24 hours of incubation

[a] is not significantly different from [a*] while [b] is significantly different from [a*] at $P<0.05$

#### 4.1.4.4 Effect of concentration and combination of enzymes at 42 hours of incubation

The yield and viability of protoplasts isolated with different combination and concentration of enzymes at 42 hours incubation are shown in figures 4.8a and 4.8b. The results showed that 2% cellulase and 0.5% macerozyme with the highest protoplast yield (4.95 ± $0.07\times10^5$/ml/g/FW) was not significantly different from protoplast yield at 4% cellulase and 0.5% macerozyme, 2% cellulase and 1% macerozyme, 4% cellulase and 1% macerozyme, 4% cellulase and 0.5% pectinase and, 2% cellulase and 1% pectinase. Furthermore, the results also showed that protoplast yield at 2% cellulase and 0.5% maceroenzyme was statistically significant ($P<0.05$) and higher than protoplast yield at 1% cellulase and 0.5% macerozyme, 2% cellulase and 0.5% macerozyme, 1% cellulase and 1% macerozyme, 1% cellulase and 0.5% pectinase, 2% cellulase and 0.5% pectinase, 1% cellulase and 1% pectinase, 4% cellulase and 1% pectinase. In addition, from the results, although 2% cellulase and 0.5% macerozyme had the highest protoplast yield, the protoplast viability (25.00 ± 1.41%) was statistically significant ($P<0.05$) and lower than 1% cellulase and 0.5% pectinase wihich had the highest protoplast viability (31.50 ± 0.70%) but with low poor protoplast yield ($1.30 \pm 0.09\times10^5$/ml/g/FW) at 42 hours incubation.

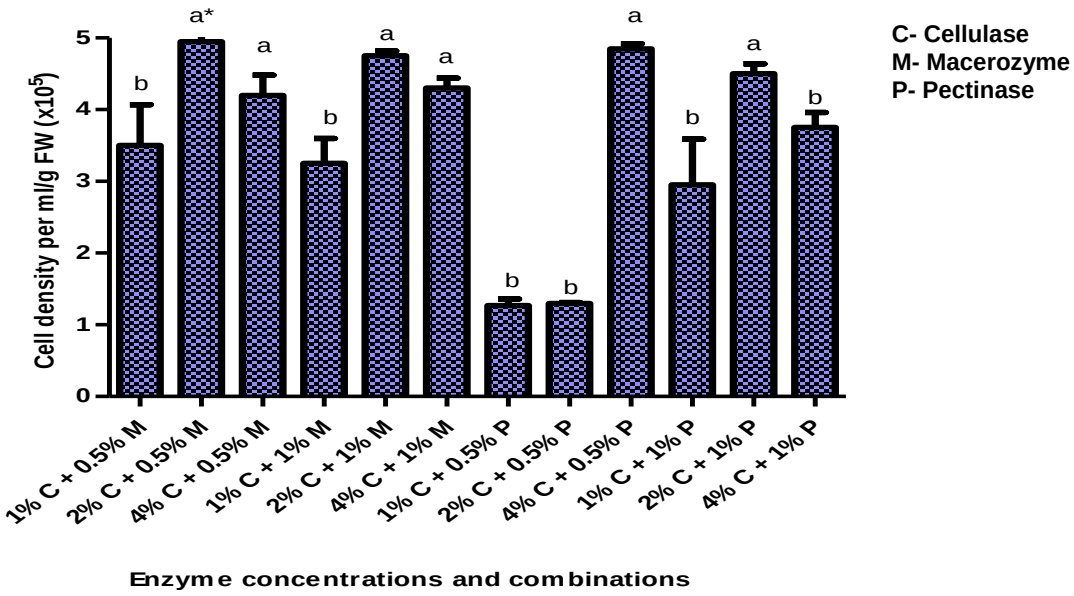


Figure 4.8a: Effect of concentration and combination of enzymes on the yield of isolated protoplasts at 42 hours of incubation

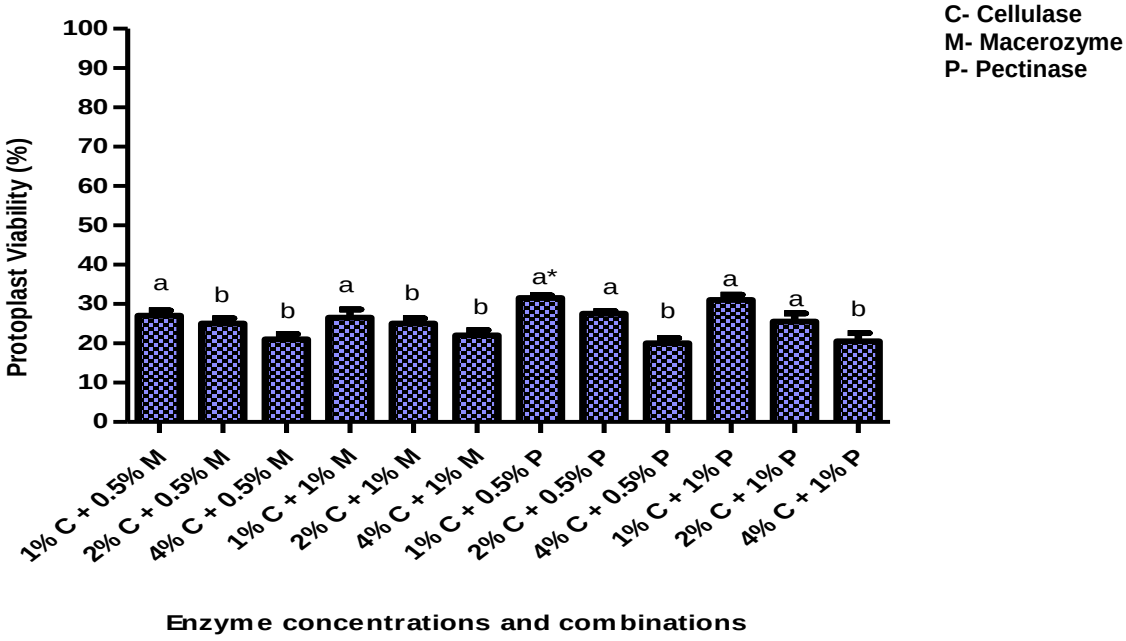


Figure 4.8b: Effect of concentration and combination of enzymes on the viability of isolated protoplasts at 42 hours of incubation

[a] is not significantly different from [a*] while [b] is significantly different from [a*] at $P<0.05$

### 4.1.5 Effect of germination and growth method on the yield and viability of isolated protoplasts

Table 4.1 shows the data obtained from the study when leaves sourced from plants grown in the green house and *invitro* in the laboratory using the optimum enzyme combination (2% cellulase and 0.5% macerozyme) and molarity (0.5 M) at different times of incubation was carried out. In the study, the result revealed no significant difference in both protoplast yield and viability. Digestion of leaves of Bambara groundnut plants from the laboratory germination for 18 hours showed consistent good yield and viability of isolated protoplasts with the data obtained from the green house germination.

Table 4.1: Comparative study of yield and viabilty of isolated protoplast from leaves of *in vitro* and green-house germinated Bambara groundnut plants using the optimum enzyme combination (2% cellulase and 0.5% macerozyme) and molarity (0.5 M) at different times of incubation.

| Incubation periods ( hours] | Green house protoplasts | Laboratory protoplasts | Green house protoplasts | Laboratory protoplasts |
|---|---|---|---|---|
| | Cell density per ml/g FW ($\times 10^5$) | | Viability (%) | |
| 4 | $0.10 \pm 0.00^{a*}$ | $0.15 \pm 1.99^{a}$ | $89.00 \pm 1.41^{a*}$ | $87.00 \pm 1.41^{a}$ |
| 18 | $3.15 \pm 0.07^{a*}$ | $3.70 \pm 0.92^{a}$ | $80.50 \pm 3.54^{a*}$ | $78.00 \pm 2.83^{a}$ |
| 24 | $3.30 \pm 0.14^{a*}$ | $3.40 \pm 1.10^{a}$ | $66.00 \pm 2.83^{a*}$ | $69.00 \pm 4.24^{a}$ |
| 42 | $5.15 \pm 0.21^{a*}$ | $5.25 \pm 0.21^{a}$ | $26.00 \pm 2.83^{a*}$ | $31.0 \pm 1.41^{a}$ |

$^{a}$ is not significantly different from $^{a*}$ in the same row for the yield and viability of isolated protoplasts at different incubation time.

**4.2 Protoplast culture.**

Figure 4.9 shows the protoplasts in culture undergoing cell division. This division was observed after 48 hours only in protoplasts cultured in the dark in liquid MS culture containing (1.0mgl-1 NAA and 0.5mgl-1 BAP, 0.2mg 2, 4-D). There was no further division beyond 48 hours. Cell division was not observed in other culture methods used in this study and cultured protoplasts died after 5 days in all the media. However regeneration experiments are still on going in parallel with proteomics studies to identify proteins whose expression correlate directly or inversely with the regeneration process (results not shown).

