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Factors influencing micropropagation and somatic embryogenesis of two, Kello and Qulle, cassava varieties

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2,4-D	2,4-Dichlorophenoxy acetic acid
BAP	6-Benzyl Amino Purine
CMD	Cassava Mosaic Disease
EU	European Union
FEC	Friable Embryogenic Calli
GA_3	Gibberrelic acid
HCN	Hydrogen Cyanide
K	„Kello“ variety
MS	Murashig and Skoog
NAA	α-naphtaline acetic acid
PGRs	Plant Growth Regulators
ppm	Parts per million
TDZ	Thidiazuron
TMS	Tropical Manioc Selection
Q	„Qulle“ variety

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Abstract

*Cassava (*Manihot esculenta *Crantz) is a perennial shrub of the Euphorbiaceae family grown in the tropics, African and Asian continents for its starchy tuberous roots. Its cultivation is constrained by the lack of high quality seed, low productivity and profit, virus and insect pests, high heterozygosity, low natural fertility, poor seed set and seed germination. These problems are difficult to be dealt with traditional breeding system. Therefore, other biotechnological methods are required to solve the above mentioned problems. The objective of this study was to overcome the above mentioned tribulations and come up with the best way to get a maximum number of shoots and morphologically fit* in vitro *materials through micropropagation and along with that to study the different factors that are associated with the micropropagation of the two cassava varieties; 'Kello' and 'Qulle'. The study also includes a method of somatic embryogenesis that is an ideal source of* in vitro *materials for large-scale propagation. In this experiment a Solid MS medium with different salt strengths, sucrose concentrations, TDZ, a two-step procedure involving pre-soak of nodal explants into a liquid MS medium prior to culture on a solid MS medium, with different pH values, repeated subcultures and somatic embryo induction on MS medium supplemented with 2,4-D alone and in combination with 2 µM CuSO4 were used. Maximum mean number of shoots per explant as well as a better morphological property of* in vitro *material was obtained at 0.2 mg/L TDZ for both 'Kello' and 'Qulle'. In both varieties, the two-step culture system resulted in a maximum mean number of shoots/explant at a TDZ concentration of 0.2 mg/L. A medium salt strength of quarter produced maximum mean number of shoots per explants in case of 'Kello' while in 'Qulle' at a full strength MS medium. The highest mean number of shoots per explant for 'Kello' and 'Qulle' was obtained at pH 5.6 and 6.6, respectively. For all parameters maximum mean numbers for both 'Kello' and 'Qulle' were obtained at 0.15 % sucrose. During somatic embryo induction the treatments with different 2,4-D concentrations induced FEC and somatic embryos but the somatic embryos failed to develop beyond the globular stage. The micropropagation property of the two varieties through successive subculturing indicated that repeated subculturing results in loss of multiplication property of the* in vitro *materials.*

Key words/phrases: *Manihot esculenta*, Salt strength, Shoot multiplication, Sucrose, FEC, 2,4-D, TDZ

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial shrub of the family Euphorbiaceae. It is mainly grown in the tropics for its starchy tuberous roots, which are used for human consumption, as animal feed, and as raw material for the starch industry. Among the most important tropical crops in terms of source of calorie which are rice, sugarcane, maize and cassava; it is more cheaply cultivated (Mathews *et al.,* 1993; Reamakers *et al.,* 1993; Nweke, 2004). It is very important for the agro-economy of several tropical countries because of its broad adaptation to a variety of soil, climate, drought tolerance, and ability to grow on marginal soil (Mathews *et al.,* 1993; Reamakers *et al.,* 1993; Le *et al.,* 2007).

Central and north eastern Brazil, south- western Mexico, and eastern Bolivia are the centers of diversity. Cassava is also grown on the African and Asian continents. The average yield in the world is only a small fraction of the potential yield. One of the reasons for this low yield is the use of cuttings infected with diseases and pests as the starting material (Raemakers *et al.,* 1993).

Cassava, also known as manioc, mandioc, tapioca, or yucca, is Africa‟s second most important crop in terms of calories consumed. Its starchy tuberous roots yield 25-35% starch which provide food for over 500 million people for small-scale and subsistence farming in developing countries (Li *et al.,* 1998; Smith *et al.,* 1986).

Cassava is the third source of calories in the tropics and could develop as a major feed and industrial crop in the coming decades, and with more than one billion people suffering from hunger and malnutrition, it is clear that staple crops like cassava must play a major role in address. Cassava production has increased by 75% since 1970 in which the large majority of this is due to increased land committed to the crop, not due to improvements in yield. However, compared to what has occurred for the major cereals, scientific and technical progress over the past periods has not substantially impacted on cassava productivity (Fauquet, 2001).

Cassava is a cheap source of carbohydrate to people in the tropics. Cassava has a low protein and high cyanogen content, which significantly impacts on its nutritional value (Khonan *et al.,* 1994). These problems including the severely reducing yield due to virus and insect pests are difficult to be dealt with traditional breeding system. The allopolyploid cassava plants show high heterozygosity and low natural fertility, poor seed set and seed germination. Therefore, other methods are required to overcome the above mentioned problems. Cassava biotechnology offers powerful tools in complementing the traditional breeding methods and can extend the genetic pool of useful gene sources beyond the species (Reamakers *et al.,* 1993; Hankoua *et al.,* 2006; Saelim *et al.,* 2006; Danso and Ford-Llyod, 2008).

Cassava‟s multiplication is mainly by stem cuttings which is a slow process relative to that of grain crops. It is mostly grown on small farms and the cuttings are usually planted at the start of the rainy season. The crop cycle depends on whether the early or late varieties are grown, which takes respective 8 and 18 months post planting to be harvested (Santana *et al.,* 2009).

According to Santana *et al.* (2009), despite its importance, the average worldwide productivity over the past 30 years has only limited to 12 -13 tonne/hectare which is far away from its potential crop productivity of 80 tonne/hectare. Its large-scale cultivation is constrained by the lack of high quality seed and the low productivity and profit. These can be overcome by tissue culture methodologies which produce high quality vegetative planting material that ensure a higher increase in productivity more than 30 tonne/hectare (Santana *et al.,* 2009). A higher increase in productivity may also be obtained by using genetic varieties resistant to pests and diseases or to environmental constraints.

Plant tissue culture as one of the major components of biotechnology plays a large role in improving different varieties of crops as well as complementing the traditional breeding method. It is always involved in ensuring better qualities of crops that we use. Plant tissue culture with the concept of cellular totipotency, media formulation along with cell, tissue, organ, and protoplast culture led many recalcitrant plants amenable to *in vitro* regeneration and to the development of haploids, somatic hybrids and pathogen free plants. Tissue culture methods have also been employed to study the basic aspects of plant growth, metabolism, differentiation and morphogenesis and provide ideal opportunity to manipulate these processes (Gupta and Ibaraki, 2006; Le *et al.,* 2007).

With the aim of overcoming the above mentioned impedes, this study aimed to come up with the best way to get a maximum number of shoots and fit *in vitro* materials through micropropagation and along with that, the different factors that are associated with the micropropagation of the two cassava varieties namely, „Qulle‟ and „Kello‟. Having the objective of coming up with a method with reduced limitations for the production of micropropagated cassava plantlets, since *in vitro* propagation could be affected by a variety of environmental factors, it is mandatory and important to know the effects of different factors that are associated with cassava micropropagation.

2. Literature Review

2.1. Origin and distribution of cassava

Cassava is a staple crop with great economic importance worldwide, yet its evolutionary and geographical origins have remained unresolved and controversial. One of the most fundamental questions about cassava that remains controversial is its evolutionary origin. The genus *Manihot* (comprising 98 species) is distributed across much of the Neotropics, and the identity of cassava‟s closest wild relatives within the genus has been a source of wide spread speculation. Most traditional domestication hypotheses have envisioned the crop to be a compile species derived from one or more species complexes either in Mexico and Central America or throughout the Neotropics (Olsen and Schaall, 1999).

Wild populations of *M.esculenta* that are likely to be the crop‟s direct progenitors have been identified in South America. There is continued speculation that the crop‟s origins may extend beyond *M.esculenta* to hybridizing *Manihot* species. Wild populations of *M.esculenta* occur primarily in west central Brazil and eastern Peru (Olsen and Schaall, 1999; Fregene and Puonti-Kaerlas, 2002).

Central and north eastern Brazil, south- western Mexico, and eastern Bolivia are the centers of diversity (Beeching *et al.,* 1993; Reamakers *et al.,* 1993). Cassava is also grown on the African and Asian continents (Reamakers *et al.,* 1993).

The origin of genus *Manihot* includes South and Central America. It was possibly first domesticated in America between 5000-7000 B.C. In the 16th century, after the conquest of America, it was introduced by Portuguese into West Africa and from there it spread across sub-Saharan Africa (Reamakers *et al.,* 1993).

2.2. Agro-economy of Cassava

Cassava is propagated vegetatively by stem cuttings which make it very advantageous in way that in periods of food shortage, the farmer doesn‟t have to save a part of the plant‟s edible crop for replanting of the field to obtain the next year‟s crop (Andersen *et al.,* 2000).

Because of its broad adaptability to a variety of soil and climate, drought tolerance and ability to grow on depleted and marginal soils, cassava is very important to the agro-economy of several tropical countries. Among tropical crops; rice, sugarcane, maize and cassava that are the most important sources of calories for human consumption, cassava is the most cheaply cultivated. It also has important uses as animal feed and in industry (Mathews *et al.,* 1993).

2.3. Production, utilization, importance and side effects

2.3.1. Production

Since 1970 worldwide production of cassava has increased by 75% in which the large majority of it is due to increased land committed to the crop, not due to improvements in yield. However, being the third source of calories in the tropics and impending to develop as a major feed and industrial crop in the coming decades, scientific and technical progresses over the last 30 years has not substantially impacted cassava productivity (Fauquet, 2001).

There was a rapid development of cassava production in Asia over the past 30 years, attaining a peak in 1989, after which it declined slightly. The increases were because of increasing exports of hard pellets from Thailand to the EU and the decrease was due to falling prices of coarse grains in Europe, which reduced cassava‟s competitiveness in that market (Howeler and Clair, 2001).

According to Mathews *et al.* (1993) the average agricultural yield varies widely, because of some contributing factors like varietal differences, losses due to diseases such as cassava bacterial blight, African cassava mosaic virus, tuber rot, and to insect pests, such as spider mites and mealy bug a wide range of productivity was observed for example: from 7.7 metric tonnes/ hectare in Zaire to 18.8 metric tonnes/ hectare in India.

