ADDIS ABABA UNIVERSITY
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SCHOOL OF GRADUATE STUDIES
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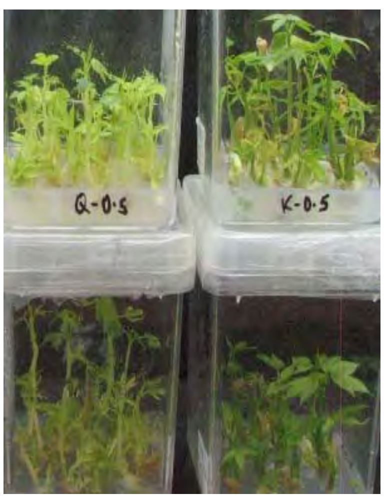
Micropropagation of Selected Cassava Varieties (*Manihot esculenta* Crantz) from Meristem Culture



By Dawit Beyene

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ABSTRACT

Cassava (*Manihot Esculenta* Crantz) is a perennial woody herb which has been mainly cultivated in different parts of Africa, Asia and Latin America for its edible starchy roots. Due to high seed dormancy and very slow germination rate farmers normally practice propagation of cassava by stem cuttings which has led to accumulation of viral and bacterial diseases that reduce productivity and may cause loss of superior genotypes. The objective of this study was to establish a protocol for in vitro micropropagation of selected cassava varieties ('Qulle' and 'Kello'). For this experiment solid MS medium supplemented with different BAP, GA_3 and NAA combinations has been used to initiate the growth of meristems and shoot induction. All the meristems were responded to a medium with 5 mg/l BAP in combination with 1 mg/l GA_3 and 0.01 mg/l NAA, but shoots from this treatment were bushy with abnormal phenotypic appearance. This might be due to supra-optimal amount of BAP. Morphologically normal appearance of shoots was observed in the growth initiation medium with 0.5 mg/l BAP, 1 mg/l GA_3 and 0.01 mg/l NAA. Among different treatments used for multiplication purpose, 0.5 mg/l BAP in combination with 1 mg/l GA_3 and 0.01 mg/l NAA was found to be the best; in this treatment a maximum of 27 shoots for 'Qulle' and 21 shoots for 'Kello' was obtained. In rooting experiment half and full strength MS media did not show statistical difference. Root induction potential of the two varieties increased with the increment of IBA concentration up to 1 mg/l. Of those shoots which were acclimatized in the greenhouse, 89.1% of 'Qulle' and 75% of 'Kello' survived.

Key words/phrases: Cassava, *Manihot esculenta*, meristem, tissue culture

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BAP	6-benzylaminopurine
g	gram
GA_3	gibberrelic acid
IBA	indol-3-Butyric acid
l	liter
mg	milligram
masl	meters above sea level
mm	millimeter
MS	Murashige and Skoog basal media
NAA	α-naphthalene Acetic Acid
s	seconds

1. INTRODUCTION

Cassava is a perennial woody herb which is grown as an annual. The name cassava is a collection name for many species belonging to the group *Manihot* of the family Euphorbiaceae (Olsen and Schaal, 1999). Different botanical studies, archeological findings and DNA based analysis shows that origin of cassava is south and east of Amazon basin (Allem, 2002). The crop was introduced to the African continent by Portuguese traders, first into West Africa via the Gulf of Benin and the Congo River during the second half of the 16th century and, secondly, into East Africa towards the end of the 18^{th} century (Christopher, 2008).

Its starchy, swollen roots are the staple food for millions of people in the tropics. It is the third most important source of calories for populations in the humid tropics, after rice and corn (Zeigler *et al*., 1980; FAO, 2001).

It is widely consumed in Sub-Saharan Africa and parts of Asia. An estimated 800 million people obtain more than 500 calories per day from cassava. The productivity of the crop on marginal soils; its ability to withstand disease, drought, and pests and flexible harvesting dates makes cassava a remarkably adaptable and hearty crop in areas where drought, poverty, and malnutrition are prevalent (Nassar *et al*., 2009).

Cassava roots can be stored in the ground for up to 24 months, and in some varieties for up to 36 months, thus harvest may be delayed until market, processing or other conditions are favorable. Cassava is classified as sweet or bitter, depending on the amount of cyanogenic compounds (Chavez *et al*., 2000).

The production of cassava in Africa and Asia continents has increased, while in Latin America it has remained relatively lower level over the past 30 years. Thailand is the main exporter of cassava with most of it going to Europe. In Ethiopia, there is higher cultivation of cassava in south and southwest part of the country mainly for the edible tubers (Amsalu Nebiyu *et al.*, 2000). In Wolaita, southwest Ethiopia (Kefa, Bench, Maji, Sheko) and Gambella areas, cassava tubers are consumed in the form of bread or injera by mixing its flour with different cereal crops (Amsalu Nebiyu and Elfinesh Firidisa, 2006).

The propagation method used in cassava is one of the bottlenecks for its production. Cassava's seeds are normally dormant and germinate very slowly, as a result farmers practice the conventional propagation method by stem cuttings. However this propagation method leads to the accumulation of viral and bacterial diseases which reduce productivity of the crop and may cause loss of superior genotypes (Nassar and Ortiz, 2007).

Such production constrains can be addressed by the application of plant biotechnology, which helps to eliminate or minimize the major limitations of traditional plant breeding (Rao, 1996). Tissue culture is one of the most commercially exploited components of biotechnology that has been used for the rapid clonal multiplication (micropropagation) of selected genotypes of diverse groups of plant species (Rani and Raina, 2000).

Plant tissue culture generally refers to the set of techniques designed for the growth and multiplication of cells, tissues, and/or organs using nutrient solutions in an aseptic and controlled environment. This is due to the fact that cells are totipotent in nature (the ability of a single cell to express the full genome and reproduce the whole plant) (Loyola-Vargas and Vázques-Flota, 2006). Culturing of an organized tissue in the form of very small shoots or meristem has allowed the most valuable application of plant tissue culture in order to eliminate virus from infected mother plant (Davies, 1981). In addition to that plant tissue culture also has so many applications, some of them are clonal propagation & somatic embryogenesis, germplasme exchange, embryo rescue, genetic transformation, etc (Rao, 1996).

During meristem culture, one should take a very small stem apex (0.2-1.0 mm in length) consisting of the apical meristem with/without one or two leaf primordial (George, 2008). The major advantage of meristem culture is its potential to eliminate pathogenic organisms that may have been present in the donor plants and also maintenance of stability in genetic inheritance (Hu *et al.,* 1984; Grout, 1990; Brian, 1999).

The aims of this research were to develop an efficient *in vitro* protocol for micropropagation of selected cassava (*Manihot esculenta* Crantz) varieties (’Qulle’ and ‘Kello’) through meristem culture. Culture initiation, shoot multiplication, rooting and acclimatization steps and survival rates were investigated.

2. LITERATURE REVIEW

2.1 Taxonomy and Description of Cassava

Cassava (*Manihot esculenta*, Cranz.) is little-branched woody herb that grows up to 5m. It has large and starchy roots and leaves are 3-5(-7) lobed (Gilbert, 1995). The genus *Manihot* belongs to the Euphorbiaceae family with 2n=36 and has about 100 species, among which *Manihot esculenta* is the only one commercially cultivated (Nassar, 1978; Alves, 2002; Ricardo *et al*., 2007). In Ethiopia, cassava is locally known as “yefurno duket zaf” (Amharic), Batata (Soomaali), Deekikaa (Afaan Oromoo) (Gilbert, 1995; Amsalu Nebiyu *et al*., 2000).

Stems, when planted, produce sprouts and adventitious roots within one week. Seeds are normally dormant and slow germinating. Seed dormancy does not break by scarifying the micropylar end; nevertheless thermal treatment is the best, i.e. a temperature of 18^0C for 16 h or 26^0C for 8 h to achieve seed germination. Seedlings ensuing from sexual seed are normally weaker than those from cuttings (Nassar and Ortiz, 2007).

2.2 Origin and Distribution of Cassava

The origin of cassava (*Manihot esculenta*, Cranz) has been obscure for long time. To know the exact origin of a given plant, its botanical and agricultural origin should be known. According to Allem (2002), a wild population of cassava that is indistinguishable on morphological ground from the domestic one was found in central Brazil which confirms the botanical origin of the plant.

Cassava is an ancient crop species, it is estimated that domestication began 5000-7000 years BC. This estimation receives support from archeological findings in the Amazon. According to this estimation, the crop was already cultivated in all Neotropical America by the time when the first European reach there (Allem, 2002).

Olsen and Schaal (1999) form their molecular studies made by DNA analysis, extracted from dried leaves of 157 individuals of *Manihot esculenta* and 35 individuals of *Manihot pruinosa* also confirmed that West Central Brazil (South and East of Amazon basin) and Eastern Peru are the center of origin of cassava. Hillock (2002) also reported that South America region is the origin of cassava.

Cassava was introduced to West Africa coast by Portuguese sailors in the 16^{th} century. Initially the crop was not accepted but now it has spread and gained prominence as a major food for many communities in Sub-Saharan Africa (Were, 2004).