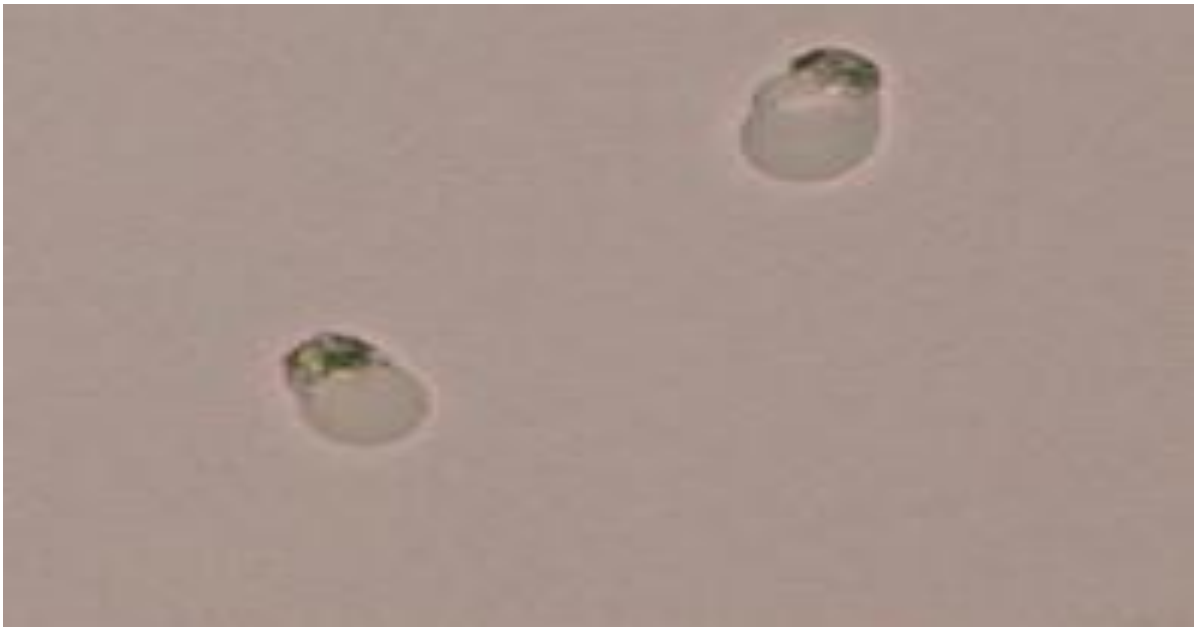

Figure 4.9: Protoplast in MS liquid culture initiating cell division

# CHAPTER FIVE

## 5.0 DISCUSSION

Development of protoplast technology has been given considerable attention and has attained noteworthy progress (Tee *et al.*, 2010). The use of protoplast systems has increased the usefulness of plants in both biochemical and genetic research (Rao and Prakash, 1995). Protoplast, among all the possible starting points for plant genetic manipulation gives the opportunity to take advantage of all the technologies now available (Rao and Prakash, 1995). Protoplasts can be isolated from plant tissues or cultured cells (Tahami *et al.,* 2014). Young seedlings are frequently used to isolate protoplasts from many plant species (Hong *et al.,* 2012). The physiological status of the source tissue affects the release of viable protoplasts (Davey *et al*., 2005). The individual cell or tissue source may involve special conditions for successful isolation or for culturing (Tahami *et al.,* 2014). In this study, the leaves of Bambara groundnut were used for protoplast isolation and this study is the first report of successful isolation of protoplast from Bambara groundnut. It has been reported that the most used sources of protoplasts are leaf mesophyll because they are commonly believed to give information about the cellular function of proteins that are usually expressed in other cell types (Sheen, 2001: Faraco *et al*., 2011).

Protoplasts are isolated primarily by mechanical or enzymatic methods. While the former is used only occasionally, it remains historically important and the later can be used to produce large quantity of protoplasts (Chamani *et al.,* 2012). Enzymatic isolation also produce less breakage and much less osmotic shrinkage than the mechanical isolation (Chamani *et al.,* 2012). In this study, both methods of isolation were used but the mechanical did not produce any protoplast unlike the enzymatic isolation that released good yield of protoplasts. The isolated protoplasts from Bambara groundnut leaves were bright, spherical in shape and well separated (Figure 4.1a). It is vital to determine optimal conditions to maximize yield and viability of protoplasts due to the fact that they are osmotically fragile and get ruptured easily during preparation procedures (Hong *et al.,* 2012). Several factors influence the protoplast release such as the extent of thickening of cell walls, temperature, duration of enzyme incubation, pH optima of the enzyme solution, gentle agitation, and nature of the osmoticum (Davey *et al.,* 2005). In this study, different conditions such as age of plant material, incubation time, osmotic stabiliser and combination/ concentration of enzymes were optimised in order to obtain large quantities of viable protoplasts. Trypan blue, a staining dye was used to monitor the viability of the protoplasts. Trypan blue is excluded from living protoplasts but densely stained dead cells and cell debris (Figure 4.1b).

It has been reported that the age of the source plants is vital for successful protoplast isolation (Guangyu *et al.,* 1997). There is difficulty in the isolation of protoplasts from older leaves because they contain thicker cuticle and more lignin accumulation in their cell walls whereas protoplasts isolated from too young leaves are also fragile due to the fact that young leaves do not contain thick deposit of cellulose or pectin (Lord *et al.*, 2010; Zhang *et al.,* 2011). Middle stage plants have a very thin cuticle and generally have low amounts of lignin and this leads to isolation of healthy protoplast (Lord *et al.*, 2010). This necessitated the comparison of the protoplast isolation from different ages of Bambara groundnut leaves i.e. 4, 6, 8 and 10 weeks in this study. The yield and viability of isolated protoplasts from the leaves of 4 weeks old Bambara groundnut plant was significantly higher ($p<0.05$) than 6, 8 and 10 weeks old plant. The results indicated that leaves of 4 week-old Bambara groundnut plant gave the optimal yield and viability of isolated protoplasts.

Osmotic values of the environment into which protoplasts are released are vitally important (Zhang *et al*., 2011). An osmotic stabiliser is necessary to give osmotic support to the protoplasts following the removal of cell wall (Tee *et al.*, 2010). The osmotic stabilizers play an essential role in the protection of the nascent protoplasts in different environments by supporting the protoplasts from being lysed (Babaoglu, 2000). The appropriate concentration of osmotic-pressure regulating agent can help in the prevention of protoplasts missing cell walls from bursting or shrinking (Zhang *et al.,* 2011). Generally, osmotic potential is adjusted by adding D-mannitol, sorbitol, glucose or sucrose to the enzyme mixture and mannitol is commonly effective (Navratilova, 2004; Zhang *et al.,* 2011).). Mannitol and sorbitol are commonly used than glucose as an osmoticum during protoplast isolation because they are believed to be relatively inert metabolically and infuse gradually into the protoplast (Guangyu *et al.,* 1997).

Hypotonic and hypertonic solutions can cause protoplasts to burst or become damaged, hence the need for the addition of mannitol to the enzyme solution and washing solution to prevent the protoplast from cell leakage or shrinking (Tudses *et al*., 2014). Due to variance in the composition of cell wall among different plant species, optimisation of osmoticum stabiliser (mannitol) at different concentrations was carried out in this study. Different mannitol concentrations (0.4, 0.5, 0.6 and 0.7 M) were tested to identify the optimal concentration for protoplast yield from Bambara groundnut leaves. The results from this study revealed that the mannitol concentration of 0.5 M gave the maximum yield of protoplast with the highest viability when compared with other concentrations of mannitol used.

Enzymatic digestion of cell wall components is necessary for protoplast production but it induces a stress reaction from which protoplasts have to recover in an artificial environment (Wiszniewska *et al.,* 2012). An appropriate choice of enzyme mixture compounds is a critical step in the optimization of protoplast isolation. The enzymes and techniques used for isolation of protoplasts have effect on their subsequent behaviour and development (Rao and Prakash, 1995). The success of protoplasts isolation is dependent especially on the tissue condition and the combination of enzymes being used (Tahami *et al.,* 2013). In this study, different enzyme concentrations and combinations were examined for the best protoplast isolation. The tested enzyme solutions include 1% cellulase and 0.5% macerozyme, 2% cellulase and 0.5% macerozyme, 4% cellulase and 0.5% macerozyme. 1% cellulase and 1% macerozyme, 2% cellulase and 1% macerozyme, 4% cellulase and 1% macerozyme, 1% cellulase and 0.5% pectinase, 2% cellulase and 0.5% pectinase, 4% cellulase and 0.5% pectinase. 1% cellulase and 1% pectinase, 2% cellulase and 1% pectinase, 4% cellulase and 1% pectinase. The results in this study revealed that all the enzyme concentrations and combinations were able to successfully isolate protoplast from the leaves of Bambara groundnut but, in varying numbers of protoplast yield and viability.

Pectinolytic enzymes such as macerozyme and pectinase were used for the separation of cells while cellulase removed their walls. Cellulase Onozuka R10 consists of three compositions that are required for complete enzymatic crystalline cellulose hydrolysis and they are i) endoglucanase - help in digestion of the crystalline cellulose; ii) exoglucanase- helps in cleaveage of cellobiose non-reducing ends of the cellulose polymer chain, and iii) ß-glucosidase- helps in breakeage of the ß-1,4 glucosidic bonds of cellobiose to produce glucose monomers (Zhang *et al.,* 2009; Jeng *et al*., 2011; Tudses *et al*., 2014).

In addition to enzyme concentrations and combinations, the different times (4, 18, 24, 42 hours) of incubation were also investigated in this study. The time of incubation that is needed for the protoplasts to be released could be influenced by the concentrations and combination of enzyme as well as the composition of protoplast isolation solution used (Tee *et al*., 2010). Tee *et al*. (2010) also reported that the incubation time, duration of incubating plant tissues in protoplast isolation solution differ among different plant species. In this study, the protoplast released after 4 hours of incubation was very low in number though very viable. An incubation time of 4 hours resulted in insufficient digestion of the tissue and cell wall material surrounding the protoplasts resulting in reduced yield, but with a high percentage of viable protoplasts (Raikar *et al*., 2008). The yield of protoplasts increased when the incubation time was increased to 18 and 24 hours. The results showed no significant increase in protoplast viability between 4 and 18 hours while there was significant decrease between 4 and 24 hours of

incubation. There was a decline in the yield and viability of protoplast produced at 42 hours of incubation due to prolonged incubation which caused the protoplasts to lyse and burst. As previously reported by Lord *et al*., (2010), prolonged incubation of leaves could lead to dysfunction and breaking of cells. This suggests that the cell wall lysis depends on the concentration and combination of lytic enzymes as well as the duration of incubation. Among all the different combination and concentration of enzymes used at the different incubation periods, digestion of Bambara groundnut's leaves with 2% cellulase and 0.5% macerozyme for 18 hours produced protoplasts with the optimum yield and viability.