In spite of development and wide dissemination of high yielding cassava varieties in other countries; other than India, the increase in yields was only marginal. This is due to displacement of cassava to ever more marginal areas and nutrient depletion and erosion caused by soil degradation evoked by continuous cassava production on sloping land (Howeler and Clair, 2001).

2.3.2. Importance and utilization

Keeping its competitiveness at a time of increasing globalization, the cost of cassava production per tonne has to be reduced, and new cassava-based products and markets should be developed. There is an increasing awareness among researchers and government officials that cassava can play an important role in improving the living standards of poor farmers in the most marginal areas (Howeler and Clair, 2001).

The crop is grown for many reasons like famine reserve, rural food staple, cash crop and urban food staple, industrial raw material and livestock feed (Nweke, 2004).

In EU, there is a rapid increase in the production of cassava starch and starch-derived products. In other countries, especially Indonesia and India, cassava remains a very important human food, whereas in Vietnam and China it is increasingly important for on-farm pig feeding and starch production (Howeler and Clair, 2001).

2.3.3. Food value

The roots and leaves are prepared as food in different ways in many parts of Africa. The roots are eaten raw, roasted or boiled. However, cassava leaves are a more convenient food product than the roots and are also edible. Similar to other dark green leaves, the leaves of cassava plant have nutritive values which are valuable sources of vitamins A, C, iron, calcium and protein (Nweke *et al.,* 2002).

Table 1. Nutritional values of fresh cassava roots and leaves per 100 grams (Nweke *et al.*, 2002)

Nutrient	Unit	Cassava roots	Cassava leaves
Energy	Calories	145	62
Water	Grams	62.5	80.5
Carbohydrate	Grams	34.7	9.6
Protein	Grams	1.2	6.8
Fat	Grams	0.3	1.3
Calcium	Milligrams	33	206
Iron	Milligrams	0.7	2.0
Vitamin A	International Unit	Tr	10000
Vitamin B1	Milligrams	0.06	0.16
Vitamin B2	Milligrams	0.03	0.30
Niacin	Milligrams	0.06	1.80
Vitamin C	Milligrams	36	265

2.3.4. Industrial use

Cassava starch has many industrial uses. It is used to manufacture many chemical products such as citric acid and is also used in papermaking, food processing, lubricants, adhesives and textiles (Nweke *et al.,* 2002). Fresh cassava roots are industrially used for the production of chips, pellets and starch. Recently, cassava has started to be used for bio-ethanol production too (Kuiper *et al.,* 2007).

2.3.5. Effect of cassava consumption on health

The high content of cyanogenic glucosides is the major concern of cassava consumption as a staple food. Cassava contains the two cyanogenic glucosides linamarin and lotaustralin in all parts of the plant which up on tissue disruption, the cyanogenic glucosides are degraded with concomitant release of hydrogen cyanide and ketones. The level of cyanide generated from the tubers differs from one variety to the other but as calculated on a dry weight basis, ranges between 0.15 and 1.5 g/kg of tuber. Acyanogenic cassava plants are not known. Use of cassava products as a staple food thus requires careful processing to remove the cyanide. Inadequate processing may result in chronic cyanide intoxication and tropical ataxic neuropathy. In more severe cases, it might cause cyanide intoxication that could correlate to outbreaks of paralytic disease konzo. Processing is labour intensive and time consuming and results in a simultaneous loss of proteins, vitamins, and minerals (Andersen *et al.,* 2000).

The toxicity of hydrogen cyanide, which occurs as a result of the hydrolysis of cyano-genic glucosides limits the use of cassava as food. The total cyanide, comprising both bound and free cyanide, indicates the potential of the cyano-genic glucosides in the roots or leaves. The cyano-genic glucoside concentration is higher under the peel and around the fiber in the middle part of the root. Several factors including soil types, soil moisture and time of maturity influence the concentration of cyano-genic glucoside. Several post-harvest practices, mainly the storage and cooking methods considerably affect the cyanide in reducing its content (Dejene, 2006).

According to Rosling (2001) cassava is a major staple food but most roots are with high levels of the potentially toxic cyanogenic glucoside linamarin which can be removed either through processing or through the work of molecular geneticist by developing acyanogenic transgenics. But from the looks of anthropological inquiry into female small-scale farmers‟

perceptions, cyanogenesis protects cassava from thievery, vermin and unplanned harvest by family members.

Among over 5000 known phenotypically distinctive cassava cultivars, all contain varying concentrations of the cyanogenic glucosides linamarin and lotaustralin, which are hydrolyzed to hydrogen cyanide (HCN) by endogenous linamarase when the tissue is damaged (Wilson, 2003; Haque and Bradbury 2004).

The potentially toxic compounds; cyanogenic glucosides can cause acute cyanide poisoning and death in humans and animals during consumption if these compounds are present in sufficient quantities. Cassava products are considered harmless at concentrations less than 50 ppm. However, consumption over long periods of time results in chronic toxicity (Food Safety Network, 2005). The cyanogenic potential of known cassava cultivars ranges from less than 10 mg kg^{-1} as HCN fresh weight basis to more than 500 mg kg^{-1} as HCN fresh weight basis (O‟Brien *et al.,* 1994). Cooking the roots slowly destroys the cyanogens (Nweke *et al.,* 2002).

Since attempts to eliminate the production of cynogenic glucoside in cassava through traditional breeding have not been successful, an alternative approach is antisense technology, which requires knowledge of the biosynthetic pathway and the enzymes involved and identification of the relevant genes (Andersen *et al.,* 2000).

2.4. Cassava in Africa

New high-yielding TMS varieties invigorated the cassava transformation that is now underway in Africa by transforming cassava from a low-yielding famine-reserve crop to a high-yielding cash crop for both rural and urban consumers. The TMS varieties have been developed by IITA (International Institute of Tropical Agriculture) scientists in the 1970s and 1980s by drawing on the pioneering research of H.H. Storey, a British researcher on cassava mosaic virus at the Amani Research Station in Tanzania from the mid 1930s to the mid 1950s. Despite this ongoing transformation, cassava is being neglected by many African governments and international donor agencies because of myths and half-truths about its nutritional value and alleged role as a soil nutrient deplete (Nweke, 2001).

Cassava is an important food security as well as income-generating crop for small-scale farmers in the sub-Saharan Africa. Production of the crop is, however, constrained by;

cultivation of low yielding varieties, pests and diseases, limited processing and utilization options, socio-economic factors (poor man‟s crop), limited commercialization and marketing (Obiero *et al.,* 2004).

Cassava is an important crop in many African farming systems by playing an important role in food security, but also as a cash crop. During the last five decades, total production in Africa has almost quadrupled from 31 to 118 million tones/ year which rather has been due to increases in land area under production than increases in yield in the last three decades. Average cassava yields in Africa have gradually increased from 6-10 tonne/ hectare over the past five decades. Nevertheless, there is 20% less cassava per hectare than the world average of 12.2 tonne/ hectare (Fermont, 2009).

Cassava has been growing in South Africa in KwaZulu-Natal, Mpumalanga and the Limpopo Provinces by small-scale and subsistence farmers. A number of characteristics like famine secure, drought tolerance, ability to grow on poor soil, relative insect and pest resistance, more production of carbohydrate per hectare than other food staple, and ability to be left in the ground for a long time before harvesting made the crop encouragingly selectable by the small scale farmers (Nweke *et al.,* 2002).

About 11% of the total production of cassava in Africa accounts for East Africa (Tanzania, Kenya, Uganda, Rwanda and Burundi). There is 6.5 to 12.0 tonne/ hectare yield estimates between countries. Within the region, cassava is the most important staple food crop in terms of total production, followed by maize, sweet potato and cooking bananas. The cassava mosaic disease (CMD) pandemic that developed in Uganda in early 1990s and which has subsequently spread out had and still severely affects its production. The pandemic is caused by simultaneous infections of cassava plants by the existing African Cassava Mosaic Virus and the new East African Cassava Mosaic Virus-Uganda, in combination with the super-abundance of the *Bemisia tabaci* whitefly vector. The pandemic causes an average yield loss of 72% in CMD affected, widely grown landraces (Fermont, 2009).

Cassava is one of the main crops in Mozambique. It is the second most important food crop in Mozambique after maize and is the most important root crop. It is almost exclusively used for human consumption. An estimated 70% of the total cassava production is utilized as human food. Roots and leaves provide a major source of carbohydrate and vitamins. In areas prone to drought and floods, cassava is the main crop (Nweke *et al.,* 2002).

To alleviate the food security problems in the continent, in most of the sub-Saharan African countries, there is a guarantee of a new wave of cassava, the effectiveness of which will depend fundamentally on the individual Government‟s positive approach towards assisting farmers in procuring improved planting materials and educating farmers on the new processing techniques to eliminate or minimize loss (Dejene, 2006).

2.5. Cassava Production in Ethiopia

The crop was introduced to Ethiopia in the 19th century. The ones identified as the bitter cultivars by locals had been introduced first, and then followed by the sweet cultivars, having high and low cyanide contents, respectively. It is known by a variety of local names like „Mita Boye‟, „Yenchet Boye‟, „Furno Tree‟ and „Mogo‟ in the southern parts of Ethiopia, where it is dominantly grown and utilized. It is primarily grown and used as food crop for about a century in southern and south-western parts of Ethiopia (Dejene, 2006).

According to Dejene (2006) a survey conducted in different parts of southern and south-western Ethiopia indicated that farmers use stem cuttings (30-100 cm length) as planting materials and leave the cassava to grow for a number of years, harvesting the tuberous roots whenever they need it. Based on the local experience, the bitter cultivars take 15 months to mature whereas the sweet cultivars mature within 8 to 10 months. Cassava grows between 480 to 1800 m.a.s.l. within the temperature range of 15 to 30 ^{0}C, and 692 to 1470 mm annual rainfall.

In southern Ethiopia, particularly in a place called Amarokelo, cassava is used as a staple food. In place known as Wolaita and Arba Minch, cassava roots are widely consumed after washing and boiling or in the form of bread and „enjera‟ by mixing its flour with that of cereal crops, like teff, maize, and sorghum. It is also used in making local alcoholic beverages such as „areke‟ and „tela‟. The importance of cassava as a major source of carbohydrate for several million people in developing countries in general, and Ethiopia in particular is enormous. However, only a limited number of researches have been conducted on this important crop in Ethiopia (Dejene, 2006).