2.3 Ecology and Agronomy of Cassava

Cassava is cultivated throughout the lowland tropics, typically between 30^0N and 30^0S of the equator, in areas where the annual mean temperature is greater than 18^0C. Cassava can also grow poorly in cold climates and growth ceases at temperatures below 10^0C. The plant grows best when rainfall is 1000–1500 mm per year, but it can survive when rainfall is as low as 500 mm per year. When moisture availability is low, cassava plant ceases growth and sheds some of its older leaves. However, it resumes growth and produces new leaves when moisture becomes available (Bolhuis, 1966).

Cassava grows best on light, sandy loam soil of medium fertility with good drainage. On clay or poorly drained soil, growth is generally poor. Cassava can grow and yield well on soils of low fertility, where production of most other crops would be uneconomical. However, on highly fertile soil, cassava produces excessive vegetation at the expense of root formation. Cassava tuberous root formation is controlled by photoperiod. Under short day conditions tuber formation occurs readily, but at a day length of 12 h. its growth delays and yield reduces. Vegetative growth and leaf area of planted cassava cuttings approach a maximum within 5 months (Williams & Gazhali, 1969).

After planting cassava in soil, root starts to enlarge with deposits of starch accumulation by the eighth week and thickening of roots stops after 7–9 months in most cultivars (Nassar and Ortiz, 2007). Cassava is usually harvested after one year. However, because of its perennial nature it continues to grow for several years if left in the ground (Nassar and Ortiz, 2007). Cassava is harvested by hand by raising the lower part of stem and pulling the roots out of the ground, then removing them from the base of the plant. The upper parts of the stems with the leaves are removed off before harvest (Nassar and Ortiz, 2007). High yield of foliage in cassava was obtained by managing the crop as a semi-perennial with repeated harvesting of the foliage at 2–3 month intervals (Foulkes & Preston, 1978).

Cassava tubers once harvested deteriorate rapidly with in 40-48 h. This deterioration in most cases is caused by physiological changes and/or mechanical damage during harvesting, transportation and handling. In order to address this problem, most manufacturers in Nigeria prefer to convert cassava into more stable forms such as chips and flours so as to prolong the shelf life of the product (Ashaye *et al*., 2005).

It is one of the major staple crops in the world with an annual production of 185 million tons. Africa accounts for more than half of the world production, whereas Asia and Latin America are the second and third respectively (Nassar and Ortiz, 2007).

2.4 Production and Utilization of Cassava

Cassava is mainly cultivated in the tropics for its starchy tuberous root. Africa now produces more cassava than the rest of the world. The largest producing nations of Africa are Nigeria (35% of the total Africa production and 19% of world production), Democratic Republic of Congo 19% of African production, Ghana 8%, Tanzania 7% and Mozambique 6% of African production. According to the report of FAO (2001) production yearbook of 1998, Africa produces 85,945 million tones of cassava in the year 1998, which is more than half of the world production of the same year that is 158,620 million tones. In 1998, Nigeria produced 30 million tones of cassava (Hillocks, 2002).

According to the estimation of FAO, 172 million tones of cassava were produced worldwide, in 2000. Africa accounted for 54%, Asia for 28%, and Latin America and the Caribbean for 19% of the total world production (IITA, 1990; IITA, 2007). In the year 2006, the estimated global cassava production was around 226 million tones (FAO, 2008).

In the year 2004, Nigeria was the leading producer of cassava in the world and an estimated cassava output from Nigeria was approximately 34 million tones. This production performance has rated Nigeria as the largest cultivator of cassava in the world (Bamidele *et al*., 2008).

In recent times, Nigerian government has encouraged the use of the cassava to produce a wide range of industrial products such as ethanol, glue, glucose syrup and bread. Moreover, the government promulgated a law making it compulsory for bakers to use composite flour of 10 percent cassava and 90 percent wheat for bread production. The new regulation which came into effect in January 2005 stipulated that the large flour mills that supply flour to bakeries and confectioneries must pre-mix cassava flour with wheat flour (Bamidele *et al.*, 2008). In Southeast Asia and Latin America, cassava has now taken on an economic role (IITA, 2007).

2.5 Importance of Cassava

For many tropical East Africa, Asia and Latina America, cassava is one of the major food source for human beings and animals, because it is the cheapest source of starch. It is the third most important source of calories in the tropics, after rice and corn (Otim-Nape, 1994; Puonti, 1998; Scott *et al*., 2000; FAO, 2001; Aerni, 2005). In the tropics, cassava is a staple food for more than 800 million people. In addition, cassava roots provide more than 60% of the daily energy intake for the population of Northeast Brazil and many countries in Africa (Coursey and Haynes, 1970; Nassar *et al*., 2009). Recently, cassava is used as a raw material in the production of ethanol, glue and glucose syrup (Bamidele *et al*., 2008). In Southeast Asia, it had become an industrial cash crop where it has been a subsistence crop (Nassar and Ortiz, 2007).

2.6 Food Value of Cassava

Table 1. Food values of cassava (Nassar and Ortiz, 2007).

Edible fleshy portion of the tuberous	80% - 90%
Water	60% - 65%
Carbohydrate	30% - 35%
Protein	1% - 2%
Fat	0% - 0.2%
Fiber	1% - 2%
Mineral	1% -1.5%
Food energy (kcal)	124

2.7 Effect of Cassava on Health

Cassava (*Manihot esculenta*) is one of the most widely distributed major human food crop with potentially toxic compounds called cyanogenic glycosides, primarily linamarin and a small amount of lotaustralin (ethyl linamarin). Linamarin is chemically similar to glucose but with cyanide (CN ion) attached to it (O'Hair, 1995; Okafor, 2004).

Unlike other root crops many varieties of cassava contain cyanogenic glucosides, which can lead to chronic toxicity if inadequately processed (IITA, 2007). High cyanide intake from the consumption of insufficiently processed cassava can cause acute toxicity such as vomiting, stomach pains, giddiness, headache, etc. Adverse effects noted in humans from long-term cassava consumption include diseases such as "Konzo" or upper motorneuron diseases, iodine deficiency disorder (goiter) and tropical ataxic neuropathy (Tylleskar *et al*., 1992; Okafor, 2004**)**.

Large scale cassava processing could also be hazardous, not by consuming residual cyanide in food, but the discharge of hydrocyanic acid into the air resulted in contamination of the atmospheric air and natural water sources in areas nearer to large scale processing as well as possible occupational exposures of humans to cyanide poisoning during large scale cassava processing (Okafor, 2004).

According to the finding of Okafor (2004) cyanide poisoning in cassava consuming populations of Nigeria mainly results if food preparation is carelessly done or is almost non-existent rather than ingestion of cassava foods. In addition, iodine deficiency and/or low protein intake could be the major problems of the communities where chronic cassava cyanide diseases were reported.

There are various processing methods that help to reduce the cyanide content and avoid toxicity of the crop, such as peeling the cover, sun drying, fermenting and heat cooking are the most commonly used methods of which fermentation and heat cooking can take the cyanide to a non-toxic level (IITA, 2007).

From the recent findings of Tilahun Abera (2009) the cyanide content of the two released varieties of cassava (‘Kello’ and ‘Qulle’) in Ethiopia which were brought from Uganda, has found to be 4.62$\pm$0.01 mg/100 g for ‘Qulle’ and 6.04$\pm$0.02 for Kello mg/100 g of cassava flour.

2.8 Cassava in Africa

Cassava is considered as an ideal food crop for the tropical Africa. This is due to the fact that crop presents easy propagation systems, high drought tolerance, satisfactory yields even growing in low-fertile soils, low exigency for sophisticated cultural requirements, potential resistance and tolerance to pests and diseases, high root starch contents and good mechanization prospects (Horton, 1988; Negeve, 1999; Ricardo *et al*., 2007).

An estimated 70 million people obtain more than 500 Kcal per day from cassava and more than 800 million people consume 100 Kcal per day (Kawano, 2003; Nassar *et al*., 2009). The ability of the crop to grow on poor soils and under difficult climate as well as the advantage of flexible root harvesting whenever there is a need, make it the ‘crop of last resort’ for farmer families and their domestic animals in the tropics (Hillocks *et al*., 2001). The importance of cassava as a food crop in Africa becomes obvious when its annual consumption per capita is compared to the rest of the world: While the World average of annual cassava consumption lies around 17 kg/capita in 2001, Africa's annual consumption is still above 80 kg/capita. Latin America's consumption has decreased by half over the past 30 years from a peak of above 40 kg/capita in the early 1970s to slightly above 20 kg/capita in 2002 (Aerni, 2005).

In Ethiopia, cassava has been cultivated and used in different parts of the country mainly for its edible tubers. A survey in different parts of South and Southwest Ethiopia, particularly in Gedo zone, showed that cassava is almost used as a staple food. In Wolaita, southwest Ethiopia (Kefa, Bench, Maji, Sheko) and Gambella areas, cassava tubers are consumed widely after washing and boiling or in the form of bread or injera by mixing its flour with different cereal crops such as maize, wheat or tef (Amsalu Nebiyu and Elfinesh Firidisa, 2006).