This study also investigated the effect of using leaves from *in vitro* and greenhouse generated plants. This was necessary to investigate if the sterilization procedure for greenhouse grown plant and also the different conditions of germinating the plants could have a significant effect on the yield and viability of isolated protoplast of Bambara groundnut. Data obtained from this experiment showed that there was no significant difference in yield and viability of protoplasts isolated from leaves obtained from the two different sources.

Protoplast culture is a rigorous culture system by which isolated protoplasts have to deal with developmental reprogramming as well as serious infringement of cell integrity (Wiszniewska *et al*., 2012). Cell wall regeneration is the first noticeable activity taken up by isolated protoplasts and the success of this process decides the succeeding actions of protoplasts in culture (Wiszniewska *et al*., 2012). In this study, protoplasts were cultured in liquid MS medium, MS medium in agar and agarose bead supplemented with growth hormones. In this study, no cell division was observed in MS medium in agar and agarose bead. However, in the liquid MS medium supplemented with NAA and 2,4 D and BAP, cell division initiation was noticed after 48 hours in very few of the plated protoplasts (Figure 4.9). Cell division was not sustained beyond 48 hours and hence, colony/callus formation and plant regeneration could not be achieved. The media were also supplemented with 200mg/l yeast extract and 250mg/l casein hydrolysate and no cell division was observed in any of the plated protoplasts. Protoplasts died after 5 days in all the media.

Regeneration of protoplasts isolated with less harsh isolation treatments (e.g 2% cellulase and 0.5% pectinase) which resulted in although very low yield but high viability may need to be evaluated for their regeneration capability in future studies. Bambara groundnut could also have the physiological tendency to fail in response to protoplast culture and application. As previously reported, grain legumes are generally recalcitrant in tissue culture applications or even less responsive to protoplast manipulation studies (Babaoglu *et al*., 2000). The failure of cultured protoplast from Bambara groundnut to further undergo cell division in this study could

also be as a result of programmed cell death (apoptosis). This necessitated a further study on the proteomic analysis of the protoplasts in culture in comparison with freshly isolated protoplasts for possible insights to the presence or absence of the proteins that can be associated with the failure of regenerating Bambara groundnut plant from the isolated protoplasts. Optimisation of the resolution of the protein samples in first dimension IEF run as well as second dimension analysis in SDS-PAGE gel electrophoresis is ongoing (results not conclusive), this experiment is being continued in a separate study. Comparison of the 2D gels of the progression of protoplast in culture / regeneration medium should lead to identification of proteins that are being expressed or shut down and thus explain why regeneration has not been achieved.

# CHAPTER SIX

## 6.0 CONCLUSION

In this study, successful isolation of protoplasts from the leaves of Bambara groundnut plant was obtained. Isolation as a crucial step of protoplast utilization was extensively optimized, taking into consideration the various factors that could affect protoplast isolation in Bambara groundnut. The optimal enzyme-buffer solution for releasing good number of viable protoplasts from the leaves of Bambara groundnut plant was composed of 2% (w/v) cellulase, 0.5% (w/v) macerozyme, 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES buffer at pH 5.7. Among the three culture methods used for Bambara groundnut plant regeneration in this study, the cultured protoplasts in the liquid MS medium that were supplemented with only NAA and 2,4 D underwent cell division but did not proceed to attain regeneration of Bambara groundnut plants. The success achieved in the determination of optimum conditions for the isolation of viable protoplasts from Bambara groundnut will provide a basis for future work on the development of a protoplast-to-plant regeneration system as well as genetic transformation of Bambara groundnut.

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**ADDENDUM 1: Research output**

**Conferences attended**

i) 24th Conference of the South African Society for Biochemistry and Molecular Biology (SASBMB).
Paper presented: Mundembe R, **Ayeleso TB**, Adetunji AT and Lewu F. Bambara groundnut: An african crop with unrealised potential.
Date: 6th-9th July, 2014

ii) U6 Consortium International Conference, Cape Town, South Africa
Paper presented: **Ayeleso TB,** Mundembe R and Lewu F. Developing a novel protocol for protoplast preparations in Bambara groundnut.
Date: 5th-10th September, 2014

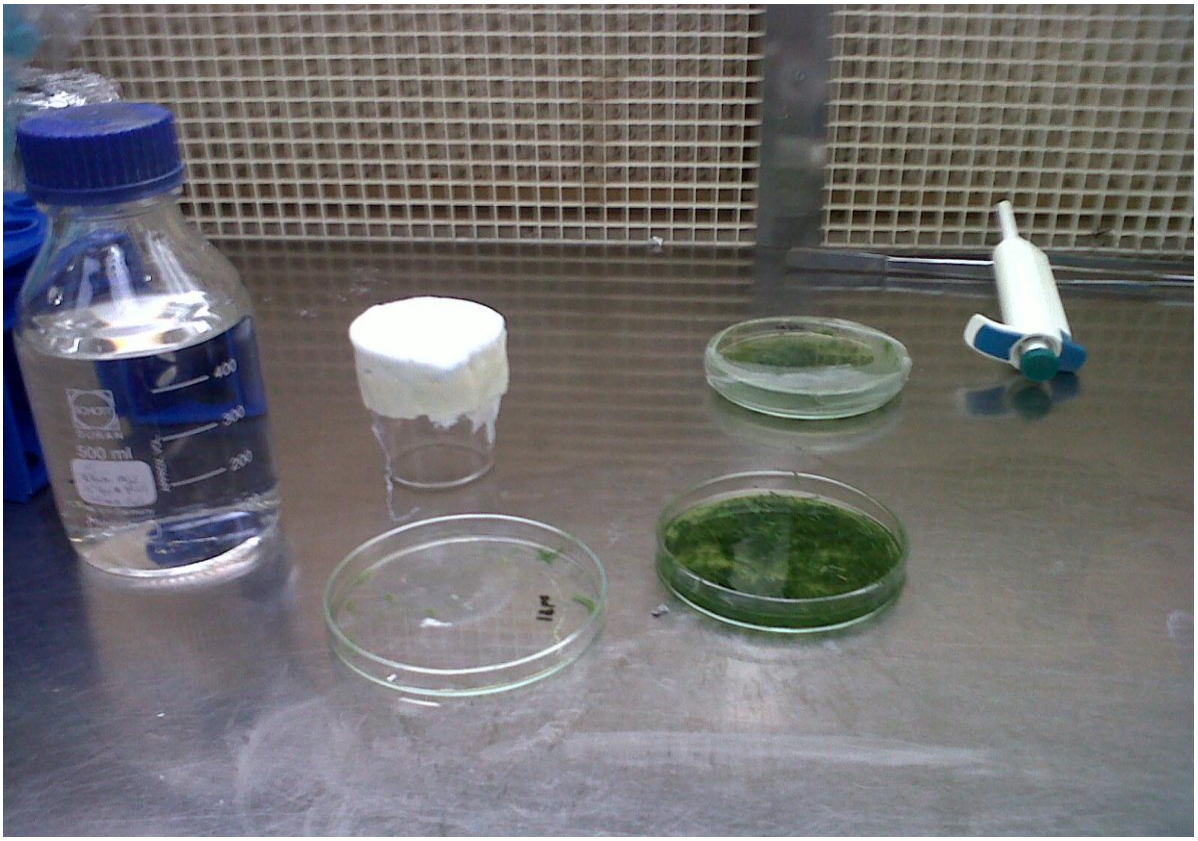
iii) Annual Symposium, Department of Botany and Plant Biotechnology, University of Johannesburg, Auckland Park, South Africa.
Paper Presented: **Ayeleso TB,** Mundembe R and Lewu F. Determination of optimal conditions for the isolation of protoplasts from Bambara groundnut.
Date: 28th October, 2014.

**Manuscript submitted for publication**

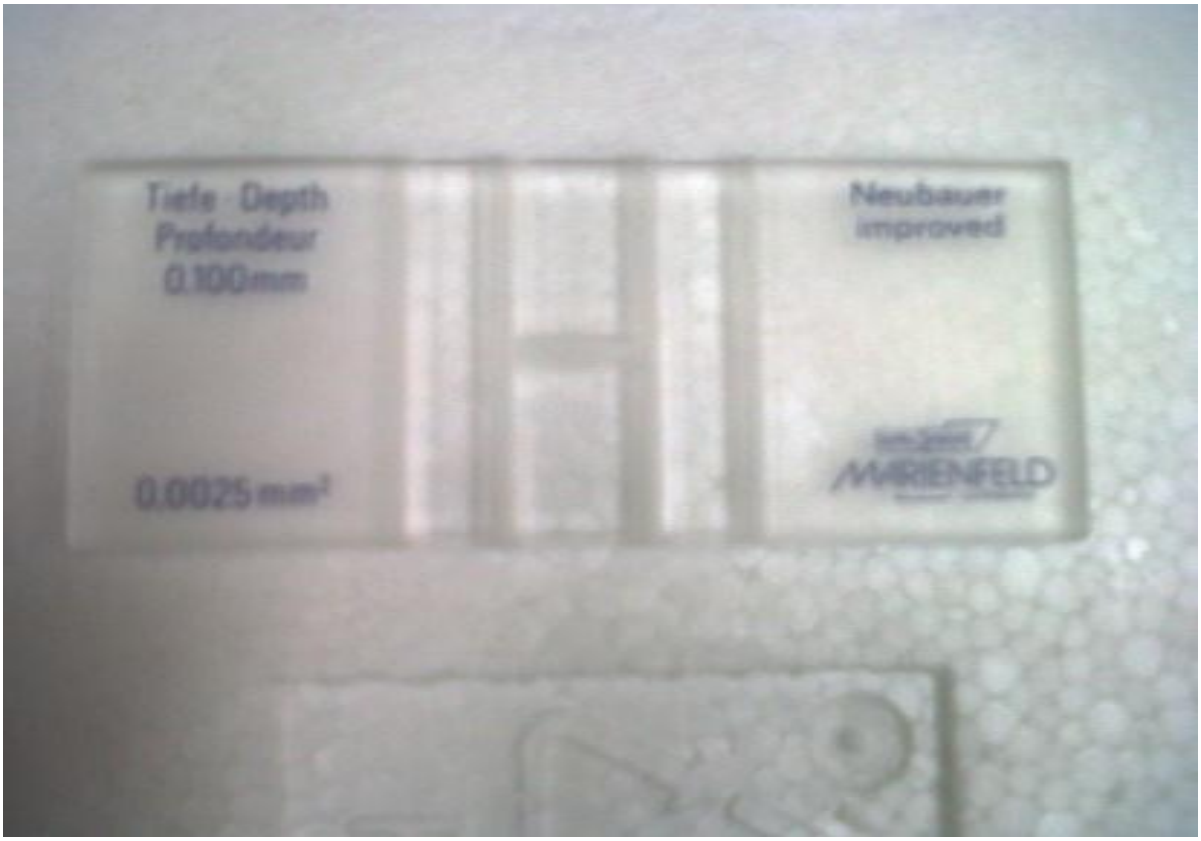
i) **Ayeleso, T.B.,** O`Riley, L., Lewu, F. and Mundembe, R. (2015). Optimisation of factors affecting protoplast yield and viablity in Bambara groundnut (*Vigna Subterranea*)**.**