2.6. Tissue culture

The average yield of cassava in the world is 8.8 tonne/ hectare which is only a fraction of the potential yield of 90 tonnes/ hectare. One of the reasons for this low yield being the use of cuttings infected with diseases and pests as the starting material. Cassava normally propagated vegetatively through cuttings, though sexual propagation via seed can occur. It is basically perennial, but the roots are harvested on an annual or biennial basis. Its wild species normally propagate by seed and produce roots of minor economic importance (Reamakers *et al.,* 1993).

About 700 million people in the world obtain more than 500 Kcal per day from the crop and more than 500 million people consume 100 Kcal per day from cassava (Taylor *et al.,* 2004). As the global dependence on the crop is expected to increase with a projected 60% boost in production by 2020 (Aerni, 2006), there is a need for using biotechnological techniques towards increasing the yield of this plant.

New and powerful biotechnological tools are available and becoming integral components of many crop improvement programs. A Global Cassava Plan is being formulated, which aims to implement a concerted and integrated approach for bringing the benefits of biotechnology and other modern improvement and delivery systems to bear on this essential crop (Fauquet, 2001).

2.6.1. Micropropagation of cassava

Tang (1984) suggested that, to improve the genetic make-up of cassava, there is a need for a fast and convenient system of cassava multiplication by means of *in vitro* micropropagation.

Even if the contribution of breeding and genetics was enormous in the improvement of crop plants recently, it‟s being observed that micropropagation has become an irreplaceable tool in the improvement and genetic manipulation of plants especially vegetatively propagated crops (Onuoch and Onwubiku, 2007).

Santana *et al.* (2009) discussed that tissue culture methodologies can be used to produce high quality vegetative planting material, which ensures greater productivity. In order to reach a technological transference, a cheap and simplified method to micropropagate cassava *in vitro* plants is required. Hence, came up with a way for the production of a cheap media made of locally ready available components.

In vitro propagation has the potential to produce millions of plants and cuttings within a year. However, propagation systems based on single leaf-bud or two-node cuttings may be the most practical and effective method of propagation in developing countries with limited technical expertise and facilities. *In vitro* propagation offers certain advantages. For instance, the time frames for propagation are considerably reduced and, by containing multiplication *in vitro* where phytosanitary conditions, are better, the development and dissemination of disease-free clones should be enhanced. Also, because the system is laboratory based, it is not affected by environmental conditions which would limit the optimum time of planting, especially for leaf-bud cuttings (Smith *et al.,* 1986)

2.6.2. Somatic embryogenesis

Li *et al.* (1998) described the development of an efficient system for production of somatic embryos by inducing primary and cycling somatic embryos.

Both primary and secondary somatic embryogenesis are characterized by the direct formation of propagules with a bipolar character in which this type of embryogenesis could be described as direct or organized somatic embryogenesis (Raemakers *et al.,* 1997).

Ibrahim *et al.* (2008) reported the induction of primary somatic embryogenesis by culturing isolated shoot apex obtained from *in vitro* plants, in Petri-dish containing cassava induction medium (CIM) which is composed of MS medium supplemented with 8 mg/L picloram. Maturation of embryos was achieved by transferring the primary embryos into cassava maturation medium (CMM) containing MS medium supplemented with 0.1 mg/L BAP.

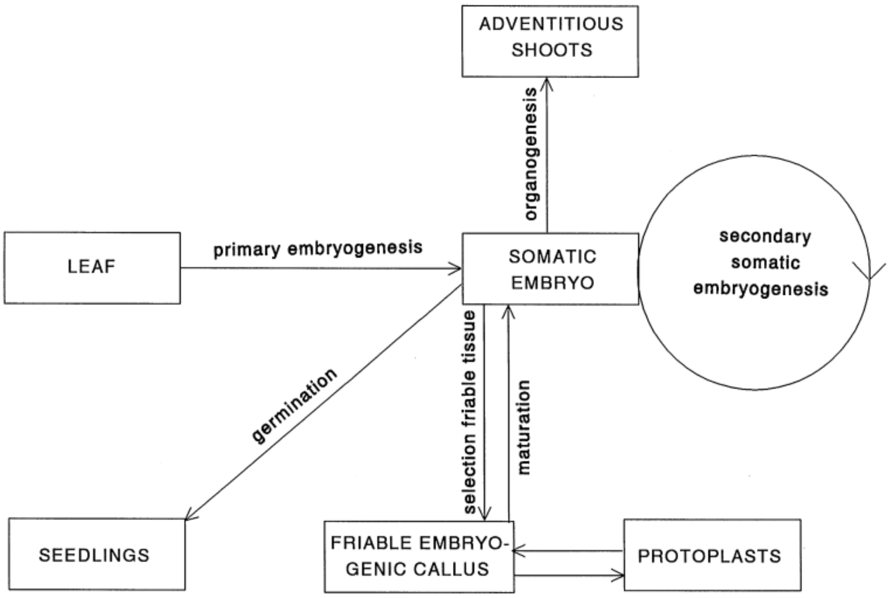

Figure 1. Schematic representation of different regeneration steps in cassava (Raemakers *et al.*, 1997)

Saelim *et al.* (2006) described the induction of somatic embryogenesis from different plant materials and various phytohormone concentrations in induction medium and their development into green cotyledons in maturation medium. The developing young green cotyledons were transferred to organogenesis medium for shoot regeneration.

3. Objectives

3.1. General objective

- To investigate the various factors associated with micropropagation of two cassava varieties in Ethiopia.

3.2. Specific objectives

- To investigate the effect of different salt strengths, sucrose concentration and pH on the efficiency of micropropagation of cassava
- To improve shoot initiation and multiplication efficiency using different concentrations of TDZ
- To improve shoot initiation and multiplication using a two-step procedure involving liquid medium containing different concentrations of TDZ pre-treatment prior to shoot initiation
- To evaluate the effect of different concentrations of 2,4-D on the efficiency of somatic embryogenesis
- To establish friable embryogenic callus lines

4. Material and Methods

4.1. Stock plant preparation

Fresh stem cuttings of two varieties of cassava namely „Kello" and „Qulle" obtained from Hawassa Agricultural Research Center, Root Crops Research Division, were planted in pots containing a 1:2:1 ratio of sand, soil and compost, respectively. The plants were maintained in a glasshouse at Addis Ababa University, College of Natural Sciences, at average temperature of 25 ± 2 ^{0}C under natural light condition.

4.2. Stock Solutions and medium preparations

4.2.1. MS medium and growth regulators stock solution preparation

Murashige and Skoog (1962) MS nutrient was used along with the proper type and concentration of plant growth regulators. A full strength macronutrients, micronutrients, Fe-Na-EDTA and $FeSO_4$ mixture and vitamins were used. A stock solution for each of the MS components (Appendix 1) were weighed in proper amounts and completely dissolved in double distilled water and stored at -20 ^{0}C.

The plant growth regulators were prepared in 1mg/ml concentration after being weighed and dissolved using drops of 1 N NaOH, allowed to be completely dissolved in double distilled water and stirred on magnetic stirrer. The stock solutions were stored at +4 ^{0}C for immediate use.

4.3. Culture medium preparation

4.3.1. MS Basal medium preparation

A full strength MS basal medium was prepared using 100 ml/L MS macronutrients, 10 ml/L MS micronutrient, 10 ml/L MS vitamin, 10 ml/L Fe-Na-EDTA and $FeSO_4$ mixture, 2 % sucrose. Then the pH was adjusted to 5.8 using 1 N HCl and 1 N NaOH and 7.0 g agar was added and melted either on a stirring hot plate or a microwave oven. When the agar became clear solution, 25 ml of the medium was dispensed into baby food jars for shoot culture initiation and 50 ml medium was dispensed on Magenta GA7 culture vessels for shoot multiplication and autoclaved at 121 ^{0}C with 0.15 Kpa pressure for 15 minutes.

4.3.2. Culture medium for the effect of TDZ on solid medium

On the MS basal medium the appropriate concentrations of TDZ (0.1 mg/L, 0.15 mg/L, 0.2 mg/L and 0.25 mg/L) was added. Four treatments containing different concentrations of TDZ with five replications were used.

4.3.3. Culture medium for the effect of TDZ on liquid medium

On the MS basal medium the appropriate amount of TDZ (0.1 mg/L, 0.2 mg/L, 0.3 mg/L and 0.4 mg/L) was added. Four treatments containing different concentrations of TDZ with five replications were used.

4.3.4. Culture medium for different salt strengths

Culture medium was prepared using 100 ml/L MS macro nutrient, 10 ml/L MS micro nutrient, 10 ml/L MS vitamin, 10 ml/L Fe-Na-EDTA and $FeSO_4$ mixture, 2 % sucrose in case of full strength. The amount of macro nutrient, micro nutrient, Fe-EDTA and $FeSO_4$ mixture and vitamin stock solutions used decreased by half and quarter in case of half and quarter salt strengths and supplemented with 0.5 mg/L BAP + 1 mg/L GA_3 + 0.01 mg/L NAA. Then the pH was adjusted to 5.8 using 1 N HCl and 1 N NaOH and 7.0 g agar was added and melted either on a stirring hot plate or a microwave oven. When the agar become clear solution, 25 ml medium was dispensed into baby food jars for shoot culture initiation and 50 ml medium was dispensed on Magenta GA7 culture vessels for shoot multiplication and autoclaved at 121 ^{0}C with 0.15 Kpa pressure for 15 minutes. Three treatments (full, half and quarter) salt strengths with five replications were used.

4.3.5. Media preparation for the different types of pH

This involved the use MS medium (prepared at 4.3.1 section of this paper without the addition of agar) then the pH was adjusted to different points as 5.0, 5.6, 5.8, 6.0, 6.6 using 1 N HCl and 1 N NaOH. After the pH adjustment 1.3 %, 1.1 %, 0.8 %, 0.7 % and 0.6 % agar was added, respectively. Five treatments with pH values of 5.0, 5.6, 5.8, 6.0 and 6.6 with five replications were used.

4.3.6. Culture medium for different sucrose concentrations

MS basal medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 1 mg/L GA_3, and addition of 2%, 1.5 % and 1% sucrose was used separately. Three treatments (with sucrose concentrations of 2%, 1.5% and 1%) with five replications were used.