In Ethiopia, there is high cultivation of cassava in South and Southwest part of the country mainly for its edible tubers. It grows in areas that are 480-1880 m above sea level with annual temperature of 15-30^{0}C and annual precipitation of 692-1470 mm. Some cultivars are quite drought resistant and even the mature roots can stay longer in the ground before being harvested (Mulugeta Taye, 1994; Gilbert, 1995; Amsalu Nebiyu *et al.*, 2000).

2.9 Breeding of Cassava

Despite its high importance to food security, cassava has long been neglected in plant breeding programmes. There are about 100 wild *Manihot* species, which provide an important genetic endowment for cassava breeding. Professional cassava breeding started in the 20th century and was spurred on by increasing population demands. The main breeding goals are high yield per unit area, particularly in marginal or pest-prone environments (Nassar and Ortiz, 2007).

Even though, the potential application of classical breeding in cassava improvement is constrained by the high heterozygosity & outcrossing nature and low natural fertility of the plants. The most notable results from cassava breeding are seen today in Sub-Saharan Africa, where it has been transformed from a poor man's crop to an urban food, and in Southeast Asia, where it has changed from a subsistence crop to an industrial cash crop. Long-term research by many international and national groups has led to the development of high-yielding cassava cultivars that increased crop yields up to 40%. Manipulation of genes from wild species has led to new cultivars that resist prevailing diseases and pests, allowing the avoidance of large-scale famine in sub-Saharan Africa (Nassar and Ortiz, 2007).

Cassava improvement continues to tap genetic variation through conventional breeding (including the use of wild species) and biotechnology, because many pathogens still take their toll and occasionally epidemics affect farmer fields significantly. However, new sources of variation are needed to genetically enhance the nutritional quality of this important food crop in Africa and other areas in the tropics of the developing world (Nassar and Ortiz, 2007).

Biotechnology could provide an efficient tool to complement the traditional breeding, e.g. by engineering disease and pest resistance, improving the protein content of cassava roots and reducing the cyanogenic glucoside content of the plants (Le *et al*., 2007).

2.10 Factors that Affect Cassava Production

Root crops such as sweet potato and cassava usually are found to be infected by one or more viruses. This virus infection causes a significant reduction in yield and tuber quality (Quak, 1977).

Throughout the twentieth century, cassava mosaic disease (African cassava mosaic virus (ACMV)) has been a hindrance to cassava production in Africa (Rossel *et al*., 1988; Were, 2004). Cassava also being affected by other viral and bacterial diseases such as Cassava bacterial blight (CBB), Cassava American latent virus (CALV), Cassava vein mosaic virus (CVMV), Cassava brown streak virus (CBSV) etc (Nichols, 1950; Lozano, 1986; Walter *et al.*, 1989).

Figure 1. Major diseases in Cassava: (A) Cassava brown streak virus,
(B) African cassava mosaic virus and (C) Cassava bacterial blight (FAO, 1991).

Cassava and many others vegetativly propagated root crops are frequently characterized by their inability to produce seed due to presence of one or more factors, such as incompatibility, abnormal seed and seedling development, seed dormancy, virus infection and environmental conditions, which affect flowering and seed setting. Presence of these factors poses limitations on the use of environmental techniques for improvement and production of these crops (Hettiarachchi, 1988; Nassar and Ortiz, 2007).

Application of biotechnological tools has significant roles in addressing different delimitations from classical breeding of cassava, for instance farmers normally practiced propagation of cassava from stem cuttings, which has led to accumulation of viral and bacterial diseases that reduce productivity and may cause loss of superior genotypes (Nassar and Ortiz, 2007). Meristem culture with thermotherapy is the best therapeutic and/or chemotherapy technique to produce virus free plants (Walter *et al.*, 1989).

2.11 Biotechnology in Cassava

The main objective of plant biotechnology is to eliminate or minimize the major limitations of traditional plant breeding (Rao, 1996). Plant biotechnology has become increasingly important at the global level, as it offers opportunities to increase sustainability, profitability and international competitiveness in agriculture and forestry. One of the most commercially exploited components of plant biotechnology has been the rapid clonal multiplication/micropropagation of selected genotypes in diverse groups of plant species through plant tissue culture (Rani and Raina, 2000).

Plant tissue culture refers to the set of techniques designed for the growth and multiplication of cells, tissues, and organs using nutrient solutions in an aseptic and controlled environment by isolating them from the mother plant, this is because cells are totipotent in nature (Loyola-Vargas and Vázques-Flota, 2006). Other practical application of biotechnology includes; somatic embryogenesis, production of virus free plantlets, embryo rescue, production of haploid plants, gene transfer & production of transgenic plants, etc (Rao, 1996; Razdon, 2005).

Commercial application of plant tissue culture began in the USA with the micropropagation of orchids in the 1970s (Govil and Gupta, 1997). Today, micropropagation is a multibillion dollar industry with an estimated annual global market of more than US$15 billion, and is practiced in thousands of nurseries and commercial biotechnology laboratories throughout the world. Since then many vegetables, ornamental plants, food crops, fruit crops, forest trees and cereals have been commercialized using tissue culture (Rani and Raina, 2000).

Use of micropropagation has many advantages over conventional methods of plant propagation of which the major once are: (I) it is possible to generate pathogen-free plants, even from explants of infected mother plants; (ii) it is important in terms of multiplying plants throughout the year, with control over most facets of production; (iii) it is possible to produce haploid organisms; (iv) it can be used for further studies in genetic transformation (v) it enables the production of a large number of plants in a short time from a selected number of genotypes, where the traditional methods of multiplication are either not available or are ineffective in large-scale multiplication systems (Rani *et al*., 1995).

There is *Cassava Biotechnology Network* (CBN) that attempts to provide information to biotechnologists on the needs of developing country cassava users (i.e. producers, processors, marketers, and consumers). The main objective of CBN is to invest in research, but for a proper direction of research efforts. In Latin America and West Africa, the network has access to information of farmers and processors through participatory research conducted by the

International Centre for Tropical Agriculture (CIAT), Colombia, and through the work of the *International Institute for Tropical Agriculture* (IITA), Nigeria. To broaden its geographical scope, CBN has recently collaborated in case studies in Tanzania and China (Aerni, 2005; Thro *et al*., 1994).

Cassava improvement for increased production necessitates addressing various factors, that beset its production. These factors include pests and diseases, genetics and breeding programs but more recently it has been observed that micropropagation has become an irreplaceable tool in the improvement and genetic manipulation of plant, especially vegetatively propagated crops (Onuoch and Onwubiku, 2007).

The ability to culture organized tissue in the form of very small shoots or meristem has allowed the most valuable application of plant tissue culture. Meristem culture has long been used for the production of virus-free plants, while the culture of small shoots and meristem is being exploited for the rapid vegetative multiplication (micropropagation) of a range of horticultural and agricultural plants (Davies, 1981).

Meristem culture is a technique which was first developed by Robbins in 1972 (Robbins, 1972). The essence of meristem culture is the excision of the organized apex of the shoot from a selected donor plant for subsequent i*n vitro* culture. The excised meristem is typically small and removed under sterile condition (Grout, 1990).

During meristem culture one should take a very small stem apex (0.2-1.0 mm in length) consisting of just the apical meristem and/or one or two leaf primordial and excludes any differentiated pro-vascular or vascular tissue (Grout, 1990; George, 2008).

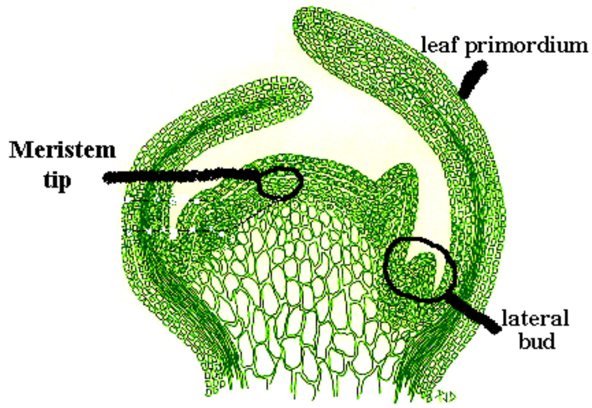


Figure 2. Apical meristem of plants (Kartha, 1974).

As it has been exploited for many other crops, tissue culture technology could offer a very valuable tool for improvement of root crops. Apart from that, meristem culture techniques have several important applications in germplasm conservation, excluding of pathogenic organisms that may have been present in the donor plants and also there is stability of genetic inheritance since plantlet production is from an already differentiated apical meristem (Robbins, 1972; Hu *et al.*, 1984; Grout, 1990; Brian, 1999; Grout, 1999).

In order to apply tissue culture technique, the establishment of culture medium and adjustment of their concentration with maintenance of aseptic condition are mandatory and the keys to success in micropropagation (Onuoch and Onwubiku, 2007). Moreover, *in vitro* propagated plantlets needs to be hardened gradually in order to improve survival upon transfer to soil, because the plantlets have small juvenile leaves with reduced photosynthetic capacity and malfunctioning stomata (Pierik, 1993; Lineberger, 2006).