**ADDENDUM 2: Thin strips of Bamabra groundnut leaf materials incubated in enzyme-buffer solution**

**ADDENDUM 3: Protoplast preparation in the laminar flow unit**



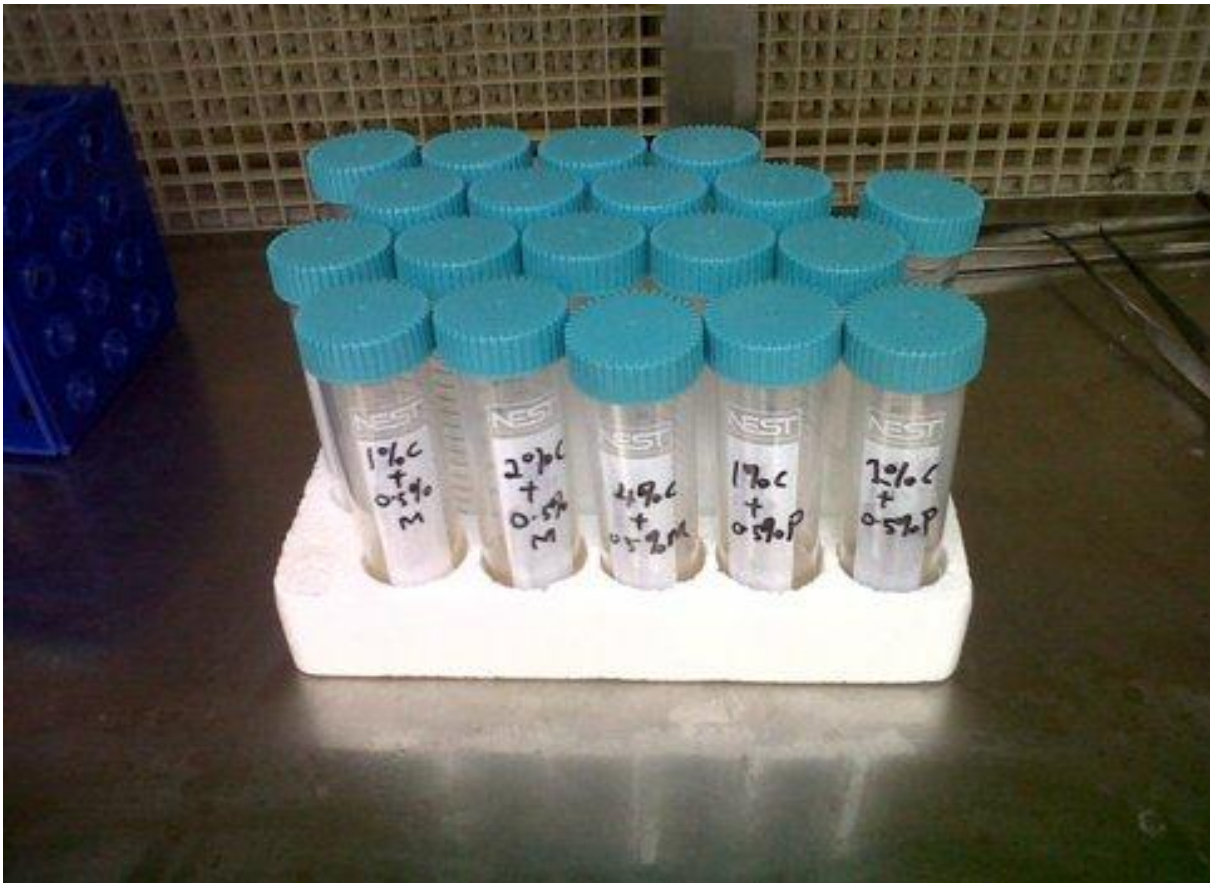
**ADDENDUM 4: Heamocytometer used for quantification of protoplast.**



**ADDENDUM 5: A swing out rotor centrifuge used to ensure horizontal separation of the digested plant materials**

**ADDENDUM 6: Weight measurement of leaf materials by weighing balance.**

**ADDENDUM 7: Enzyme-buffer solutions**



**ADDENDUM 8: ANOVA table for age of leaves on the yield of isolated protoplasts**

| | SS | Df | MS |
|---|---|---|---|
| Treatment (between columns) | 10.63 | 3 | 3.545 |
| Residual (within columns) | 0.0286 | 4 | 0.00715 |
| Total | 10.66 | 7 | |

| Tukey's Multiple Comparison Test | Mean Diff. | Q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | 1.650 | 27.60 | Yes | *** |
| Column A vs Column C | 1.700 | 28.43 | Yes | *** |
| Column A vs Column D | 3.260 | 54.52 | Yes | *** |
| Column B vs Column C | 0.05000 | 0.8362 | No | ns |
| Column B vs Column D | 1.610 | 26.93 | Yes | *** |
| Column C vs Column D | 1.560 | 26.09 | Yes | *** |

**ADDENDUM 9: ANOVA table for age of leaves on the viability of isolated protoplasts**

| | SS | df | MS | |
|---|---|---|---|---|
| Treatment (between columns) | 931.0 | 3 | 310.3 | |
| Residual (within columns) | 45.04 | 4 | 11.26 | |
| Total | 976.0 | 7 | | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -0.5000 | 0.2107 | No | ns |
| Column A vs Column C | 15.50 | 6.532 | Yes | * |
| Column A vs Column D | 25.00 | 10.54 | Yes | ** |
| Column B vs Column C | 16.00 | 6.743 | Yes | * |
| Column B vs Column D | 25.50 | 10.75 | Yes | ** |
| Column C vs Column D | 9.500 | 4.004 | No | ns |

**ADDENDUM 10: ANOVA table for incubation time on the yield of isolated protoplasts**

| | SS | df | MS | |
|---|---|---|---|---|
| Treatment (between columns) | 31.68 | 3 | 10.56 | |
| Residual (within columns) | 0.1749 | 4 | 0.04373 | |
| Total | 31.86 | 7 | | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -4.250 | 28.74 | Yes | *** |
| Column A vs Column C | -4.400 | 29.76 | Yes | *** |
| Column A vs Column D | -5.000 | 33.82 | Yes | *** |
| Column B vs Column C | -0.1500 | 1.014 | No | ns |
| Column B vs Column D | -0.7500 | 5.072 | No | ns |
| Column C vs Column D | -0.6000 | 4.058 | No | ns |

**ADDENDUM 11: ANOVA table for incubation time on the viability of isolated protoplasts**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 4991 | 3 | 1664 |
| Residual (within columns) | 136.9 | 4 | 34.23 |
| Total | 5128 | 7 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | 3.000 | 0.7251 | No | ns |
| Column A vs Column C | 19.00 | 4.592 | No | ns |
| Column A vs Column D | 62.55 | 15.12 | Yes | ** |
| Column B vs Column C | 16.00 | 3.867 | No | ns |
| Column B vs Column D | 59.55 | 14.39 | Yes | ** |
| Column C vs Column D | 43.55 | 10.53 | Yes | ** |

**ADDENDUM 12: ANOVA table for molarity of mannitol on the yield of isolated protoplasts**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 3.870 | 3 | 1.290 |
| Residual (within columns) | 0.0340 | 4 | 0.0085 |
| Total | 3.904 | 7 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -1.200 | 18.41 | Yes | *** |
| Column A vs Column C | 0.5500 | 8.437 | Yes | * |
| Column A vs Column D | 0.4500 | 6.903 | Yes | * |
| Column B vs Column C | 1.750 | 26.84 | Yes | *** |
| Column B vs Column D | 1.650 | 25.31 | Yes | *** |
| Column C vs Column D | -0.1000 | 1.534 | No | ns |

**ADDENDUM 13: ANOVA table for molarity of mannitol on the viability of isolated protoplasts**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 556.0 | 3 | 185.3 |
| Residual (within columns) | 13.97 | 4 | 3.493 |
| Total | 570.0 | 7 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -16.00 | 12.11 | Yes | ** |
| Column A vs Column C | -3.000 | 2.270 | No | ns |
| Column A vs Column D | 7.000 | 5.297 | No | ns |
| Column B vs Column C | 13.00 | 9.836 | Yes | ** |
| Column B vs Column D | 23.00 | 17.40 | Yes | *** |
| Column C vs Column D | 10.00 | 7.567 | Yes | * |