4.3.7. Media preparation for somatic embryo induction

CIM (Cassava induction medium) was prepared using MS basal medium supplemented with different concentrations of 2,4-D (4.0 mg/L, 6.0 mg/L, 8.0 mg/L, 10.0 mg/L and 12.0 mg/L) or 7.0 mg/L, 10.0 mg/L, 12.0 mg/L, 16.0 mg/ L 2,4-D with 2 µM $CuSO_4$ mixed in a volumetric flask to make a final volume of 1000 ml. Then the pH was adjusted to 5.8 using 1 N HCl and 1 N NaOH. After the pH adjustment, 8.0 g agar was added and melted either on a stirring hot plate or a microwave oven. When the agar became clear solution, 25 ml of the medium was dispensed into baby food jars and 50 ml of the medium was dispensed into Magenta GA7 culture vessels, and autoclaved at 121 ^{0}C with 0.15 Kpa pressure for 15 minutes.

For somatic embryo induction ten treatments (4.0, 6.0, 8.0, 10.0 and 12.0) mg/L 2,4-D and (7.0, 10.0, 12.0, 16.0) mg/L 2,4-D along with 2 µM $CuSO_4$ with six replications were used.

CMM (Cassava maturation medium) was prepared in half MS medium supplemented with (0.1, 0.2 and 0.3) mg/L BAP alone or with 0.01 mg/L 2,4-D. After pH adjustment to 5.8, the medium was poured into 100 ml Erlenmeyer flask and Magenta GA7 culture vessels with membrane rafts and autoclaved at 121 ^{0}C with 0.15 Kpa pressure for 15 minutes. In this case six treatments (0.1 mg/L, 0.2 mg/L and 0.3 mg/L BAP alone or along with 0.01 mg/L 2,4-D) with six replications were used.

4.4. Explant Collection and surface sterilization

Newly sprouted shoot tips about 5-10 cm which are going to be trimmed into 2-3 cm of explants were collected from „Qulle" and „Kello" varieties every four to seven days from the glasshouse. The explants were then washed once to remove dirt and mud with tap water alone and again washed with OMO powder detergent and rinsed well until all the detergent was removed, and then rinsed twice with double distilled water. This was followed by disinfection with 70 % ethanol for a minute and rinsed three times with sterile double distilled water. The explants were further disinfected with 1% Clorox bleach containing 5.25 % active chlorine and 1 drop of tween 20 for 10 minutes in case of „Qulle" and 11 Minutes in case of „Kello" followed by five times rinsing with sterile double distilled water. While treating with Clorox, the explants were gently shaken using hand and all the disinfection activities starting from 70 % ethanol was performed in the laminar airflow cabinet.

4.5. Culture Initiation

Shoot explants were trimmed to about 2-3 cm long after sterilization and used for shoot culture while explants containing at least two nodes were used in the node cultures. All leaves except the youngest two leaf lobes at the tip and scales were removed using sterile forceps and scalpel. The explants were then cultured in the different types of shoot initiation media that were used to investigate the different factors and the vessels were sealed with parafilm. Four explants per culture vessel were used and the experiment was repeated five times for all the different factors except to that of liquid medium treatment in which case is single explant per test tube with twenty replications.

In the two-step culture system nodal explants were soaked in a liquid MS medium supplemented with the different concentrations of TDZ and the cultures were maintained on an orbital shaker at 110 rpm for a week. Then transferred to a solid MS medium supplemented with 0.5 mg/L BAP + 1 mg/L GA_3 + 0.01 mg/L NAA on which is stayed for four weeks.

4.6. Shoot Multiplication

Shoot and node derived cultures were used as a source of explants for shoot multiplication. Five explants (either 5 shoots or 5 nodes) were cultured in each of the Magenta GA7 culture vessels with six replications.

4.7. Culture Conditions

The cultures were kept in the culture room under 16 h / day lights and 8 h/ day of darkness cycles (light intensity of 2000 lux) at 29 ± 2 ^{0}C and subculture was carried out every four weeks.

4.8. Somatic embryogenesis

4.8.1. Induction of somatic embryos

Young leaf lobes were used to induce somatic embryos from „Kello" and „Qulle" varieties of *in vitro* propagated cassava shoots. Different hormone composition 4 mg/L, 6 mg/L, 8 mg/L, 10 mg/L and 12 mg/L 2,4-D and 7 mg/L, 10 mg/L, 12 mg/L and 16 mg/ L 2,4-D with 2 µM $CuSO_4$ were used. Five explants per treatment were used and each treatment was replicated six times. The leaf lobe explants after being wounded using sterile scalpels were cultured in such a way that the adaxial part of the leaf is in contact with the medium for 60 days (those with 4 mg/L, 6 mg/L, 8 mg/L 2,4-D) and 40 days (those with 10 mg/L and 12 mg/L 2,4-D). The cultures were then transferred to maturation medium consisting of half MS supplemented with 0.1 mg/L BAP alone and in combination with 0.01 mg/L 2,4-D to induce maturation and somatic cotyledon formation which are useful for generating cyclic somatic embryos (Saelim *et al.,* 2006)

4.8.2. Culture conditions

Somatic embryo induction was done using baby food jars and the explants were maintained in a dim light for a week and transferred to a condition in which there is 16 h/day light and 8 h/day darkness cycles with light intensity of 2000 lux at a temperature of 29±2 ^{0}C.

4.9. Micropropagation scheme of the two cassava varieties

The Micropropagation scheme of the two cassava varieties „Qulle" and "Kello" were studied while subsequently subculturing the above varieties. Each of the initially used subcultured shoots and multiplied shoots were recorded at the beginning of each subculture to investigate the pattern of the shoot multiplication. The medium used in this case is full strength MS medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA and 1 mg/L GA_3.

4.10. Rooting and Acclimatization

Propagated shoots were transferred to a hormone free MS medium in order to sprout roots. Rooted shoots were then transferred to glasshouse by rinsing off the agar from the plantlets. The pots were covered with polyethylene bags and kept for 1- 2 weeks.

4.11. Study design

The study was conducted at Addis Ababa University Plant propagation and Tissue Culture Laboratory. The statistical method used to study this particular problem was CRD (Completely Randomized Design) and the data was analysed using the statistical data analysis software SPSS 17.0 and the resulting output was fed into Sigma plot 10.0 to get graphical interpretation of the results. One-way ANOVA was used for mean separation at a probability level of 5%.

5. Results

5.1. Shoot induction using solid MS medium with different concentrations of TDZ

The shoot explants in all treatments started responding five to seven days after culture and by the end of the second week shoot elongation and development of leaves were clearly observed. After four weeks, both varieties displayed a fully developed *in vitro* material (Fig. 2). Statistical data analysis based on number of shoots per explants indicated that all treatments except the ones with 0.1 mg/L TDZ and 0.2 mg/L TDZ were significantly different in case of „Kello‟. In case of „Qulle‟ all treatments are significantly different to each other but not with that of the control (0.5 mg/L BAP, 0.01 mg/L NAA and 1 mg/L GA_3).

Maximum mean number of shoots and number of leaves per explant were attained (2.05 and 5.7, respectively) at a concentration of 0.25 mg/L TDZ and maximum mean length of shoot per explant (4.55 cm) was obtained at a concentration of 0.2 mg/L TDZ in case of „Kello‟. In contrast, maximum mean number of shoots, leaf and length of shoot per explants 6.1, 9.45 and 1.90 cm, respectively at a TDZ concentration of 0.2 mg/L, 0.1 and that of the control respectively, in case of „Qulle‟ were attained.

Table 2. Mean number of shoots, leaves, nodes and length of shoot per explant of variety „Kello‟ on medium supplemented with different concentrations of TDZ after 4 weeks

Mg/L of TDZ	**Mean**		
	No. of shoots	No. of leaves	Length of shoots
Control	1.95 ± 1.05^{d}	3.0000 ± 0.85^{a}	4.2250 ± 0.78^{c}
0.10	1.45 ± 0.68^{abd}	2.3500 ± 2.03^{bc}	3.2750 ± 0.95^{abc}
0.15	1.60 ± 1.04^{c}	3.9000 ± 3.83^{d}	4.0750 ± 1.55^{d}
0.20	1.55 ± 0.68^{a}	4.6500 ± 3.13^{b}	4.5500 ± 2.15^{a}
0.25	2.05 ± 0.75^{b}	5.7000 ± 4.36^{ac}	4.4250 ± 1.41^{b}

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

Table 3. Mean number of shoots, leaves, nodes and length of shoot per explant of variety „Qulle‟ on medium supplemented with different concentrations of thidiazuron after 4 weeks

Mg/L of TDZ	**Mean**		
	No. of shoots	No. of leaves	Length of shoots
Control	2.9500 ± 1.16^{c}	2.3000 ± 1.45^{c}	1.9000 ± 1.16^{d}
0.10	4.1500 ± 0.51^{b}	9.4500 ± 5.13^{a}	0.9500 ± 0.51^{c}
0.15	4.8250 ± 0.63^{b}	8.6000 ± 2.60^{a}	1.2500 ± 0.63^{bc}
0.20	6.1000 ± 0.96^{a}	8.6000 ± 2.32^{a}	1.1000 ± 0.96^{a}
0.25	4.8500 ± 0.60^{b}	5.1500 ± 1.63^{b}	0.9500 ± 0.60^{b}

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

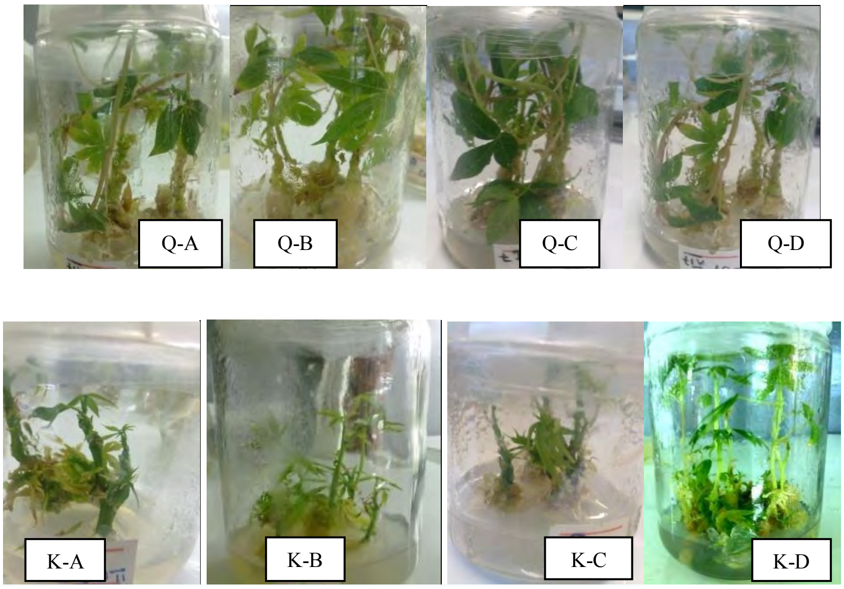

Figure 2. Culture initiation of the two cassava varieties „Qulle‟ (Q) and „Kello‟ (K) on MS medium supplemented with different concentrations of TDZ 0.1, 0.15, 0.2, 0.25 mg/L TDZ for A, B, C and D, respectively.