3. OBJECTIVES

3.1. General objective

- To develop *in vitro* micropropagation protocol for selected cassava (*Manihot esculenta* Crantz) varieties ('Qulle' and 'Kello').

3.2. Specific objectives

- To generate optimum medium for *in vitro* shoot induction from meristem culture
- To optimize suitable medium for shoot multiplication of *in vitro* cultured cassava
- To optimize rooting medium for *in vitro* propagated cassava shoots
- To acclimatize *in vitro* grown cassava plantlets
- To evaluate the survival rate of plantlets in the glasshouse

4. MATERIALS AND METHODS

4.1 Donor Plant Preparation and Growth Condition

Two varieties of cassava namely 'Qulle' and 'Kello' were taken as plant materials for this study. Freshly harvested stem cuttings of these varieties were obtained from Hawassa Agricultural Research Center, Root Crops Research Division. The stems were planted in a pot containing red soil, compost and sand at a ratio of 1:1:2 respectively and allowed to sprout in glasshouse, at Holeta Agricultural Research Center (HARC), at average temperature of 25^0 C ± 2^0 C under natural light condition.

4.2 Stock Solutions and Medium Preparation

4.2.1 MS stock preparation

The Murashige and Skoog (1962) MS nutrient with its full macro, micro and vitamin compositions were used as the basic components of the medium (Appendix 1). All the macro, micro and vitamin components of MS stock solution was prepared by weighing and dissolving the powder in double distilled water as recommended and stored in refrigerator at a temperature of $+4^0$C. The prepared stock solutions were used for a maximum of one month.

4.2.2 Growth regulators stock preparation

Different growth regulators were used for this thesis research 6-benzyl aminopurine (BAP), gibberrelic acid (GA_3), α-naphthalene acetic acid (NAA) and indol-3-butyric acid (IBA). All growth regulator stock solutions were prepared by weighing and dissolving the powder in double distilled water at the ratio of 1mg/ml. To begin the dissolving process 3-4 drops of 1N NaOH, 1N HCl or 94% ethanol were added based on the requirement of the growth regulators (NaOH for auxin, HCl for cytokinin and ethanol for gibbereline were used). Then, the volume was adjusted by adding double distilled water. Finally, growth regulators stock solutions were stored in a refrigerator at a temperature of $+4^0$C for one month.

4.2.3 Culture medium preparation

Culture medium was prepared by taking the proper amount of MS stock solutions (100 ml/L macro, 10 ml/L micro and 10 ml/L vitamin) then 3% sucrose (w/v) was added. After mixing all the components and adjusting the volume, growth regulators were added as required and pH was adjusted to 5.8 using 1N NaOH or 1N HCl. Finally, 7.5 g of agar was added and the medium was sterilized by autoclaving at a temperature of 121 ^{0}C with a pressure of 0.15 kpa for 15 min.

For shoot induction from meristem, 20 ml of the medium was poured onto 90 mm sterilized glass Petri dish. For shoot multiplication, 40 ml of the medium was poured into Magenta culture vessels and for rooting 10 ml of the medium was pored into 20 mm diameter test tube. Different concentration and combination of growth regulators were considered to optimize shoot initiation, shoot multiplication and rooting of the two varieties.

4.3 Explants Collection and Surface Sterilization

Shoot tips (15-20 mm) from both varieties were taken from two-month-old glasshouse grown plants. The explants were washed twice with tap water and rinsed with double distilled water once. Then, the explants were sterilized in 70% alcohol for 1 min and rinsed three times with sterile double distilled water and also further disinfected with 1% sodium hypochlorite solution containing one drop of tween 20 for 7 min followed by rinsing five times in sterile distilled water.

4.4 Meristem Isolation and Shoot Induction

Apical and lateral meristems consisting of the meristematic dome with one or two leaf primordia were isolated using dissecting microscope, sterile hypodermic needle and scalpel. The excised meristem was immediately inoculated onto freshly prepared culture medium to avoid dehydration. Five meristems were cultured onto each Petri dish (90 mm) containing 20 ml basal medium supplemented with 0.01 mg/l NAA, 1 mg/l GA_3 and different concentration of BAP. The Petri dishes were sealed with Para film. (Table 3).

4.5 Culture Conditions

All cultures were kept in a growth room under 16 h light (2700 lux light intensity) and 8 h dark cycle at 25^0C± 2^0 C. Sub-culturing was carried out every four weeks.

4.6 Treatment combination for shoot induction

The effects of five different concentration of BAP (0.1, 0.5, 1, 2, and 5 mg/l) with 0.01 mg/l NAA and 1 mg/l GA_3 was compared on shoot induction from meristem culture of the two selected cassava varieties. Growth regulator free MS basal medium was also used as a control.

For each treatment a total of 60 sample units were used, 30 explants for each variety, 6 replication per treatment and 5 explants per Petri dish were used. On every five day bases the number of dead meristems, the number of meristems that give shoot and those which form callus were counted.

4.7 Shoot Multiplication

Meristem-derived shoots were used as explants and inoculated onto freshly prepared MS basal medium containing different concentrations of growth regulators. For shoot multiplication 19 different concentrations and combinations of growth regulators were used (Table 2). In addition, growth regulators free MS basal medium was used as a control. Magenta culture vessel with three shoots was considered as a unit of replication and there were five replications for each treatment. The cultures were incubated at 25^0C± 2^0 C with 16 h photoperiod in a growth room. The number of shoots per explants, shoot height of the main shoot, number of nodes and number of leaves was recorded after one month.

Table 2 Different growth regulators combinations for shoot multiplication

Treatments						
BAP	GA_3	BAP × GA_3		BAP × GA_3 × NAA		
0	0	0	0	0	0	0
0.1	0.1	0.1	1	0.5	1	0.01
0.5	0.5	0.5	1	1	1	0.01
1	1	1	1	_	_	_
2	2	2	1	_	_	_
5	5	5	1	_	_	_
10	10	_	_	_	_	_

Growth regulators used in mg/l.

4.8 Rooting

Rooting experiment was conducted using shoots of both varieties on rooting medium supplemented with half and full strength of MS consisting of different IBA concentration (0.01, 0.1, 0.5. 1 mg/l). In both cases, full and half MS basal medium without growth regulator were used as a control to examine the rooting potential of the two varieties. A single shoot with 1cm and more was cultured in a test tube. Each unit was considered as a unit of replication and there were 20 replications for each treatment's per variety. The cultures were maintained in a growth room of 16 h photoperiod at 25^0C± 2^0 C. The number of main roots per shoot and length of longest root were recorded after two weeks.

4.9 Acclimatization and Growing of Plantlets in the Glasshouse

After being in rooting medium for two weeks, all shoots were transferred to a pot containing red soil, compost and sand in the ratio of 1:1:2. Each pot with plantlet was covered with plastic bags and transferred to glasshouse, the plastic bag gradually removed after a week. The number of surviving plants in the glasshouse was recorded after being in the glass house for three weeks.

4.10 Experimental Design and Data Analysis

For this experiment completely randomized design (CRD) were used. Statistical analysis of quantitative data was carried out by JMP/SAS computer software of version 8.0.1. A difference at probability level of $p \leq 0.05$ was considered significant for all analyses. The whole experimental activities were done in Holeta Agricultural Research Center (HARC).

5. RESULTS

5.1 Meristem Survival and Growth Initiation

Survived meristems start to respond within 5-10 days after culturing. The responses include swelling of the meristem, color change, callus formation and direct shoot induction (Figure 3 and Figure 4). For both varieties, after being in growth initiation medium for 25 days, all the meristems cultured were survived and responded on the media containing 5 mg/l BAP, 1 mg/l GA_3 and 0.01 mg/l NAA. On contrast the lowest meristem survival (76.7%) for 'Kello' variety was observed on MS containing 0.5 mg/l BAP with 1 mg/l GA3 and 0.01 mg/l NAA, for 'Qulle' 80% survival on the control (Table 3).