**ADDENDUM 14: ANOVA table for concentration and combination of enzymes on the yield of isolated protoplasts at 4 hours of incubation**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 0.04738 | 11 | 0.004308 |
| Residual (within columns) | 0.00343 | 12 | 0.0002858 |
| Total | 0.05081 | 23 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -0.0600 | 5.019 | No | ns |
| Column A vs Column C | -0.0200 | 1.673 | No | ns |
| Column A vs Column D | 0.0200 | 1.673 | No | ns |
| Column A vs Column E | -0.03000 | 2.509 | No | ns |
| Column A vs Column F | -0.04000 | 3.346 | No | ns |
| Column A vs Column G | 0.07000 | 5.855 | Yes | * |
| Column A vs Column H | 0.07000 | 5.855 | Yes | * |
| Column A vs Column I | -0.07000 | 5.855 | Yes | * |
| Column A vs Column J | -0.0500 | 4.182 | No | ns |
| Column A vs Column K | -0.04000 | 3.346 | No | ns |
| Column A vs Column L | -0.0200 | 1.673 | No | ns |
| Column B vs Column C | 0.04000 | 3.346 | No | ns |
| Column B vs Column D | 0.08000 | 6.692 | Yes | * |
| Column B vs Column E | 0.03000 | 2.509 | No | ns |
| Column B vs Column F | 0.02000 | 1.673 | No | ns |
| Column B vs Column G | 0.1300 | 10.87 | Yes | *** |
| Column B vs Column H | 0.1300 | 10.87 | Yes | *** |
| Column B vs Column I | -0.01000 | 0.8365 | No | ns |
| Column B vs Column J | 0.01000 | 0.8365 | No | ns |
| Column B vs Column K | 0.02000 | 1.673 | No | ns |
| Column B vs Column L | 0.04000 | 3.346 | No | ns |
| Column C vs Column D | 0.0400 | 3.346 | No | ns |
| Column C vs Column E | -0.01000 | 0.8365 | No | ns |
| Column C vs Column F | -0.0200 | 1.673 | No | ns |
| Column C vs Column G | 0.0900 | 7.528 | Yes | ** |
| Column C vs Column H | 0.0900 | 7.528 | Yes | ** |
| Column C vs Column I | -0.0500 | 4.182 | No | ns |
| Column C vs Column J | -0.0300 | 2.509 | No | ns |
| Column C vs Column K | -0.0200 | 1.673 | No | ns |
| Column C vs Column L | 0.0000 | 0.0000 | No | ns |
| Column D vs Column E | -0.0500 | 4.182 | No | ns |
| Column D vs Column F | -0.06000 | 5.019 | No | ns |
| Column D vs Column G | 0.0500 | 4.182 | No | ns |
| Column D vs Column H | 0.0500 | 4.182 | No | ns |
| Column D vs Column I | -0.0900 | 7.528 | Yes | ** |
| Column D vs Column J | -0.0700 | 5.855 | Yes | * |

| | | | | |
|---|---|---|---|---|
| Column D vs Column K | -0.06000 | 5.019 | No | ns |
| Column D vs Column L | -0.0400 | 3.346 | No | ns |
| Column E vs Column F | -0.01000 | 0.8365 | No | ns |
| Column E vs Column G | 0.1000 | 8.365 | Yes | ** |
| Column E vs Column H | 0.1000 | 8.365 | Yes | ** |
| Column E vs Column I | -0.0400 | 3.346 | No | ns |
| Column E vs Column J | -0.0200 | 1.673 | No | ns |
| Column E vs Column K | -0.01000 | 0.8365 | No | ns |
| Column E vs Column L | 0.01000 | 0.8365 | No | ns |
| Column F vs Column G | 0.1100 | 9.201 | Yes | ** |
| Column F vs Column H | 0.1100 | 9.201 | Yes | ** |
| Column F vs Column I | -0.0300 | 2.509 | No | ns |
| Column F vs Column J | -0.01000 | 0.8365 | No | ns |
| Column F vs Column K | 0.0000 | 0.0000 | No | ns |
| Column F vs Column L | 0.0200 | 1.673 | No | ns |
| Column G vs Column H | 0.0000 | 0.0000 | No | ns |
| Column G vs Column I | -0.1400 | 11.71 | Yes | *** |
| Column G vs Column J | -0.1200 | 10.04 | Yes | *** |
| Column G vs Column K | -0.1100 | 9.201 | Yes | ** |
| Column G vs Column L | -0.0900 | 7.528 | Yes | ** |
| Column H vs Column I | -0.1400 | 11.71 | Yes | *** |
| Column H vs Column J | -0.1200 | 10.04 | Yes | *** |
| Column H vs Column K | -0.1100 | 9.201 | Yes | ** |
| Column H vs Column L | -0.0900 | 7.528 | Yes | ** |
| Column I vs Column J | 0.0200 | 1.673 | No | ns |
| Column I vs Column K | 0.0300 | 2.509 | No | ns |
| Column I vs Column L | 0.0500 | 4.182 | No | ns |
| Column J vs Column K | 0.01000 | 0.8365 | No | ns |
| Column J vs Column L | 0.0300 | 2.509 | No | ns |
| Column K vs Column L | 0.0200 | 1.673 | No | ns |

**ADDENDUM 15: Anova table for concentration and combination of enzymes on the viability of isolated protoplasts at 4 hours of incubation**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 121.8 | 11 | 11.08 |
| Residual (within columns) | 49.90 | 12 | 4.158 |
| Total | 171.7 | 23 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | 1.000 | 0.6935 | No | ns |
| Column A vs Column C | 4.000 | 2.774 | No | ns |
| Column A vs Column D | -2.000 | 1.387 | No | ns |
| Column A vs Column E | 0.0000 | 0.0000 | No | ns |
| Column A vs Column F | 3.000 | 2.081 | No | ns |
| Column A vs Column G | 0.0000 | 0.0000 | No | ns |
| Column A vs Column H | -3.000 | 2.081 | No | ns |
| Column A vs Column I | 4.000 | 2.774 | No | ns |
| Column A vs Column J | 0.0000 | 0.0000 | No | ns |
| Column A vs Column K | 2.000 | 1.387 | No | ns |
| Column A vs Column L | 4.000 | 2.774 | No | ns |
| Column B vs Column C | 3.000 | 2.081 | No | ns |
| Column B vs Column D | -3.000 | 2.081 | No | ns |
| Column B vs Column E | -1.000 | 0.6935 | No | ns |
| Column B vs Column F | 2.000 | 1.387 | No | ns |
| Column B vs Column G | -1.000 | 0.6935 | No | ns |
| Column B vs Column H | -4.000 | 2.774 | No | ns |
| Column B vs Column I | 3.000 | 2.081 | No | ns |
| Column B vs Column J | -1.000 | 0.6935 | No | ns |
| Column B vs Column K | 1.000 | 0.6935 | No | ns |
| Column B vs Column L | 3.000 | 2.081 | No | ns |
| Column C vs Column D | -6.000 | 4.161 | No | ns |
| Column C vs Column E | -4.000 | 2.774 | No | ns |
| Column C vs Column F | -1.000 | 0.6935 | No | ns |
| Column C vs Column G | -4.000 | 2.774 | No | ns |
| Column C vs Column H | -7.000 | 4.855 | No | ns |
| Column C vs Column I | 0.0000 | 0.0000 | No | ns |
| Column C vs Column J | -4.000 | 2.774 | No | ns |
| Column C vs Column K | -2.000 | 1.387 | No | ns |
| Column C vs Column L | 0.0000 | 0.0000 | No | ns |
| Column D vs Column E | 2.000 | 1.387 | No | ns |
| Column D vs Column F | 5.000 | 3.468 | No | ns |
| Column D vs Column G | 2.000 | 1.387 | No | ns |
| Column D vs Column H | -1.000 | 0.6935 | No | ns |
| Column D vs Column I | 6.000 | 4.161 | No | ns |
| Column D vs Column J | 2.000 | 1.387 | No | ns |

| | | | | |
|---|---|---|---|---|
| Column D vs Column K | 4.000 | 2.774 | No | ns |
| Column D vs Column L | 6.000 | 4.161 | No | ns |
| Column E vs Column F | 3.000 | 2.081 | No | ns |
| Column E vs Column G | 0.0000 | 0.0000 | No | ns |
| Column E vs Column H | -3.000 | 2.081 | No | ns |
| Column E vs Column I | 4.000 | 2.774 | No | ns |
| Column E vs Column J | 0.0000 | 0.0000 | No | ns |
| Column E vs Column K | 2.000 | 1.387 | No | ns |
| Column E vs Column L | 4.000 | 2.774 | No | ns |
| Column F vs Column G | -3.000 | 2.081 | No | ns |
| Column F vs Column H | -6.000 | 4.161 | No | ns |
| Column F vs Column I | 1.000 | 0.6935 | No | ns |
| Column F vs Column J | -3.000 | 2.081 | No | ns |
| Column F vs Column K | -1.000 | 0.6935 | No | ns |
| Column F vs Column L | 1.000 | 0.6935 | No | ns |
| Column G vs Column H | -3.000 | 2.081 | No | ns |
| Column G vs Column I | 4.000 | 2.774 | No | ns |
| Column G vs Column J | 0.0000 | 0.0000 | No | ns |
| Column G vs Column K | 2.000 | 1.387 | No | ns |
| Column G vs Column L | 4.000 | 2.774 | No | ns |
| Column H vs Column I | 7.000 | 4.855 | No | ns |
| Column H vs Column J | 3.000 | 2.081 | No | ns |
| Column H vs Column K | 5.000 | 3.468 | No | ns |
| Column H vs Column L | 7.000 | 4.855 | No | ns |
| Column I vs Column J | -4.000 | 2.774 | No | ns |
| Column I vs Column K | -2.000 | 1.387 | No | ns |
| Column I vs Column L | 0.0000 | 0.0000 | No | ns |
| Column J vs Column K | 2.000 | 1.387 | No | ns |
| Column J vs Column L | 4.000 | 2.774 | No | ns |
| Column K vs Column L | 2.000 | 1.387 | No | ns |