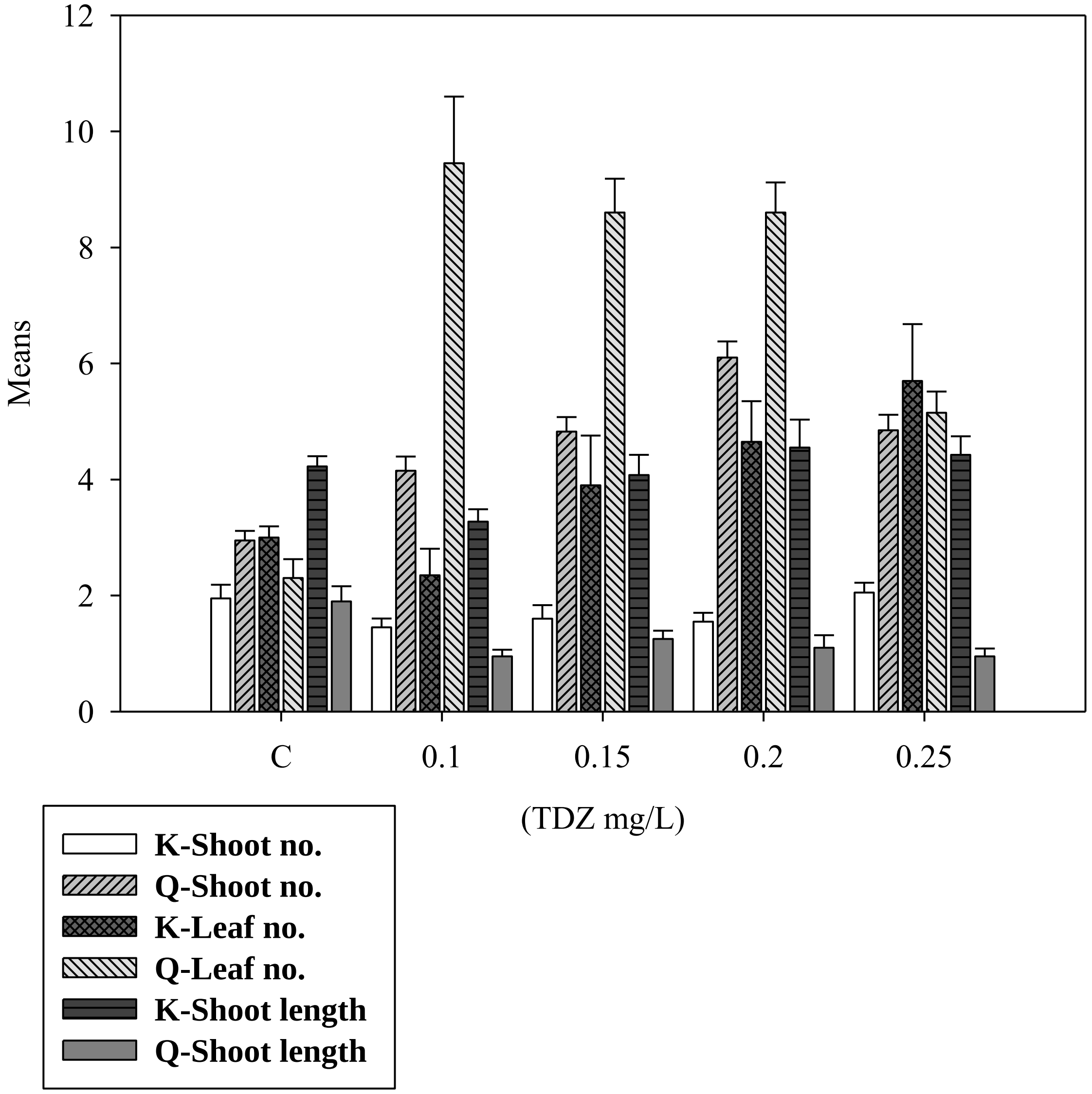

Figure 3. Mean number shoots, leaves and nodes and mean shoot length of „Kello“ (K) and „Qulle“ (Q) varieties based on different TDZ concentrations

5.2. Shoot induction using a two-step procedure

The nodal explants which were soaked in different concentrations of TDZ pre-culture treatment displayed nodal expansion and sometimes shoot initiation by the end of the week. Furthermore, shoot proliferation was observed after they have been transferred to the solid medium. The proliferation was best for those explants treated with 0.2 mg/L TDZ in both varieties with a maximum mean no of shoot per explants of 3.6 and 3.2 in case of „Kello“ and „Qulle“, respectively.

The *in vitro* materials were also better in leaf proliferation as compared to that of the control which was cultured on MS medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA and 1 mg/L GA_3.

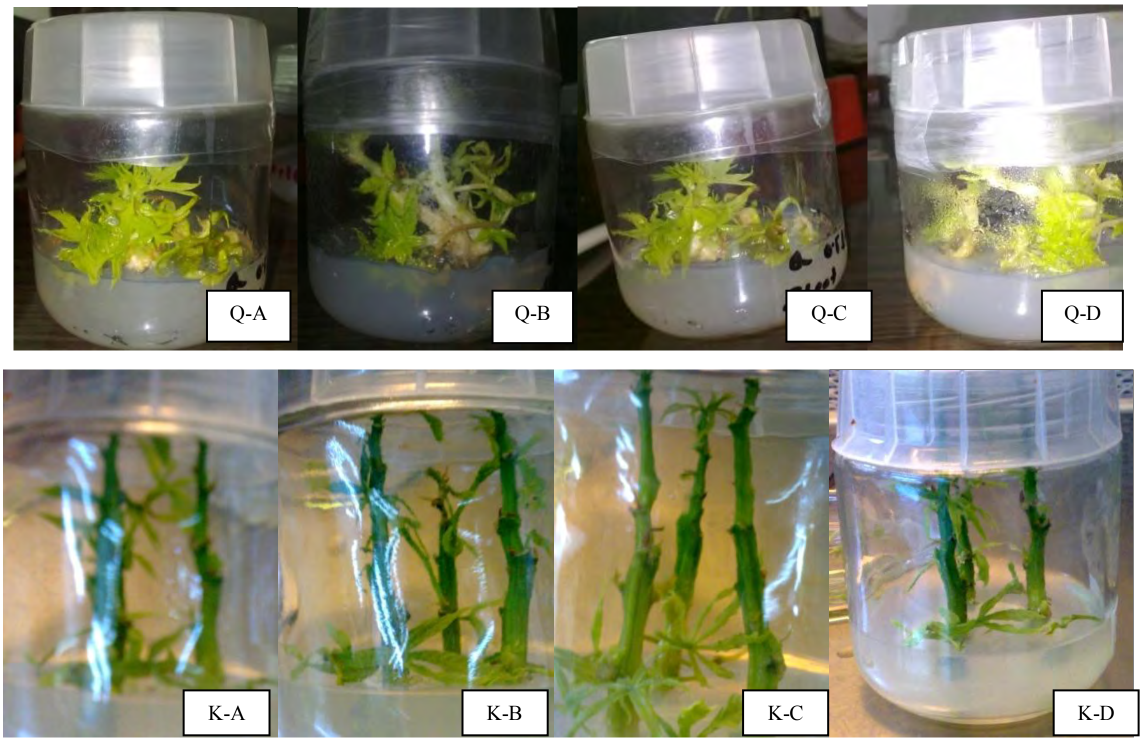

Figure 4. Nodal cultures maintained in liquid MS medium containing different concentrations of TDZ prior to shoot induction A = 0.1 mg/L, B = 0.15 mg/L, C = 0.2 mg/L D = 0.25 mg/L of TDZ

Table 4. Mean number of shoots per explant of the two varieties „Kello" and „Qulle" pre-soaked in liquid medium supplemented with different concentrations of thidiazuron followed by culture initiation on medium supplemented with 0.5 mg/L BAP, 1 mg/L GA3, and 0.01 mg/L NAA after 4 weeks

Mg/L of TDZ	**Mean no. of shoots/explant**	
	„Kello"	„Qulle"
Control	1.9500 ± 1.05^{cbd}	1.9000 ± 1.16^{bc}
0.1	2.2000 ±1.00^{b}	1.9000 ± 0.71^{bc}
0.2	3.6500 ±0.87^{a}	3.2000 ± 0.89^{a}
0.3	2.2000 ±0.83^{c}	2.3500 ± 0.67^{b}
0.4	1.5000 ±0.60^{d}	1.8000 ± 0.69^{c}

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

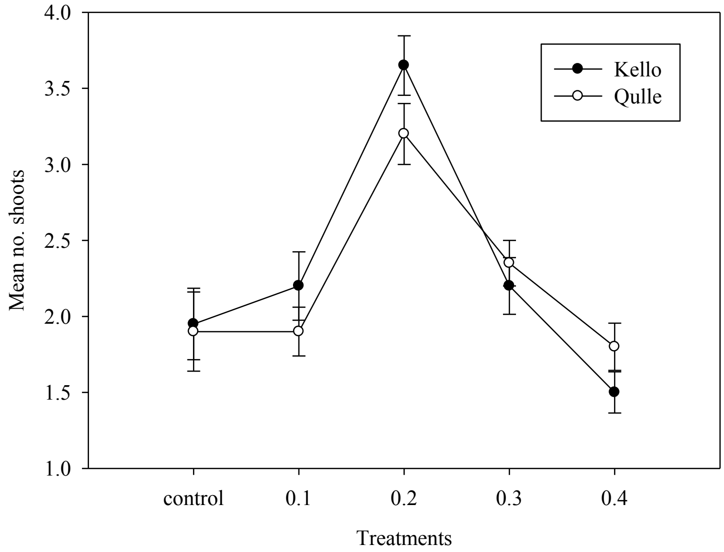

Figure 5. Mean number of shoots induced per explant on a liquid MS medium supplemented with different concentrations of TDZ prior to transfer to a solid medium containing 0.5 mg/L BAP, 1 mg/L GA3, and 0.01 mg/L NAA

5.3. Salt strength

Shoot explants on different salt strength MS medium were responding during the first week of culture. Developed cultures of both „Kello‟ and „Qulle‟ varieties were obtained by the end of the month on each of the three different salt strengths.