Table 3. Percentage of survived meristems of two cassava varieties ('Kello' and 'Qulle') on different growth initiation medium supplemented with different growth regulator concentrations after 25 days

Growth regulators (mg/l)			Survived meristems (%)		Dead meristems(%)	
BAP	GA_3	NAA	'Kello'	'Qulle'	'Kello'	'Qulle'
0	0	0	96.67^a	80.00^a	3.33^a	20.00^a
0.1	1	0.01	90.00^a	96.67^a	10.00^a	3.33^a
0.5	1	0.01	76.67^a	95.65^a	23.33^a	4.35^a
1	1	0.01	96.67^a	100.00^a	3.33^a	0.00^a
2	1	0.01	83.33^a	96.67^a	16.67^a	3.33^a
5	1	0.01	100.00^a	100.00^a	0.00^a	0.00^a

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

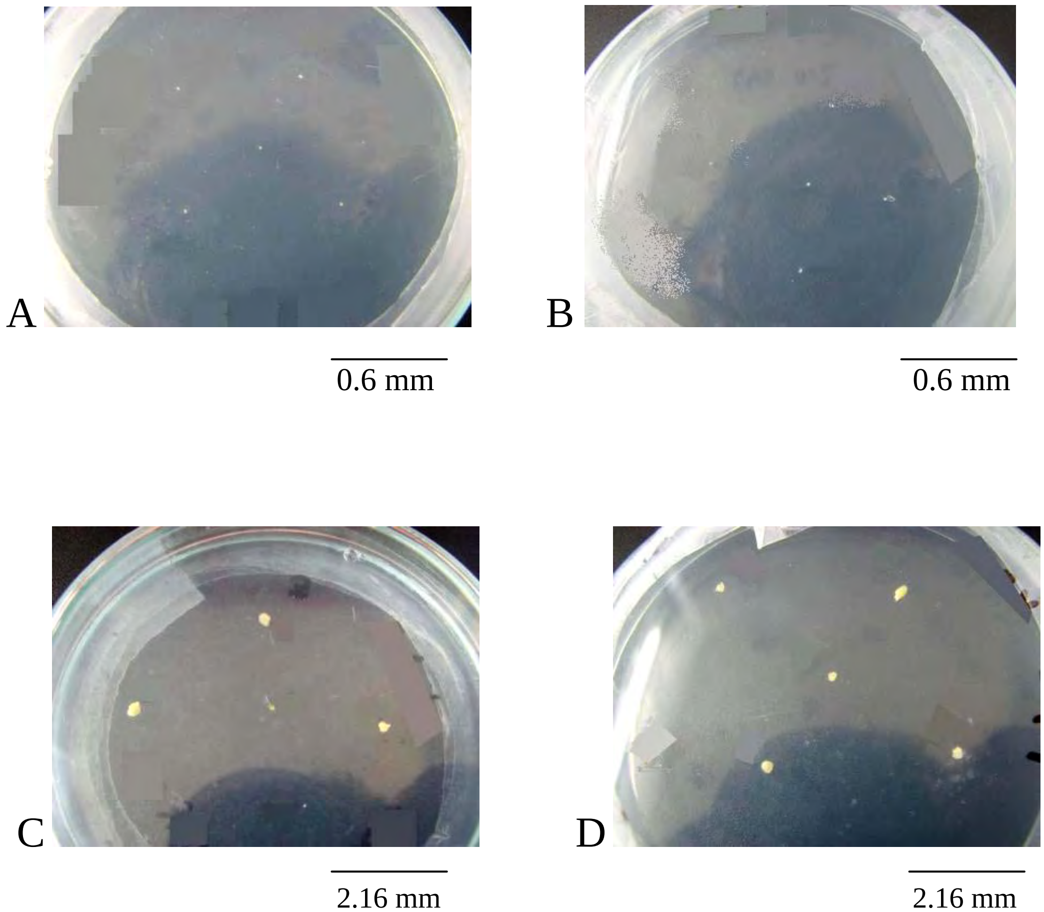


Figure 3 The response of meristem cultures of the two cassava varieties in 0.5 mg/l BAP with 1 mg/l GA3 and 0.01 mg/l NAA
(A) ‘Kello’ and (B) ‘Qulle’ after one day of culture, starts to become white in color
(C) ‘Kello’ and (D) ‘Qulle’ after 10 days of culture showing increment in size

5.2 Shoot Induction

Those meristems that responded to the initiation medium were maintained in the same medium. After 2-3 weeks, they were started to induce shoot and/or form callus (Fig.4). The control for both varieties remains at callus stage even after two months. The maximum average shoot, 84.0 % for ‘Kello’ and 82.76% for ‘Qulle’, were induced on the media supplemented with 2mg/l BAP, 1mg/l GA_3 and 0.01mg/l NAA for both varieties (Figure 5). As BAP concentration increases the percentage of shoot induction also increases except on medium supplemented with 5 mg/l BAP and the same GA_3 and NAA concentrations for ‘Qulle’, which forms undifferentiated green callus like structure. For both varieties, morphologically, best shoot proliferation was observed on MS medium supplemented with 0.5 and 1 mg/l BAP in combination with 1 mg/l GA_3 and 0.01 mg/l NAA. However, at 2 and 5 mg/l BAP levels with 1 mg/l GA_3 and 0.01 mg/l NAA; the shoots were become bushy and remain very short with abnormal leave structures (Fig 8). The percentage of callus formation also varies with variation in BAP concentration; at 0.1 mg/l of BAP all cultures of 'Kello' and 96.7% of 'Qulle' induce callus.

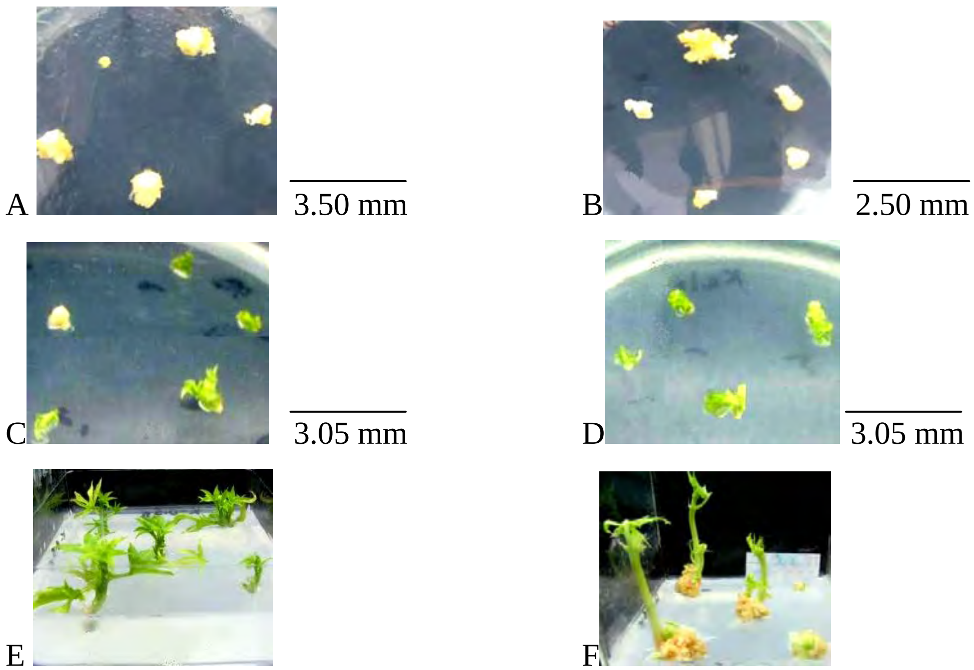


Figure 4. Callus formation and shoot induction from the meristem cultures in different medium combinations.

(A) and (B) Callus of 'Kello' and 'Qulle' respectively, after three weeks in 0.1 mg/l BAP with 1 mg/l GA_3 & 0.01 mg/l NAA

(C) and (D) Shoot induced in 'Kello' and 'Qulle' respectively, after four weeks in 0.5 mg/l BAP with 1mg/l GA_3 and 0.01 mg/l NAA

(E) and (F) Shoots of 'Kello'and 'Qulle' respectively, after seven weeks in 0.5 mg/l BAP with 1mg/l GA_3 and 0.01 mg/l NAA

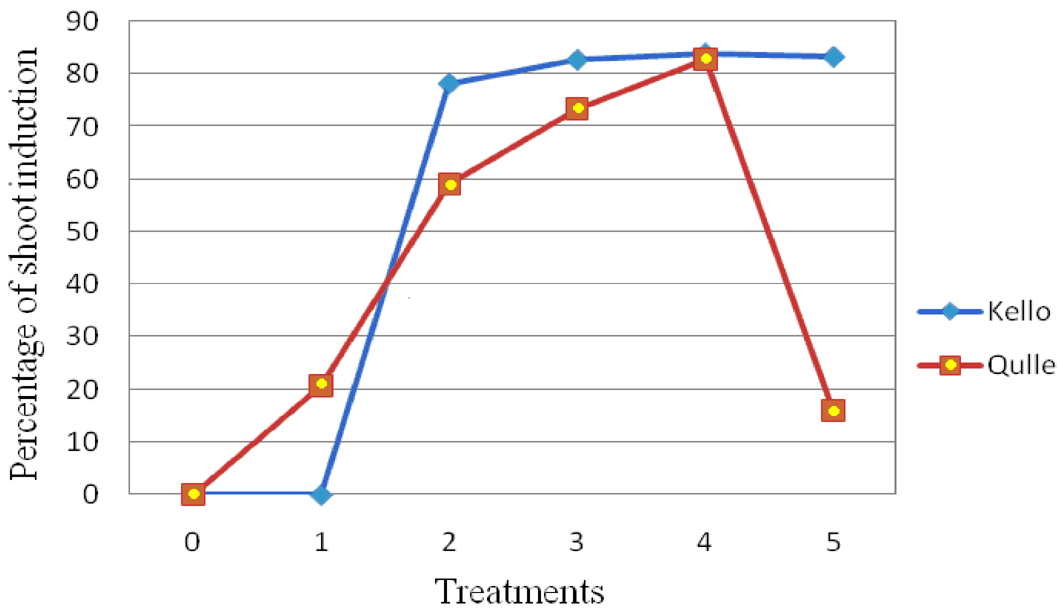


Figure 5. Shoot induction rate (%) of the two cassava varieties from survived merisems cultures after one month

0: growth regulators free,
1: 0.1 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA
2: 0.5 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA
3: 1 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA
4: 2 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA
5: 5 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA

A

B

Figure 6. Shoot proliferation rate of two cassava varieties on different shoot induction medium after three month.
(A) 'Kello' and (B) 'Qulle'.