**ADDENDUM 16: ANOVA table for concentration and combination of enzymes on the yield of isolated protoplasts at 18 hours of incubation**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 3878 | 11 | 352.6 |
| Residual (within columns) | 34.43 | 12 | 2.869 |
| Total | 3913 | 23 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -11.00 | 9.184 | Yes | ** |
| Column A vs Column C | 24.00 | 20.04 | Yes | *** |
| Column A vs Column D | 12.50 | 10.44 | Yes | *** |
| Column A vs Column E | -5.500 | 4.592 | No | ns |
| Column A vs Column F | 21.50 | 17.95 | Yes | *** |
| Column A vs Column G | -1.000 | 0.8349 | No | ns |
| Column A vs Column H | -7.500 | 6.262 | Yes | * |
| Column A vs Column I | 24.50 | 20.46 | Yes | *** |
| Column A vs Column J | 14.00 | 11.69 | Yes | *** |
| Column A vs Column K | -1.500 | 1.252 | No | ns |
| Column A vs Column L | 20.50 | 17.12 | Yes | *** |
| Column B vs Column C | 35.00 | 29.22 | Yes | *** |
| Column B vs Column D | 23.50 | 19.62 | Yes | *** |
| Column B vs Column E | 5.500 | 4.592 | No | ns |
| Column B vs Column F | 32.50 | 27.13 | Yes | *** |
| Column B vs Column G | 10.00 | 8.349 | Yes | ** |
| Column B vs Column H | 3.500 | 2.922 | No | ns |
| Column B vs Column I | 35.50 | 29.64 | Yes | *** |
| Column B vs Column J | 25.00 | 20.87 | Yes | *** |
| Column B vs Column K | 9.500 | 7.932 | Yes | ** |
| Column B vs Column L | 31.50 | 26.30 | Yes | *** |
| Column C vs Column D | -11.50 | 9.602 | Yes | *** |
| Column C vs Column E | -29.50 | 24.63 | Yes | *** |
| Column C vs Column F | -2.500 | 2.087 | No | ns |
| Column C vs Column G | -25.00 | 20.87 | Yes | *** |
| Column C vs Column H | -31.50 | 26.30 | Yes | *** |
| Column C vs Column I | 0.5000 | 0.4175 | No | ns |
| Column C vs Column J | -10.00 | 8.349 | Yes | ** |
| Column C vs Column K | -25.50 | 21.29 | Yes | *** |
| Column C vs Column L | -3.500 | 2.922 | No | ns |
| Column D vs Column E | -18.00 | 15.03 | Yes | *** |
| Column D vs Column F | 9.000 | 7.514 | Yes | ** |
| Column D vs Column G | -13.50 | 11.27 | Yes | *** |
| Column D vs Column H | -20.00 | 16.70 | Yes | *** |
| Column D vs Column I | 12.00 | 10.02 | Yes | *** |
| Column D vs Column J | 1.500 | 1.252 | No | ns |

| | | | | |
|---|---|---|---|---|
| Column D vs Column K | -14.00 | 11.69 | Yes | *** |
| Column D vs Column L | 8.000 | 6.679 | Yes | * |
| Column E vs Column F | 27.00 | 22.54 | Yes | *** |
| Column E vs Column G | 4.500 | 3.757 | No | ns |
| Column E vs Column H | -2.000 | 1.670 | No | ns |
| Column E vs Column I | 30.00 | 25.05 | Yes | *** |
| Column E vs Column J | 19.50 | 16.28 | Yes | *** |
| Column E vs Column K | 4.000 | 3.340 | No | ns |
| Column E vs Column L | 26.00 | 21.71 | Yes | *** |
| Column F vs Column G | -22.50 | 18.79 | Yes | *** |
| Column F vs Column H | -29.00 | 24.21 | Yes | *** |
| Column F vs Column I | 3.000 | 2.505 | No | ns |
| Column F vs Column J | -7.500 | 6.262 | Yes | * |
| Column F vs Column K | -23.00 | 19.20 | Yes | *** |
| Column F vs Column L | -1.000 | 0.8349 | No | ns |
| Column G vs Column H | -6.500 | 5.427 | No | ns |
| Column G vs Column I | 25.50 | 21.29 | Yes | *** |
| Column G vs Column J | 15.00 | 12.52 | Yes | *** |
| Column G vs Column K | -0.5000 | 0.4175 | No | ns |
| Column G vs Column L | 21.50 | 17.95 | Yes | *** |
| Column H vs Column I | 32.00 | 26.72 | Yes | *** |
| Column H vs Column J | 21.50 | 17.95 | Yes | *** |
| Column H vs Column K | 6.000 | 5.010 | No | ns |
| Column H vs Column L | 28.00 | 23.38 | Yes | *** |
| Column I vs Column J | -10.50 | 8.767 | Yes | ** |
| Column I vs Column K | -26.00 | 21.71 | Yes | *** |
| Column I vs Column L | -4.000 | 3.340 | No | ns |
| Column J vs Column K | -15.50 | 12.94 | Yes | *** |
| Column J vs Column L | 6.500 | 5.427 | No | ns |
| Column K vs Column L | 22.00 | 18.37 | Yes | *** |

**ADDENDUM 17: ANOVA table for concentration and combination of enzymes on the viability of isolated protoplasts at 18 hours of incubation**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 3748 | 11 | 340.7 |
| Residual (within columns) | 34.43 | 12 | 2.869 |
| Total | 3782 | 23 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -9.150 | 7.640 | Yes | ** |
| Column A vs Column C | 24.00 | 20.04 | Yes | *** |
| Column A vs Column D | 12.50 | 10.44 | Yes | *** |
| Column A vs Column E | -5.500 | 4.592 | No | ns |
| Column A vs Column F | 21.50 | 17.95 | Yes | *** |
| Column A vs Column G | -1.000 | 0.8349 | No | ns |
| Column A vs Column H | -7.500 | 6.262 | Yes | * |
| Column A vs Column I | 24.50 | 20.46 | Yes | *** |
| Column A vs Column J | 14.00 | 11.69 | Yes | *** |
| Column A vs Column K | -1.500 | 1.252 | No | ns |
| Column A vs Column L | 20.50 | 17.12 | Yes | *** |
| Column B vs Column C | 33.15 | 27.68 | Yes | *** |
| Column B vs Column D | 21.65 | 18.08 | Yes | *** |
| Column B vs Column E | 3.650 | 3.047 | No | ns |
| Column B vs Column F | 30.65 | 25.59 | Yes | *** |
| Column B vs Column G | 8.150 | 6.805 | Yes | * |
| Column B vs Column H | 1.650 | 1.378 | No | ns |
| Column B vs Column I | 33.65 | 28.10 | Yes | *** |
| Column B vs Column J | 23.15 | 19.33 | Yes | *** |
| Column B vs Column K | 7.650 | 6.387 | Yes | * |
| Column B vs Column L | 29.65 | 24.76 | Yes | *** |
| Column C vs Column D | -11.50 | 9.602 | Yes | *** |
| Column C vs Column E | -29.50 | 24.63 | Yes | *** |
| Column C vs Column F | -2.500 | 2.087 | No | ns |
| Column C vs Column G | -25.00 | 20.87 | Yes | *** |
| Column C vs Column H | -31.50 | 26.30 | Yes | *** |
| Column C vs Column I | 0.5000 | 0.4175 | No | ns |
| Column C vs Column J | -10.00 | 8.349 | Yes | ** |
| Column C vs Column K | -25.50 | 21.29 | Yes | *** |
| Column C vs Column L | -3.500 | 2.922 | No | ns |
| Column D vs Column E | -18.00 | 15.03 | Yes | *** |
| Column D vs Column F | 9.000 | 7.514 | Yes | ** |
| Column D vs Column G | -13.50 | 11.27 | Yes | *** |
| Column D vs Column H | -20.00 | 16.70 | Yes | *** |
| Column D vs Column I | 12.00 | 10.02 | Yes | *** |
| Column D vs Column J | 1.500 | 1.252 | No | ns |

| | | | | |
|---|---|---|---|---|
| Column D vs Column K | -14.00 | 11.69 | Yes | *** |
| Column D vs Column L | 8.000 | 6.679 | Yes | * |
| Column E vs Column F | 27.00 | 22.54 | Yes | *** |
| Column E vs Column G | 4.500 | 3.757 | No | ns |
| Column E vs Column H | -2.000 | 1.670 | No | ns |
| Column E vs Column I | 30.00 | 25.05 | Yes | *** |
| Column E vs Column J | 19.50 | 16.28 | Yes | *** |
| Column E vs Column K | 4.000 | 3.340 | No | ns |
| Column E vs Column L | 26.00 | 21.71 | Yes | *** |
| Column F vs Column G | -22.50 | 18.79 | Yes | *** |
| Column F vs Column H | -29.00 | 24.21 | Yes | *** |
| Column F vs Column I | 3.000 | 2.505 | No | ns |
| Column F vs Column J | -7.500 | 6.262 | Yes | * |
| Column F vs Column K | -23.00 | 19.20 | Yes | *** |
| Column F vs Column L | -1.000 | 0.8349 | No | ns |
| Column G vs Column H | -6.500 | 5.427 | No | ns |
| Column G vs Column I | 25.50 | 21.29 | Yes | *** |
| Column G vs Column J | 15.00 | 12.52 | Yes | *** |
| Column G vs Column K | -0.5000 | 0.4175 | No | ns |
| Column G vs Column L | 21.50 | 17.95 | Yes | *** |
| Column H vs Column I | 32.00 | 26.72 | Yes | *** |
| Column H vs Column J | 21.50 | 17.95 | Yes | *** |
| Column H vs Column K | 6.000 | 5.010 | No | ns |
| Column H vs Column L | 28.00 | 23.38 | Yes | *** |
| Column I vs Column J | -10.50 | 8.767 | Yes | ** |
| Column I vs Column K | -26.00 | 21.71 | Yes | *** |
| Column I vs Column L | -4.000 | 3.340 | No | ns |
| Column J vs Column K | -15.50 | 12.94 | Yes | *** |
| Column J vs Column L | 6.500 | 5.427 | No | ns |
| Column K vs Column L | 22.00 | 18.37 | Yes | *** |