In case of „Kello‟, the highest mean number of shoots, leaves, nodes and length of shoot per explants (1.60, 3.50, 3.30 and 4.10 cm, respectively) were obtained at quarter, half, and full salt strengths, respectively. Whereas, maximum mean number of shoots, leaves, nodes and length of shoot per explants (1.80, 5.20, 3.40 and 3.40 cm, respectively) were obtained at full (control), and half salt strength, respectively in case of „Qulle‟.

Table 5. Mean number of shoots, leaves, nodes and length of shoot per explant of variety „Kello‟ on medium supplemented with 0.5 mg/L BAP, 0.01mg/L NAA, 1 mg/L GA3 with different salt strengths after 4 weeks

Medium salt strength	**Mean**			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.5667 ± 1.10^{a}	3.1667 ± 1.01^{a}	2.9000 ± 0.71^{a}	4.1667 ± 4.16^{a}
Half	1.5333 ± 0.89^{a}	3.5333 ± 1.92^{a}	3.3333 ± 0.92^{a}	3.5000 ± 3.5^{ac}
Quarter	1.6667 ± 0.88^{a}	2.9667 ± 2.25^{a}	2.1333 ± 1.22^{b}	3.3500 ± 3.35^{bc}

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, leaves, nodes and length of shoot per explant of variety „Qulle‟ on medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 1 mg/L GA3 with different salt strengths after 4 weeks

Medium salt strength	**Mean**			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.8667 ± 1.07^{a}	2.8333 ± 1.68^{b}	2.8000 ± 0.76^{b}	3.1333 ± 0.88^{ab}
Half	1.0667 ± 0.63^{b}	5.2000 ± 1.60^{a}	3.4000 ± 1.06^{a}	3.4667 ± 0.93^{a}
Quarter	0.6333 ± 0.49^{c}	3.6000 ± 1.49^{b}	2.8333 ± 1.28^{b}	2.9333 ± 1.17^{b}

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

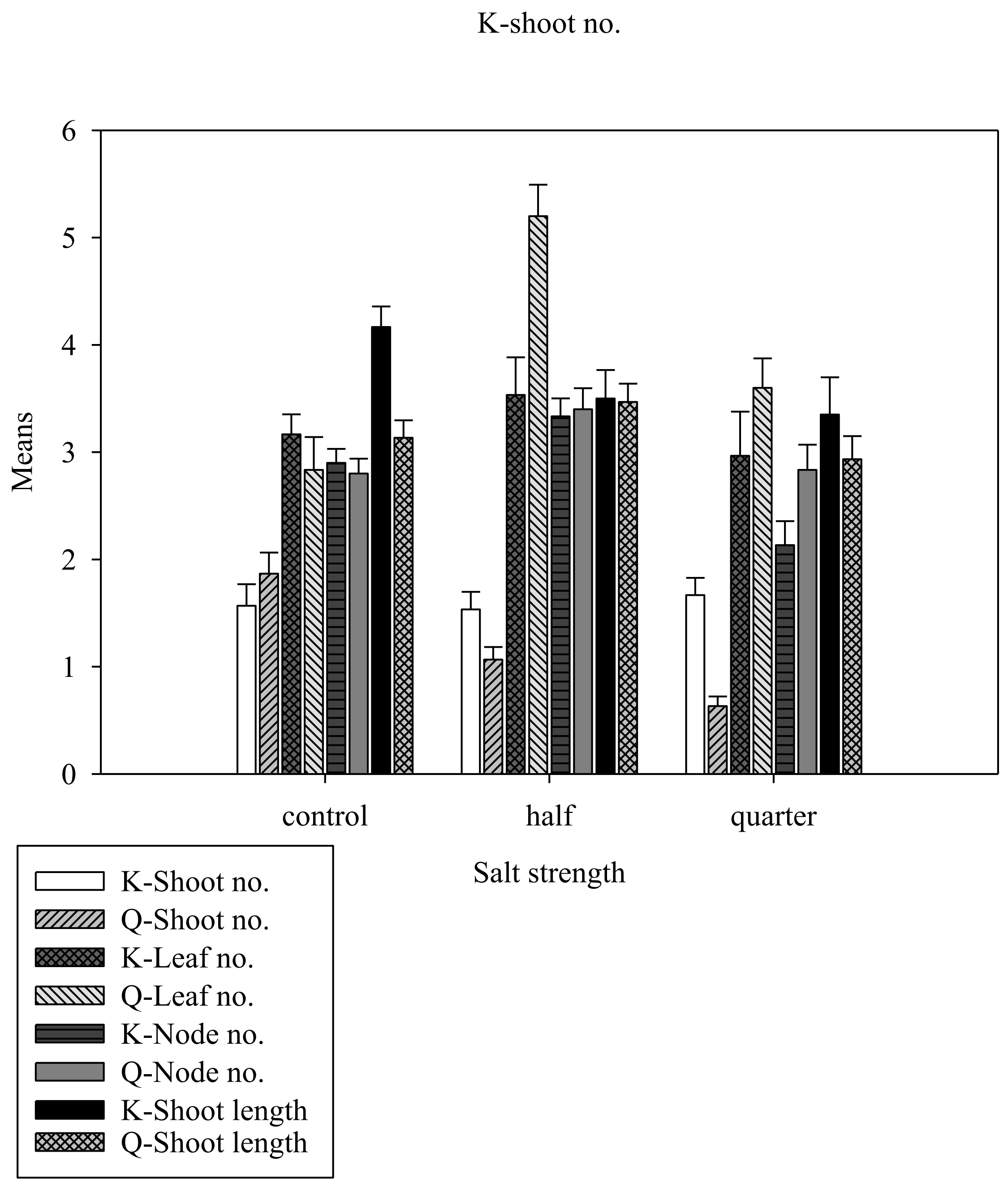

Figure 6. Mean number shoots, leaves and nodes and mean shoot length of „Kello" (K) and „Qulle" (Q) varieties based on different salt strengths

5.4. Response to medium with different pH

The cultures on different pH conditions started responding 5-7 days post culture initiation. All explants on the different pH ranges responded well and gave rise to a matured *in vitro* material by the end of the month. Maximum mean number of shoots, number of leaves, number of nodes and length of shoots were attained at a pH level of 5.6 in case of „Kello" which are 4.10, 12.10, 4.30 and 5.00, respectively. While in „Qulle‟ Maximum mean number of shoots, number of leaves, number of nodes and length of shoots were attained at a pH level of 6.6 which are 2.40, 9.80, 4.12 and 4.90, respectively.

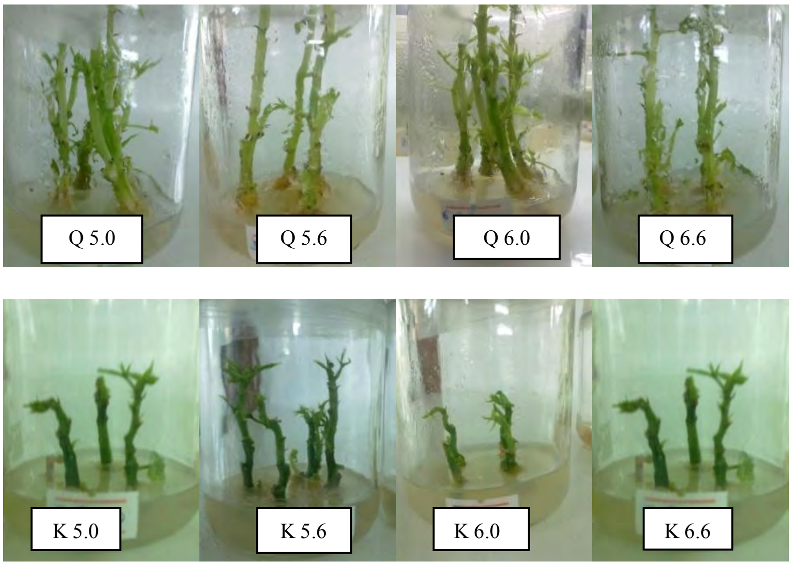

Figure 7. Shoot induction in a medium containing 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA3 at different pH levels (5.0, 5.6, 6.0 and 6.6 pH). Q = Qulle, K = Kello

Table 7. Mean number of shoots, leaves, nodes and length of shoot per explant of variety „Kello‟ on medium supplemented with 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA3 with different pH after 4 weeks

pH	**Mean**			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.95 ± 1.05^{b}	3.00 ± 0.85^{c}	3.050 ± 0.75^{c}	4.225 ± 0.78^{b}
5.0	3.80 ± 1.47^{a}	10.20 ± 2.09^{b}	3.850 ± 0.81^{ab}	4.300 ± 0.89^{b}
5.6	4.10 ± 1.33^{a}	12.15 ± 1.95^{a}	4.300 ± 0.86^{a}	5.000 ± 0.94^{a}
6.0	3.45 ± 0.68^{a}	11.85 ± 2.41^{a}	4.050 ± 0.82^{a}	4.950 ± 0.80^{a}
6.6	2.40 ± 1.09^{b}	9.00 ± 1.94^{b}	3.450 ± 0.75^{bc}	4.750 ± 1.14^{ab}

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

Table 8. Mean number of shoots, leaves, nodes and length of shoot per explant of variety „Qulle" on medium supplemented with 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA3 with different pH after 4 weeks

pH	**Mean**			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.90 ± 1.16^{ab}	2.30 ± 1.45^{c}	2.80 ± 0.76^{d}	2.95 ± 0.74^{d}
5.0	1.70 ± 0.80^{ab}	8.05 ± 1.35^{b}	3.90 ± 0.64^{ab}	3.55 ± 0.53^{c}
5.6	2.30 ± 1.12^{ab}	9.05 ± 2.98^{ab}	3.50 ± 1.00^{bc}	5.075 ± 0.81^{a}
6.0	1.70 ± 0.80^{b}	9.50 ± 2.87^{ab}	2.95 ± 1.09^{cd}	4.300 ± 0.78^{b}
6.6	2.40 ± 0.82^{a}	9.80 ± 2.85^{a}	4.10 ± 0.91^{a}	4.9250 ± 0.83^{a}