5.3 Shoot Multiplication

After three sub-culturing, callus and spontaneous roots of the induced shoots were removed and shoot-tips were transferred to Magenta culture vessels containing 40 ml shoot multiplication medium consisting of MS supplemented with different growth regulators concentration and combinations (Table 2).

The cultured shoot-tips were responded differently to the difference in multiplication medium (Table 4-7 and Figure 7). For both varieties the maximum mean number of shoots per explants was obtained on multiplication medium supplemented with 0.5 mg/l BAP in combination with

1mg/l GA_3 and 0.01 mg/l NAA, 12.23 and 7.22 for ‘Qulle’ and ‘Kello’ varieties respectively (Table 7). However, only a maximum of 1.88 and 1.62 mean numbers of shoots per explants were produced on best responding multiplication medium supplemented with GA_3 only for ‘Qulle’ and ‘Kello’ varieties respectively (Table 5). In medium with BAP only the maximum mean number of shoot per explants for ‘Qulle’ and ‘Kello’ was counted to be 6.42 and 4.75 respectively in MS medium with 0.5 mg/l BAP. But at high concentration of BAP shoots become very dwarf and bushy. The use of BAP and GA_3 in combination for shoot multiplication was found to be better than use of GA_3 alone, but lower than BAP only and the combination of BAP, GA_3 and NAA. Hence 4.10 and 3.90 mean number of shoots were produced per explants for ‘Qulle’ and ‘Kello’ respectively, on MS containing 0.5 mg/l BAP in combination with 1 mg/l GA_3 (Table 6).

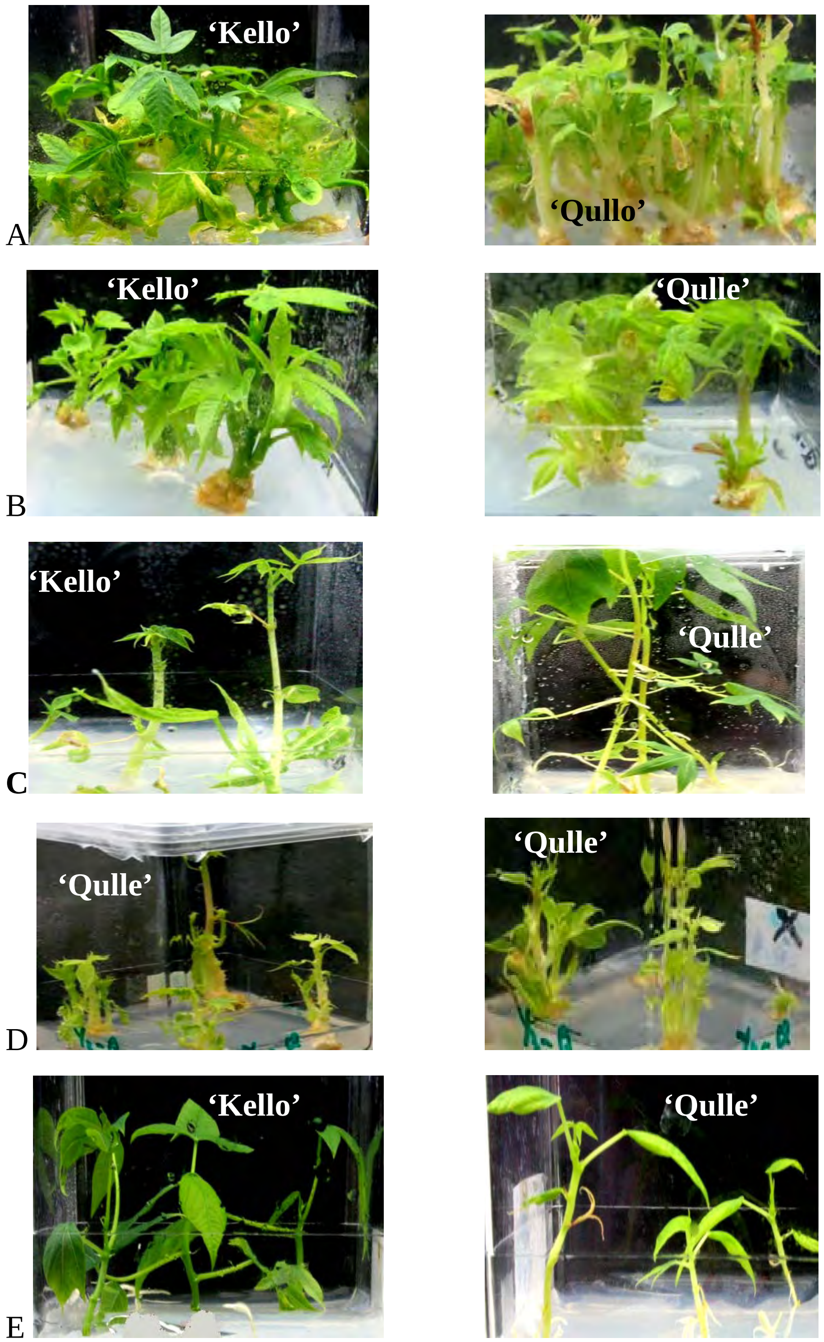


Figure 7.Multiplication rate of two cassava varieties on different multiplication medium.
(A) 0.5 mg/l BAP, 1 mg/l GA_3 and 0.01 mg/l NAA (B) 0.5 mg/l, BAP
(C) 1 mg/l, GA_3 (D) 2 mg/l BAP and 1 mg/l GA_3 and
(E) Control (without Growth regulators).

Table 4. Mean number of shoots per explants in different BAP consecration medium.

BAP (mg/l)	Mean No. of shoot/explants	
	'Kello'	'Qulle'
0.1	3.04^{ab}	5.57^{ab}
0.5	4.76^{a}	6.42^{a}
1	4.23^{ab}	5.82^{a}
2	3.66^{ab}	4.27^{bcd}
5	3.31^{ab}	2.85^{CDE}
10	2.36^{bc}	2.43^{cd}
0	1.40^{c}	1.00^{d}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Table 5. Mean number of shoots per explants in different GA_3 consecration medium.

GA_3(mg/l)	Mean No. of shoot/explants	
	'Kello'	'Qulle'
0.1	1.12^{a}	1.66^{ab}
0.5	1.37^{a}	1.61^{ab}
1	1.41^{a}	1.70^{ab}
2	1.41^{a}	1.70^{ab}
5	1.27^{a}	1.46^{ab}
10	1.62^{a}	1.88^{ab}
0	1.40^{a}	1.00^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Table 6. Mean number of shoots per explants in different BAP and GA_3 combinations medium.

BAP(mg/l)	GA_3(mg/l)	Mean No. of shoot/explants	
		'Kello'	'Qulle'
0.1	1	2.33^{cd}	2.79^{b}
0.5	1	3.90^{a}	4.10^{a}
1	1	3.66^{ab}	3.39^{ab}
2	1	3.56^{ab}	3.03^{b}
5	1	2.72^{bc}	1.41^{c}
0	0	1.40^{d}	1.00^{c}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Table 7. Mean number of shoots per explants in different BAP, GA_3 and NAA combinations medium.

BAP(mg/l)	GA_3(mg/l)	NAA (mg/l)	Mean No. of shoot/explants	
			'Kello'	'Qulle'
0.5	1	0.01	7.22[a]	12.23[a]
1	1	0.01	2.07[b]	3.79[b]
0	0	0	1.40[b]	1.00[c]

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Figure 8. Bushy and dwarf shoots developed from shoot-tip culture of 'Qulle' variety at 5 mg/l of BAP.

5.4 Rooting

Those shoots with 1cm and more in height were taken and transferred onto rooting medium that contains full and half strength MS supplemented with different IBA concentrations with (Table 8). The mean number of main roots developed per shoots in full and half strength MS rooting medium with the same IBA concentration doesn't show a significant difference at probability level of 0.05. For 'Kello' a maximum of 15 roots were counted in both half and full MS strength medium with IBA concentration of 0.5 mg/l and 1mg/l respectively and in 'Qulle' a maximum of 18 roots were counted in half MS medium and 17 roots in full MS medium both with 1mg/l IBA.

Table 8. Comparison of mean number of roots per explants in full and half strength MS rooting medium with different IBA concentration.

Treatment	MS	IBA (mg/l)	Mean No. of roots/explant
RH0	Half	0	2.87^{c}
RH1	Half	0.01	3.39^{c}
RH2	Half	0.1	4.16^{bc}
RH3	Half	0.5	5.89^{ab}
RH4	Half	1	7.02^{a}
RF0	Full	0	3.60^{c}
RF1	Full	0.01	3.69^{c}
RF2	Full	0.1	4.41^{bc}
RF3	Full	0.5	7.29^{a}
RF4	Full	1	7.26^{a}

Numbers connected by the same superscript letters in the same column are not significantly different at 0.05% probability level.