**ADDENDUM 18: ANOVA table for concentration and combination of enzymes on the yield of isolated protoplasts at 24 hours of incubation**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 32.58 | 11 | 2.962 |
| Residual (within columns) | 0.2816 | 12 | 0.02347 |
| Total | 32.87 | 23 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -1.850 | 17.08 | Yes | *** |
| Column A vs Column C | -1.050 | 9.693 | Yes | *** |
| Column A vs Column D | 0.1500 | 1.385 | No | ns |
| Column A vs Column E | -1.350 | 12.46 | Yes | *** |
| Column A vs Column F | -1.100 | 10.16 | Yes | *** |
| Column A vs Column G | 1.920 | 17.73 | Yes | *** |
| Column A vs Column H | 1.650 | 15.23 | Yes | *** |
| Column A vs Column I | -1.550 | 14.31 | Yes | *** |
| Column A vs Column J | 0.1500 | 1.385 | No | ns |
| Column A vs Column K | -1.200 | 11.08 | Yes | *** |
| Column A vs Column L | -0.6500 | 6.001 | Yes | * |
| Column B vs Column C | 0.8000 | 7.385 | Yes | ** |
| Column B vs Column D | 2.000 | 18.46 | Yes | *** |
| Column B vs Column E | 0.5000 | 4.616 | No | ns |
| Column B vs Column F | 0.7500 | 6.924 | Yes | * |
| Column B vs Column G | 3.770 | 34.80 | Yes | *** |
| Column B vs Column H | 3.500 | 32.31 | Yes | *** |
| Column B vs Column I | 0.3000 | 2.770 | No | ns |
| Column B vs Column J | 2.000 | 18.46 | Yes | *** |
| Column B vs Column K | 0.6500 | 6.001 | Yes | * |
| Column B vs Column L | 1.200 | 11.08 | Yes | *** |
| Column C vs Column D | 1.200 | 11.08 | Yes | *** |
| Column C vs Column E | -0.3000 | 2.770 | No | ns |
| Column C vs Column F | -0.05000 | 0.4616 | No | ns |
| Column C vs Column G | 2.970 | 27.42 | Yes | *** |
| Column C vs Column H | 2.700 | 24.93 | Yes | *** |
| Column C vs Column I | -0.5000 | 4.616 | No | ns |
| Column C vs Column J | 1.200 | 11.08 | Yes | *** |
| Column C vs Column K | -0.1500 | 1.385 | No | ns |
| Column C vs Column L | 0.4000 | 3.693 | No | ns |
| Column D vs Column E | -1.500 | 13.85 | Yes | *** |
| Column D vs Column F | -1.250 | 11.54 | Yes | *** |
| Column D vs Column G | 1.770 | 16.34 | Yes | *** |
| Column D vs Column H | 1.500 | 13.85 | Yes | *** |
| Column D vs Column I | -1.700 | 15.69 | Yes | *** |
| Column D vs Column J | 0.0000 | 0.0000 | No | ns |

| | | | | |
|---|---|---|---|---|
| Column D vs Column K | -1.350 | 12.46 | Yes | *** |
| Column D vs Column L | -0.8000 | 7.385 | Yes | ** |
| Column E vs Column F | 0.2500 | 2.308 | No | ns |
| Column E vs Column G | 3.270 | 30.19 | Yes | *** |
| Column E vs Column H | 3.000 | 27.70 | Yes | *** |
| Column E vs Column I | -0.2000 | 1.846 | No | ns |
| Column E vs Column J | 1.500 | 13.85 | Yes | *** |
| Column E vs Column K | 0.1500 | 1.385 | No | ns |
| Column E vs Column L | 0.7000 | 6.462 | Yes | * |
| Column F vs Column G | 3.020 | 27.88 | Yes | *** |
| Column F vs Column H | 2.750 | 25.39 | Yes | *** |
| Column F vs Column I | -0.4500 | 4.154 | No | ns |
| Column F vs Column J | 1.250 | 11.54 | Yes | *** |
| Column F vs Column K | -0.1000 | 0.9232 | No | ns |
| Column F vs Column L | 0.4500 | 4.154 | No | ns |
| Column G vs Column H | -0.2700 | 2.493 | No | ns |
| Column G vs Column I | -3.470 | 32.03 | Yes | *** |
| Column G vs Column J | -1.770 | 16.34 | Yes | *** |
| Column G vs Column K | -3.120 | 28.80 | Yes | *** |
| Column G vs Column L | -2.570 | 23.73 | Yes | *** |
| Column H vs Column I | -3.200 | 29.54 | Yes | *** |
| Column H vs Column J | -1.500 | 13.85 | Yes | *** |
| Column H vs Column K | -2.850 | 26.31 | Yes | *** |
| Column H vs Column L | -2.300 | 21.23 | Yes | *** |
| Column I vs Column J | 1.700 | 15.69 | Yes | *** |
| Column I vs Column K | 0.3500 | 3.231 | No | ns |
| Column I vs Column L | 0.9000 | 8.309 | Yes | ** |
| Column J vs Column K | -1.350 | 12.46 | Yes | *** |
| Column J vs Column L | -0.8000 | 7.385 | Yes | ** |
| Column K vs Column L | 0.5500 | 5.078 | No | ns |

**ADDENDUM 19: ANOVA table for concentration and combination of enzymes on the viability of isolated protoplasts at 24 hours of incubation**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 2369 | 11 | 215.3 |
| Residual (within columns) | 89.02 | 12 | 7.418 |
| Total | 2458 | 23 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -6.500 | 3.375 | No | ns |
| Column A vs Column C | 9.500 | 4.933 | No | ns |
| Column A vs Column D | 1.000 | 0.5192 | No | ns |
| Column A vs Column E | -17.00 | 8.827 | Yes | ** |
| Column A vs Column F | 8.000 | 4.154 | No | ns |
| Column A vs Column G | -12.00 | 6.231 | Yes | * |
| Column A vs Column H | -19.00 | 9.865 | Yes | *** |
| Column A vs Column I | 10.00 | 5.192 | No | ns |
| Column A vs Column J | 0.0000 | 0.0000 | No | ns |
| Column A vs Column K | -8.000 | 4.154 | No | ns |
| Column A vs Column L | 9.000 | 4.673 | No | ns |
| Column B vs Column C | 16.00 | 8.308 | Yes | ** |
| Column B vs Column D | 7.500 | 3.894 | No | ns |
| Column B vs Column E | -10.50 | 5.452 | No | ns |
| Column B vs Column F | 14.50 | 7.529 | Yes | ** |
| Column B vs Column G | -5.500 | 2.856 | No | ns |
| Column B vs Column H | -12.50 | 6.490 | Yes | * |
| Column B vs Column I | 16.50 | 8.567 | Yes | ** |
| Column B vs Column J | 6.500 | 3.375 | No | ns |
| Column B vs Column K | -1.500 | 0.7788 | No | ns |
| Column B vs Column L | 15.50 | 8.048 | Yes | ** |
| Column C vs Column D | -8.500 | 4.413 | No | ns |
| Column C vs Column E | -26.50 | 13.76 | Yes | *** |
| Column C vs Column F | -1.500 | 0.7788 | No | ns |
| Column C vs Column G | -21.50 | 11.16 | Yes | *** |
| Column C vs Column H | -28.50 | 14.80 | Yes | *** |
| Column C vs Column I | 0.5000 | 0.2596 | No | ns |
| Column C vs Column J | -9.500 | 4.933 | No | ns |
| Column C vs Column K | -17.50 | 9.087 | Yes | ** |
| Column C vs Column L | -0.5000 | 0.2596 | No | ns |
| Column D vs Column E | -18.00 | 9.346 | Yes | *** |
| Column D vs Column F | 7.000 | 3.635 | No | ns |
| Column D vs Column G | -13.00 | 6.750 | Yes | * |
| Column D vs Column H | -20.00 | 10.38 | Yes | *** |
| Column D vs Column I | 9.000 | 4.673 | No | ns |
| Column D vs Column J | -1.000 | 0.5192 | No | ns |

| | | | | |
|---|---|---|---|---|
| Column D vs Column K | -9.000 | 4.673 | No | ns |
| Column D vs Column L | 8.000 | 4.154 | No | ns |
| Column E vs Column F | 25.00 | 12.98 | Yes | *** |
| Column E vs Column G | 5.000 | 2.596 | No | ns |
| Column E vs Column H | -2.000 | 1.038 | No | ns |
| Column E vs Column I | 27.00 | 14.02 | Yes | *** |
| Column E vs Column J | 17.00 | 8.827 | Yes | ** |
| Column E vs Column K | 9.000 | 4.673 | No | ns |
| Column E vs Column L | 26.00 | 13.50 | Yes | *** |
| Column F vs Column G | -20.00 | 10.38 | Yes | *** |
| Column F vs Column H | -27.00 | 14.02 | Yes | *** |
| Column F vs Column I | 2.000 | 1.038 | No | ns |
| Column F vs Column J | -8.000 | 4.154 | No | ns |
| Column F vs Column K | -16.00 | 8.308 | Yes | ** |
| Column F vs Column L | 1.000 | 0.5192 | No | ns |
| Column G vs Column H | -7.000 | 3.635 | No | ns |
| Column G vs Column I | 22.00 | 11.42 | Yes | *** |
| Column G vs Column J | 12.00 | 6.231 | Yes | * |
| Column G vs Column K | 4.000 | 2.077 | No | ns |
| Column G vs Column L | 21.00 | 10.90 | Yes | *** |
| Column H vs Column I | 29.00 | 15.06 | Yes | *** |
| Column H vs Column J | 19.00 | 9.865 | Yes | *** |
| Column H vs Column K | 11.00 | 5.712 | Yes | * |
| Column H vs Column L | 28.00 | 14.54 | Yes | *** |
| Column I vs Column J | -10.00 | 5.192 | No | ns |
| Column I vs Column K | -18.00 | 9.346 | Yes | *** |
| Column I vs Column L | -1.000 | 0.5192 | No | ns |
| Column J vs Column K | -8.000 | 4.154 | No | ns |
| Column J vs Column L | 9.000 | 4.673 | No | ns |
| Column K vs Column L | 17.00 | 8.827 | Yes | ** |