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

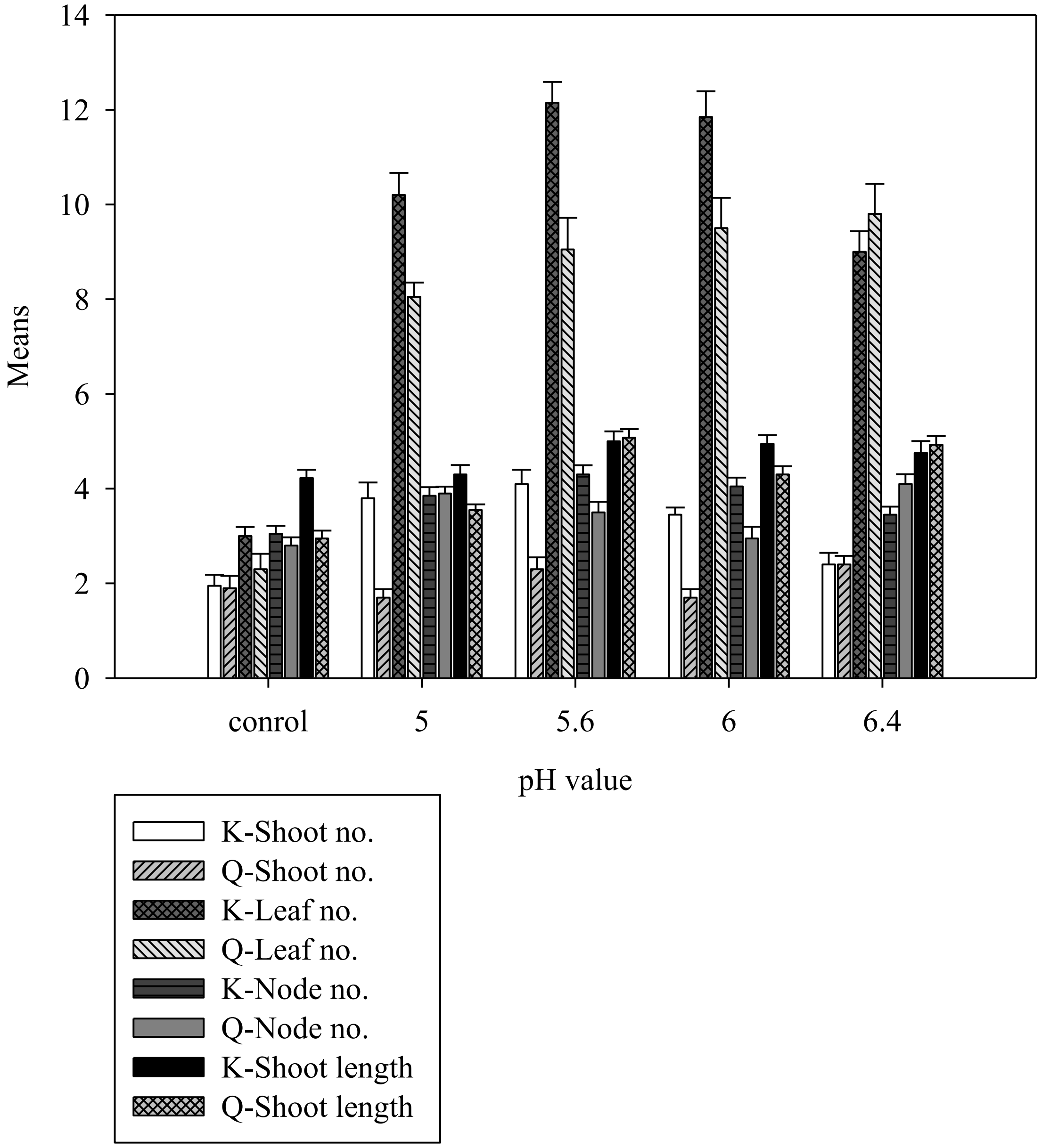

Figure 8. Mean number shoots, leaves and nodes and mean shoot length of „Kello‟ (K) and „Qulle‟ (Q) varieties based on different pH values

5.5. Response to different sucrose concentrations

In accordance with the results of the previous treatments, the explants started responding during the first week of culture initiation showing leaf emergence, shoot elongation and shoot multiplication.

In both „Kello‟ and „Qulle‟ varieties sucrose concentration of 0.15 % showed the highest mean number of shoots, number of leaves, number of nodes and length of shoot per explant.

As compared to that of the control which comprises a 0.2 % sucrose concentration, the explants were in a better appearance and there was almost no sign of necrosis which was frequently seen on a medium supplemented with a higher sucrose concentration, in this case the control.

Table 9. Mean number of shoots, leaves, nodes and length of shoot per explant of variety „Kello" on medium with different sucrose concentrations supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 1 mg/L GA3 after 4 weeks

Sucrose concentration	**Mean**			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.9500 ± 1.05^b	3.0000 ± 0.85^c	3.0500 ± 0.75^b	4.2250 ± 0.78^a
0.15%	3.7000 ± 1.55^a	12.1500 ± 6.57^a	5.8000 ± 1.57^a	5.1000 ± 1.22^a
0.10%	2.0000 ± 1.55^b	7.2000 ± 3.86^b	3.5000 ± 1.76^a	2.8500 ± 1.33^b

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

Table 10. Mean number of shoots, leaves, nodes and length of shoot per explant of variety „Qulle" on medium with different sucrose concentrations supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 1 mg/L GA3 after 4 weeks

Sucrose concentration	**Mean**			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.9000 ± 1.16^a	2.3000 ± 1.45^b	2.8000 ± 0.76^{ab}	2.9500 ± 0.74^b
0.15%	2.1500 ± 1.03^a	4.7500 ± 1.48^a	3.5000 ± 1.76^a	3.9500 ± 1.29^a
0.10%	1.4500 ± 1.27^a	3.6500 ± 2.58^a	2.3500 ± 1.38^b	2.9600 ± 1.69^b

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

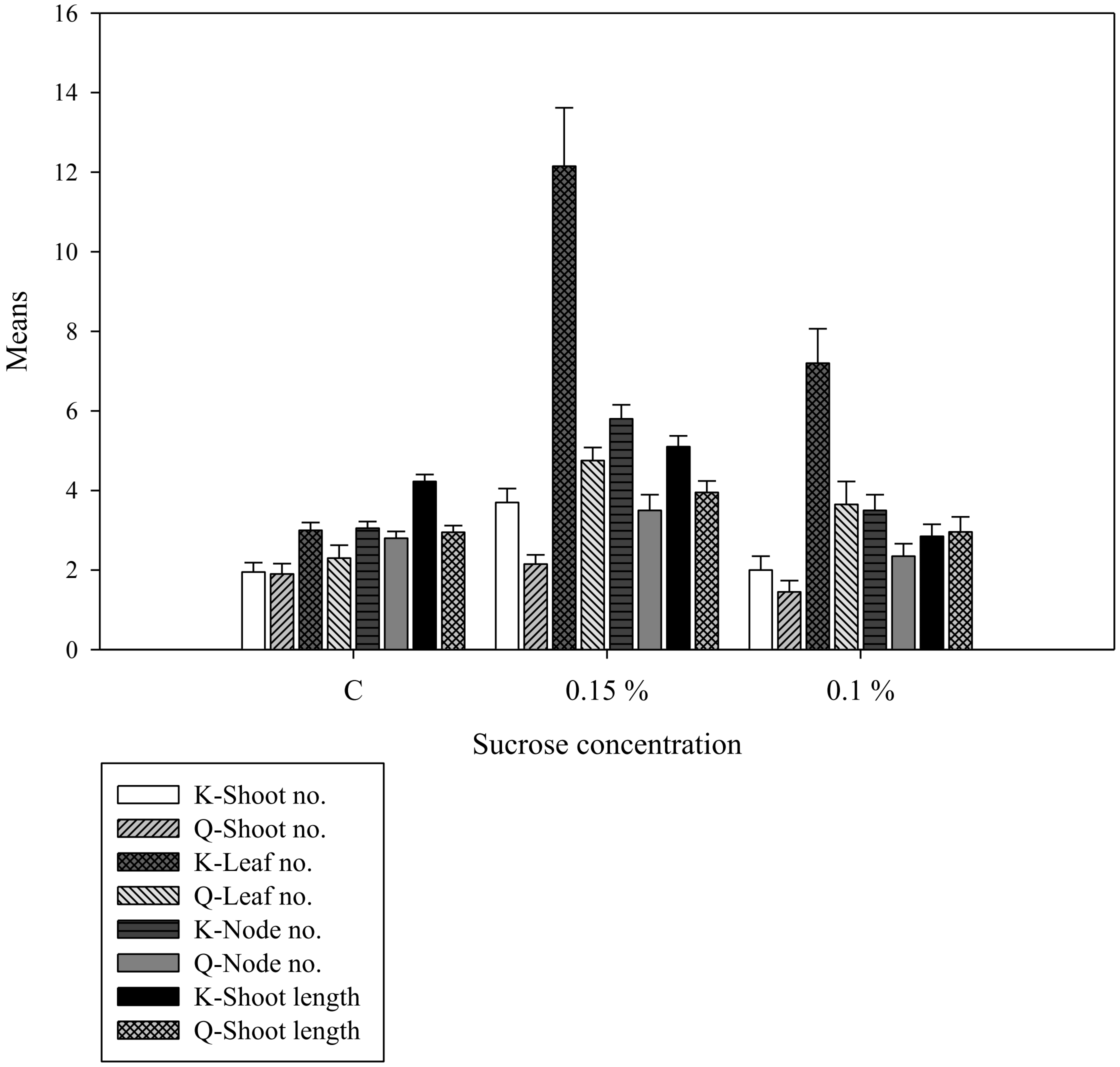

Figure 9. Mean number shoots, leaves and nodes and mean shoot length of „Kello‟ (K) and „Qulle‟ (Q) varieties based on different sucrose concentrations

5.6. Somatic embryo induction

Those treatments containing (4.0 mg/L, 6.0 mg/L, and 8.0 mg/L) 2,4-D showed the formation of a yellowish friable callus after 60 days and after 40 days of culture for those of (10.0 and 12.0). All the other treatments containing 2 µM $CuSO_4$ didn‟t produce a FEC and turned brown after a month.