In general, there was a variation in the number of main roots per explants with variation in IBA concentration. For ‘Qulle’ the highest mean number of roots per shoot (9.35) was recorded in half MS medium with 1 mg/l IBA and for ‘Kello’ a maximum of 7.00 mean roots per shoot was observed in full MS medium with IBA concentration of 1 mg/l (Figure 9 and 10).

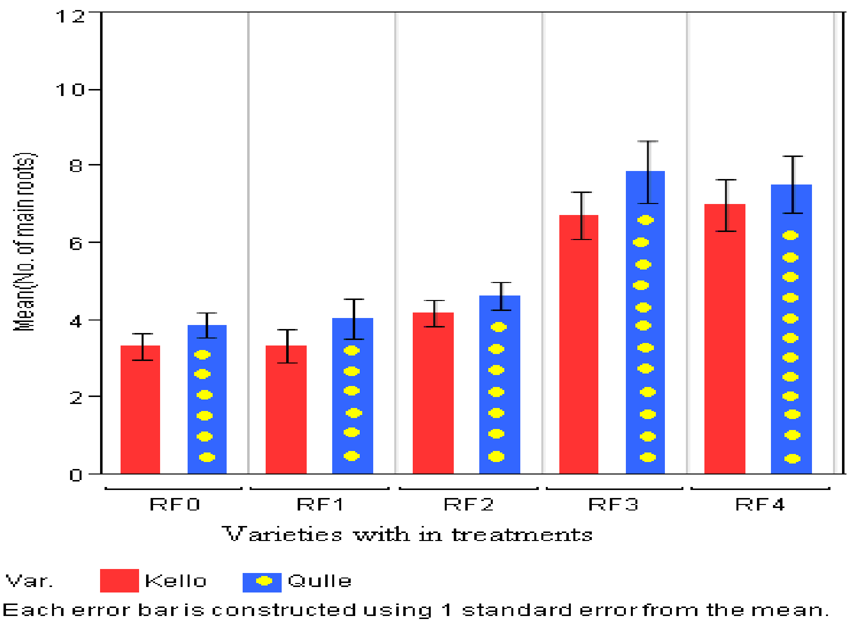


Figure 9. Rooting frequency of 'Kello' and 'Qulle' in full strength MS with various IBA concentration.

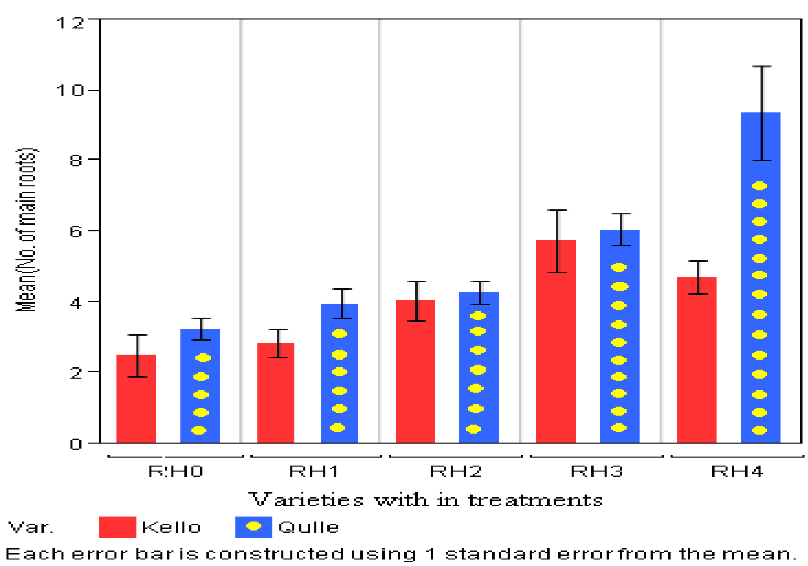


Figure 10. Rooting frequency of 'Kello' and 'Qulle' in half strength MS with various IBA concentration.

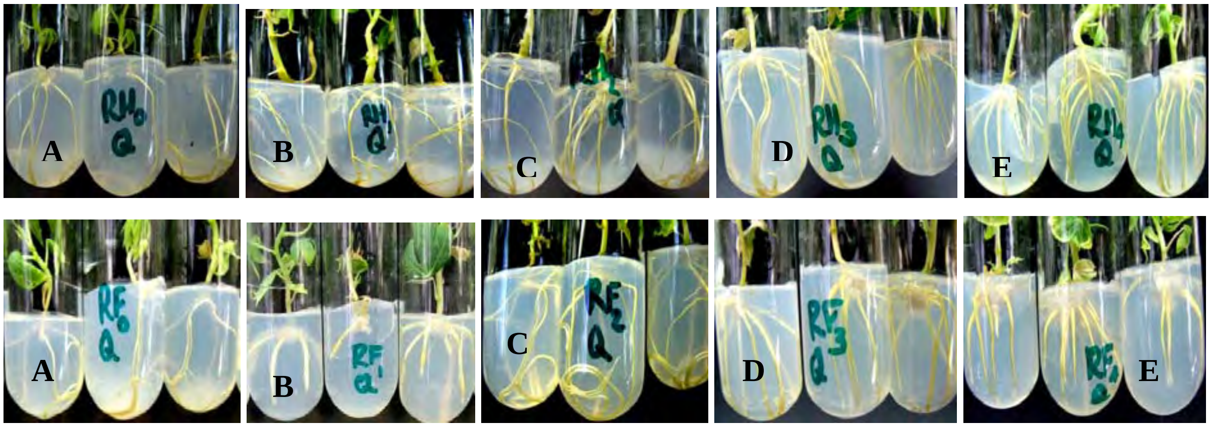


Figure 11. Variation in rooting frequency of 'Qulle' in half strength MS (RH0-RH4) and full strength MS (RF0-RF4) with different IBA concentration.
(0, 0.01, 0.1, 0.5 and 1 mg/l of IBA in respect to the letters A,B,C,D,E).

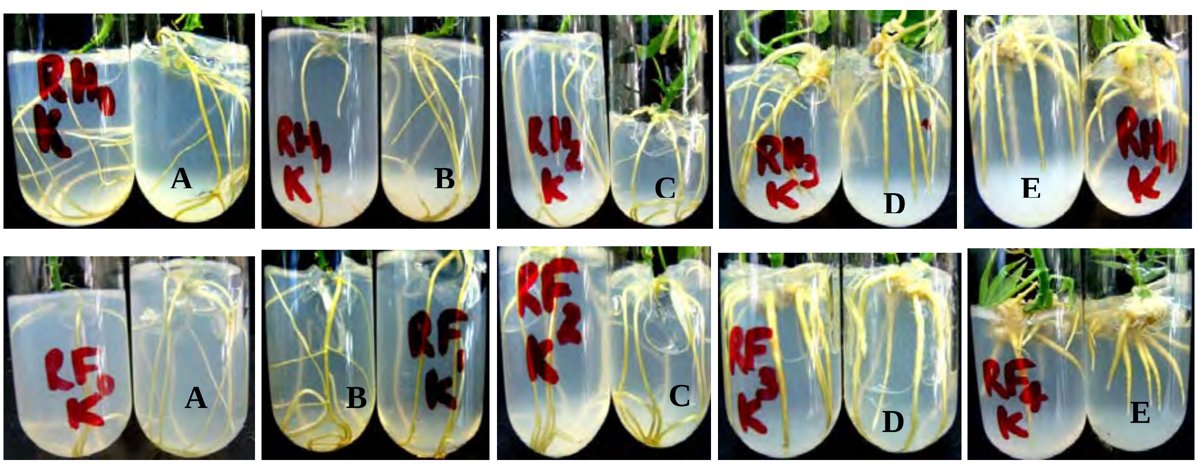
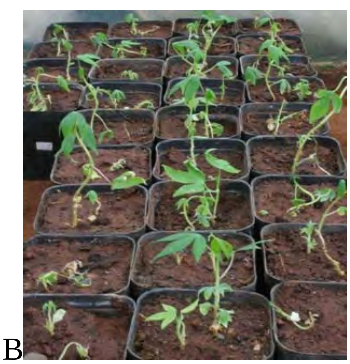


Figure 12. Variation in rooting frequency of Kulle in half strength MS (RH0-RH4) and full strength MS (RF0-RF4) with different IBA concentration.
(0, 0.01, 0.1, 0.5 and 1 mg/l of IBA in respect to the letters A,B,C,D,E).

5.5 Acclimatization

After being in the rooting medium for 15 days, those shoots that develop roots were transferred to a soil mixture of red soil, compost and sand in the ratio of 1:1:2. For those shoots which acclimatized in the screen house with a sterile soil mix the survival rate was found to be 72.8% and 67% for ‘Qulle’ and ‘Kello’ varieties respectively and for those in the glass house with a sterile soil mix the survival rate increases to 89.1% and 75% for respective ‘Qulle’ and ‘Kello’ varieties. But plantlets which acclimatized in unsterile soil mix the survival rate was severely affected up to 0% by cutworms which cut down the stem from the bottom. Further observation of survived individual plantlets in the greenhouse revealed no aberrant phenotypes.