**ADDENDUM 20: ANOVA table for concentration and combination of enzymes on the yield of isolated protoplasts at 48 hours of incubation**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 321.5 | 11 | 29.22 |
| Residual (within columns) | 28.41 | 12 | 2.367 |
| Total | 349.9 | 23 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | 2.000 | 1.838 | No | ns |
| Column A vs Column C | 6.000 | 5.515 | No | ns |
| Column A vs Column D | 0.5000 | 0.4596 | No | ns |
| Column A vs Column E | 2.000 | 1.838 | No | ns |
| Column A vs Column F | 5.000 | 4.596 | No | ns |
| Column A vs Column G | -4.500 | 4.136 | No | ns |
| Column A vs Column H | -0.5000 | 0.4596 | No | ns |
| Column A vs Column I | 7.000 | 6.434 | Yes | * |
| Column A vs Column J | -4.000 | 3.677 | No | ns |
| Column A vs Column K | 1.500 | 1.379 | No | ns |
| Column A vs Column L | 6.500 | 5.974 | Yes | * |
| Column B vs Column C | 4.000 | 3.677 | No | ns |
| Column B vs Column D | -1.500 | 1.379 | No | ns |
| Column B vs Column E | 0.0000 | 0.0000 | No | ns |
| Column B vs Column F | 3.000 | 2.757 | No | ns |
| Column B vs Column G | -6.500 | 5.974 | Yes | * |
| Column B vs Column H | -2.500 | 2.298 | No | ns |
| Column B vs Column I | 5.000 | 4.596 | No | ns |
| Column B vs Column J | -6.000 | 5.515 | No | ns |
| Column B vs Column K | -0.5000 | 0.4596 | No | ns |
| Column B vs Column L | 4.500 | 4.136 | No | ns |
| Column C vs Column D | -5.500 | 5.055 | No | ns |
| Column C vs Column E | -4.000 | 3.677 | No | ns |
| Column C vs Column F | -1.000 | 0.9191 | No | ns |
| Column C vs Column G | -10.50 | 9.651 | Yes | *** |
| Column C vs Column H | -6.500 | 5.974 | Yes | * |
| Column C vs Column I | 1.000 | 0.9191 | No | ns |
| Column C vs Column J | -10.00 | 9.191 | Yes | ** |
| Column C vs Column K | -4.500 | 4.136 | No | ns |
| Column C vs Column L | 0.5000 | 0.4596 | No | ns |
| Column D vs Column E | 1.500 | 1.379 | No | ns |
| Column D vs Column F | 4.500 | 4.136 | No | ns |
| Column D vs Column G | -5.000 | 4.596 | No | ns |
| Column D vs Column H | -1.000 | 0.9191 | No | ns |
| Column D vs Column I | 6.500 | 5.974 | Yes | * |
| Column D vs Column J | -4.500 | 4.136 | No | ns |

| | | | | |
|---|---|---|---|---|
| Column D vs Column K | 1.000 | 0.9191 | No | ns |
| Column D vs Column L | 6.000 | 5.515 | No | ns |
| Column E vs Column F | 3.000 | 2.757 | No | ns |
| Column E vs Column G | -6.500 | 5.974 | Yes | * |
| Column E vs Column H | -2.500 | 2.298 | No | ns |
| Column E vs Column I | 5.000 | 4.596 | No | ns |
| Column E vs Column J | -6.000 | 5.515 | No | ns |
| Column E vs Column K | -0.5000 | 0.4596 | No | ns |
| Column E vs Column L | 4.500 | 4.136 | No | ns |
| Column F vs Column G | -9.500 | 8.732 | Yes | ** |
| Column F vs Column H | -5.500 | 5.055 | No | ns |
| Column F vs Column I | 2.000 | 1.838 | No | ns |
| Column F vs Column J | -9.000 | 8.272 | Yes | ** |
| Column F vs Column K | -3.500 | 3.217 | No | ns |
| Column F vs Column L | 1.500 | 1.379 | No | ns |
| Column G vs Column H | 4.000 | 3.677 | No | ns |
| Column G vs Column I | 11.50 | 10.57 | Yes | *** |
| Column G vs Column J | 0.5000 | 0.4596 | No | ns |
| Column G vs Column K | 6.000 | 5.515 | No | ns |
| Column G vs Column L | 11.00 | 10.11 | Yes | *** |
| Column H vs Column I | 7.500 | 6.894 | Yes | * |
| Column H vs Column J | -3.500 | 3.217 | No | ns |
| Column H vs Column K | 2.000 | 1.838 | No | ns |
| Column H vs Column L | 7.000 | 6.434 | Yes | * |
| Column I vs Column J | -11.00 | 10.11 | Yes | *** |
| Column I vs Column K | -5.500 | 5.055 | No | ns |
| Column I vs Column L | -0.5000 | 0.4596 | No | ns |
| Column J vs Column K | 5.500 | 5.055 | No | ns |
| Column J vs Column L | 10.50 | 9.651 | Yes | *** |
| Column K vs Column L | 5.000 | 4.596 | No | ns |

**ADDENDUM 21: ANOVA table for concentration and combination of enzymes on the viability of isolated protoplasts at 48 hours of incubation**

| | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 35.30 | 11 | 3.210 |
| Residual (within columns) | 1.042 | 12 | 0.08680 |
| Total | 36.35 | 23 | |

| Tukey's Multiple Comparison Test | Mean Diff. | q | Significant? P < 0.05? | Summary |
|---|---|---|---|---|
| Column A vs Column B | -1.450 | 6.960 | Yes | * |
| Column A vs Column C | -0.7000 | 3.360 | No | ns |
| Column A vs Column D | 0.2500 | 1.200 | No | ns |
| Column A vs Column E | -1.250 | 6.000 | Yes | * |
| Column A vs Column F | -0.8000 | 3.840 | No | ns |
| Column A vs Column G | 2.230 | 10.70 | Yes | *** |
| Column A vs Column H | 2.200 | 10.56 | Yes | *** |
| Column A vs Column I | -1.350 | 6.480 | Yes | * |
| Column A vs Column J | 0.5500 | 2.640 | No | ns |
| Column A vs Column K | -1.000 | 4.800 | No | ns |
| Column A vs Column L | -0.2500 | 1.200 | No | ns |
| Column B vs Column C | 0.7500 | 3.600 | No | ns |
| Column B vs Column D | 1.700 | 8.160 | Yes | ** |
| Column B vs Column E | 0.2000 | 0.9600 | No | ns |
| Column B vs Column F | 0.6500 | 3.120 | No | ns |
| Column B vs Column G | 3.680 | 17.66 | Yes | *** |
| Column B vs Column H | 3.650 | 17.52 | Yes | *** |
| Column B vs Column I | 0.1000 | 0.4800 | No | ns |
| Column B vs Column J | 2.000 | 9.600 | Yes | *** |
| Column B vs Column K | 0.4500 | 2.160 | No | ns |
| Column B vs Column L | 1.200 | 5.760 | Yes | * |
| Column C vs Column D | 0.9500 | 4.560 | No | ns |
| Column C vs Column E | -0.5500 | 2.640 | No | ns |
| Column C vs Column F | -0.1000 | 0.4800 | No | ns |
| Column C vs Column G | 2.930 | 14.06 | Yes | *** |
| Column C vs Column H | 2.900 | 13.92 | Yes | *** |
| Column C vs Column I | -0.6500 | 3.120 | No | ns |
| Column C vs Column J | 1.250 | 6.000 | Yes | * |
| Column C vs Column K | -0.3000 | 1.440 | No | ns |
| Column C vs Column L | 0.4500 | 2.160 | No | ns |
| Column D vs Column E | -1.500 | 7.200 | Yes | ** |
| Column D vs Column F | -1.050 | 5.040 | No | ns |
| Column D vs Column G | 1.980 | 9.504 | Yes | *** |
| Column D vs Column H | 1.950 | 9.360 | Yes | *** |
| Column D vs Column I | -1.600 | 7.680 | Yes | ** |
| Column D vs Column J | 0.3000 | 1.440 | No | ns |

| | | | | |
|---|---|---|---|---|
| Column D vs Column K | -1.250 | 6.000 | Yes | * |
| Column D vs Column L | -0.5000 | 2.400 | No | ns |
| Column E vs Column F | 0.4500 | 2.160 | No | ns |
| Column E vs Column G | 3.480 | 16.70 | Yes | *** |
| Column E vs Column H | 3.450 | 16.56 | Yes | *** |
| Column E vs Column I | -0.1000 | 0.4800 | No | ns |
| Column E vs Column J | 1.800 | 8.640 | Yes | ** |
| Column E vs Column K | 0.2500 | 1.200 | No | ns |
| Column E vs Column L | 1.000 | 4.800 | No | ns |
| Column F vs Column G | 3.030 | 14.54 | Yes | *** |
| Column F vs Column H | 3.000 | 14.40 | Yes | *** |
| Column F vs Column I | -0.5500 | 2.640 | No | ns |
| Column F vs Column J | 1.350 | 6.480 | Yes | * |
| Column F vs Column K | -0.2000 | 0.9600 | No | ns |
| Column F vs Column L | 0.5500 | 2.640 | No | ns |
| Column G vs Column H | -0.03000 | 0.1440 | No | ns |
| Column G vs Column I | -3.580 | 17.18 | Yes | *** |
| Column G vs Column J | -1.680 | 8.064 | Yes | ** |
| Column G vs Column K | -3.230 | 15.50 | Yes | *** |
| Column G vs Column L | -2.480 | 11.90 | Yes | *** |
| Column H vs Column I | -3.550 | 17.04 | Yes | *** |
| Column H vs Column J | -1.650 | 7.920 | Yes | ** |
| Column H vs Column K | -3.200 | 15.36 | Yes | *** |
| Column H vs Column L | -2.450 | 11.76 | Yes | *** |
| Column I vs Column J | 1.900 | 9.120 | Yes | ** |
| Column I vs Column K | 0.3500 | 1.680 | No | ns |
| Column I vs Column L | 1.100 | 5.280 | No | ns |
| Column J vs Column K | -1.550 | 7.440 | Yes | ** |
| Column J vs Column L | -0.8000 | 3.840 | No | ns |
| Column K vs Column L | 0.7500 | 3.600 | No | ns |