A


B


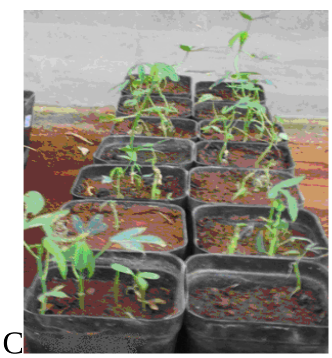
C


Figure 13. Acclimatization of cassava *in vitro* plantlets in the greenhouse:
(A) During acclimatization (B) ‘Qulle’ after three weeks (C) ‘Kello’ after three weeks

6. DISCUSSION

6.1 Meristem culture and growth initiation

After passing through standard tissue culture sterilization techniques meristems were isolated and cultured onto MS medium supplemented with different combination and concentration of BAP, GA_3 and NAA. The use of this three growth regulators during growth initiate from meristem culture of different cassava varieties were also recommended by Kartha *et al*. (1974). In addition Razdan (2005) reported the use of BAP, GA_3 and NAA is essential for plant regeneration form excised meristem culture of cassava.

In Acedo (2006) better response for rapid growth initiation from meristem culture of cassava (60-80%) was obtained in MS medium supplemented with BAP, GA_3 and NAA, in present study 'Kello' and 'Qulle' varieties were also responded to the medium, 76.67-100% and 80.0-100% respectively.

6.2 Shoot induction

In the work of Acedo (2006) the highest percentage of shoot induction (80%) from meristem was obtained on MS medium supplemented with 0.25 mg/l GA_3, 0.1 mg/l BAP and 0.2 mg/l NAA. In the present study, MS medium with 2 mg/l BAP, 1 mg/l GA_3 and 0.01 mg/l NAA gave 84.0% shoot induction for 'Kello' and 82.7% for 'Qulle'.

From the present study it was observed that, as BAP concentration increases to 2 mg/l all survived meristems gave shoot but they become very bushy and short. In contrast on 0.01mg/l BAP with 1 mg/l GA_3 and 0.01mg/lNAA except one meristem the rest gave callus and dies after 45 days by turning into dark brown color. On 5 mg/l BAP, 1 mg/l GA_3 and 0.01 mg/l MS medium meristems enlarged and give undifferentiated shoot like green structure that doesn't grow any further. But on 0.5 and 1 mg/l BAP in combination with 1mg/l GA3 and 0.01mg/l NAA both varieties gives morphologically good looking shoots. These differences on the growth morphology of cultures may be related with the physiological effect of BAP that is, at high concentration, synthetic BAP has inhibitory effect on shoot growth.

6.3 Shoot multiplication

In this work, shoot multiplication potential of the two varieties was compared using solid MS medium with various growth regulators combinations. For both varieties the highest number of shoot per explants was obtained in multiplication medium with 0.5 mg/l BAP in combination with 1 mg/l GA_3 and 0.01 mg/l NAA, where 27 shoots for 'Qulle' and 21 soots for 'Kello' have been counted from. This might be due to the combination effect of the three growth regulators (Staden *et al*., 2008).

In the work Konan *et al*. (1997) they have counted a maximum of 25 shoots per explants in solid MS medium with 10 mg/l BAP. The multiplication data for the present study was collected on the first subculture of plantlets after four weeks in the multiplication medium. Thus the number might be improved much in the second and third subculture.

Konan *et al*. (2006) in their work using nodal explants of cassava with axillary meristems cultured on MS medium supplemented with 0.1 mg/1 NAA, 1 mg/1 BAP and 0.1 mg/1 GA_3, they have reported that this combination of growth regulators is best to produced multiple shoots and Smith *et al*. (1986) in their report from *in vitro* propagation of cassava using nodal culture, they recommended that the use of 1.0mg/l BAP, supplemented with 0.25mg/l NAA induced multiple-shoot formation

As BAP concentration increases to 5mg/l and 10mg/l, in multiplication medium with BAP only shoots become very dwarf and starts to develop abnormal morphological appearance of stem and leaves. This might be resulted from the supra-optimal amount of the hormone. This observation is consistent with the physiological effect of BAP on plants; Berrie (1984) reported that synthetic cytokinins are inhibitory to shoot growth at high concentration. Similar finding with the present study in the relationship BAP concentration and shoot growth was also reported by Onuoch and Onwubiku (2007).

However, in medium with GA_3 only and in the control the lowest number of shoot per explants were obtained. In GA_3 medium rather than multiplying the number, shoots become very thin and long as the concentration of the hormone increases; similar result was also obtained in the work of Acedo (2006). This might be due to the physiological effect of the hormone, that GA_3 increase stem elongation and inhibit formation of adventitious root and shoot formation (Moshkvo *et al*., 2008).

But in multiplication medium supplemented with combination of BAP and GA_3 shoots with very good morphological appearance (reasonable shoot height, stem thickness and leaf structure in comparison with the other multiplication medium combinations) were obtained, which might be related with the combination effect of the two growth regulators.

Spontaneous rooting was observed in multiplication medium with GA_3 and the control but there was no rooting in medium with BAP only. But very few rooting were observed in medium with BAP and GA_3 combinations. This result was consistent with the work of Acedo (2006).

6.4 Rooting and acclimatization

In this study, the effect of full and half MS strength medium for rooting and the relationship between different IBA concentrations and root induction potential of the two varieties were observed. Statistically no significant difference was observed in full and half strength MS medium of the same IBA concentration at a probability level of 0.05. However, rooting frequency of plantlets varies with variation in the concentration of IBA. In both half and full strength MS at 0, 0.01 and 0.1mg/l of IBA there is less rooting frequency as compared to the other two treatments and roots are long and fragile with very few number of secondary roots nevertheless, at high concentration of IBA (0.5 and 1mg/l of IBA) roots become shorten and thick without secondary roots. The work of Smith *et al*. (1986) also shows that use of 2.5 mg/l of IBA is good to improve root initiation of cassava plantlets which agrees with the present finding that rooting increases with high IBA concentration.

The survival rate of the two cassava varieties in the green house was found to be 89.1% and 75% for ‘Qulle’ and ‘Kello’ respectively.

7. CONCLUSION

Meristematic cells are cells which are at the state of active cell division, thus use of meristem culture enable to increase the multiplication potential of cassava plantlets. According to the findings of the present experiment

- The establishments of culture medium by adjusting their concentration and maintenance of aseptic conditions are the keys to success for *in vitro* multiplication of cassava.
- Meristem response was best for both varieties on 5 mg/l BAP with 1 mg/l GA_3 and 0.01 mg/l NAA.
- Best shoot induction potential was observed on 1 and 2 mg/l BAP with 1 mg/l GA_3 and 0.01 mg/l NAA for 'Kello' and on 5 mg/l BAP with 1mg/l GA_3 and 0.01 mg/l NAA for 'Qulle', but shoots exhibited abnormal physiological appearance.
- In both varieties very good morphological appearance of shoots were observed on 0.5 and 1 mg/l BAP with 1 mg/l GA_3 and 0.01 mg/l NAA.
- For multiplication, 0.5 mg/l of BAP in combination with 1 mg/l GA_3 and 0.01 mg/l NAA was found to be the best for both varieties.
- Statistically no significant difference was observed in full and half strength MS medium for rooting, as a result using of half strength MS is enough and economical for rooting of cassava.
- To promote better root induction potential, it is best to use 1 mg/l of IBA for 'Qulle' and 0.5mg/l for 'Kello'.
- Good rate of survival of plantlets was achieved when acclimatization was done in the glasshouse with sterile soil mix.
- In this investigation as such a significant difference was not observed in the response of the two varieties to different initiation, multiplication and rooting medium combinations.

8. RECOMMENDATIONS

- It is important to develop meristem culture micropropagation protocol for other cassava varieties.
- More subculturing should be done to examine the subculture stages at which maximum shoot multiplication takes place and the stage when the multiplication rate starts to decline.
- One advantage of meristem culture is production of virus free plantlets so in the future studies using meristem culture virus indexing should be incorporated.
- Using the developed protocol, cassava plantlets should be multiplied and distributed to farmers.
- Promotion activities should be done in order to familiarize the crop with different parts of the country

9. REFERENCE

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Appendix 1.

Table 9.Nutrient composition and concentration of full strength MS basal medium.

Components	Concentration (gm/L)
Macronutrients	
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_2O$	3.7
KH_2PO_4	1.7
Micronutrients	
Fe-Na-EDTA	4.0
$ZnSO_4.7H_2O$	0.86
H_3BO_3	0.62
$MnSO_4.4H_2O$	2.23
$CuSO_4.5H_2O$	0.0025
KI	0.083
$Na_2MoO_4.2H_2O$	0.025
$CoCl_2.6H_2O$	0.0025
Organic supplements	
Myo-inositol	1.0
Glycin (Glycocoll)	0.2
Nicotinic acid	0.05
Pyridoxin (B6)	0.05
Thiamin (B1)	0.01