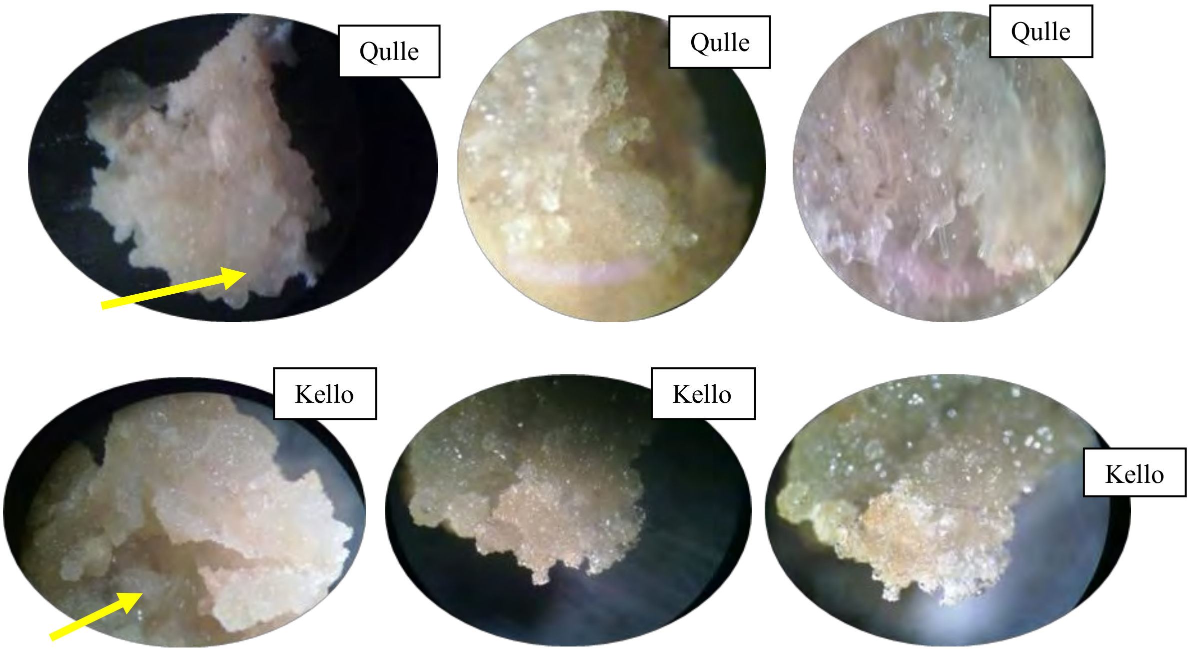

Figure 10. Structure of globular somatic embryos as seen under a dissecting microscope

Even though all the leaf lobes were able to induce somatic embryos, none of the somatic embryos obtained from the induced callus on 2,4 D medium showed embryo maturation and shoot induction on a medium containing different concentrations of BAP alone as well as along with a drastically lowered concentration of 2,4- D as compared to the ones used while inducing the somatic embryos.

5.7. Micropropagation scheme

Shoot tips obtained from induced shoots were subcultured every four weeks on a full MS medium containing 2% sucrose and supplemented with 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA_3. Cultured shoot tips responded very well at the early subcultures in case of „Qulle‟ and as the subculturing became subsequently increased; the mean number of shoots started to drop. In contrast, „Kello‟ displayed quite a different trend; the mean number of shoots linearly increased up to the 3rd month and suddenly reached an exponential peak at month 4 and again showed a drastic decline in the fifth subculture.

Table 11. Mean number of shoots per explants on different successive subculturing stages

Monthly sub culture	Mean no. of shoots/explant	
	„Kello"	„Qulle"
Subculture 1	1.5667 ± 1.10^{b}	1.8667 ± 1.07^{b}
Subculture 2	1.7000 ± 1.26^{b}	2.9333 ± 1.33^{a}
Subculture 3	1.8667 ± 1.10^{b}	1.6333 ± 1.47^{b}
Subculture 4	3.5333 ± 1.83^{a}	1.3000 ± 0.98^{b}
Subculture 5	2.0333 ± 1.35^{b}	1.2667 ± 2.22^{b}

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

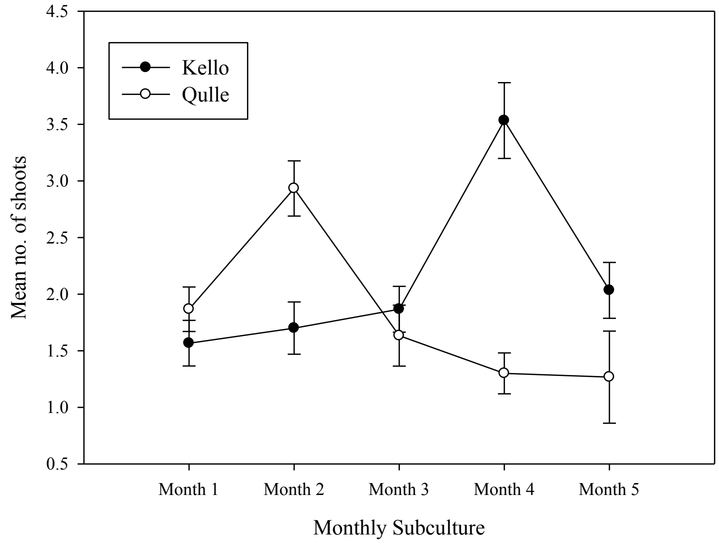

Figure 11. Subculturing schemes of the two cassava varieties in successive subcultures

5.8. Rooting and acclimatization

The plantlets were hardened and ready for field transfer by the end of the 3rd week. Among the acclimatized plantlets 50% of „Qulle" and 66.6% of „Qello" survived.

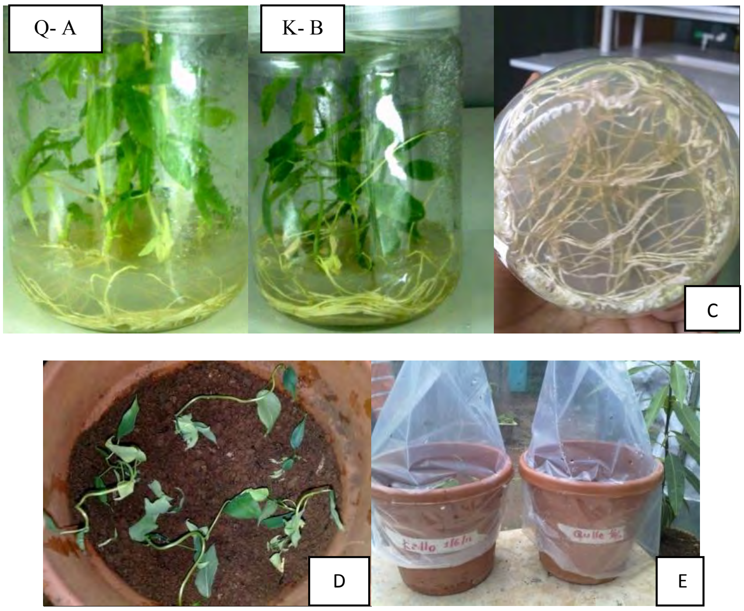

Figure 12. Rooting and acclimatization. Rooting in „Qulle‟ (A), rooting in „Kello‟ (B), roots at the bottom of the culture vessels (C), rooted plants after being planted (D), Plants covered in polyethylene bag (E)

6. Discussion

6.1. Shoot induction using solid MS medium with different concentrations of TDZ

The results obtained from shoot induction on MS medium containing different concentrations of TDZ was consistent with that of Escobar *et al.* (2001) and Siddique and Anis (2006). Their results also showed a multiple shoot proliferation using different concentration of TDZ alone and along with IAA from cotyledonary node explants which was manifested by emergence of multiple shoots from the explants as early as 15 days. This is quite similar to the results of this particular study which displayed an excellent shoot elongation at the end of the first week of culture and continued growth and shoot multiplication observed as early as the 2nd week of culture. They also reported that, at higher concentrations of TDZ which is higher than 5.0 μM, there was a considerable reduction of number of shoot buds and regeneration frequency which as they put it might be due to excessive callus growth while its specific concentration supports the maximum shoot bud induction. Similarly the result of this study (in case of „Qulle‟) showed that at a concentration of 0.25 mg/L the mean no of shoots produced per explant declined and the explants showed a mass of callus at the base.

Sajid and Aftab (2009) reported TDZ stimulates growth when added to a tissue culture medium at a low concentration (10-1000 times lower than the concentration of other PGRs) and their results are in consistent with the present study. The same applies to the results of Huetteman & Preece (1993). Aasim *et al.* (2009) described TDZ as the most active cytokinin substance which induces greater *in vitro* shoot proliferation than many other cytokinins in many plant species.

The result of Konann *et al.* (1997) which involved cassava shoot induction on MS medium supplemented with BAP, kinetin, thidiazuron and zeatin disagree with this study. In their results they found that, among the above mentioned cytokinins, BAP showed the most effective shoot induction and development.

6.2. Shoot induction using a two-step procedure

The results of Bhagwat *et al.* (1996) indicated an open-ended shoot proliferation process which yielded a maximum number of shoots per nodal explant after 10 weeks based on a protocol which involves a two-step procedure involving exposure of the nodal explants in TDZ containing liquid MS-medium for 6-8 days and a subsequent culture on agar solidified medium supplemented with 2.2 µM BAP and 1.6 µM GA_3. These results are consistent with that of the present study which involved a liquid MS-medium pre-treatment for a week and subsequent culture on agar solidified MS-medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA and 1 mg/L GA_3.

Bhagwat *et al.* (1996) described that TDZ caused the nodal explants to expand and this expansion (growth) continues during the second stage of the culture on agar solidified medium. From the expanded explant, clusters of buds and fasciated stems develop continuously and these gave rise to shoots.

Singh and Syamal (2001) suggest that a short exposure of explants to TDZ before subculture on to normal shoot proliferation medium can significantly enhance axillary shoot proliferation in hybrid tea rose cultivars.

6.3. Salt strength

The different parameters showed different response towards the medium mineral strength in such a way that, variety „Kello" produced the highest mean number of shoots, leaves, nodes and length of shoot per explant (1.60, 3.50, 3.30 and 4.10, respectively) at quarter-MS , half-MS, half-MS and full-MS medium salt strengths. Whereas, variety „Qulle" displayed maximum mean number of shoots, leaves, nodes and length of shoot per explants (1.80, 5.20, 3.40 and 3.40, respectively) at full (control), half, half and half salt strength of the medium, respectively.

The results obtained for mean number of nodes for „Kello" was inconsistent with the result of Mantell and Hugo (1989) which rather obtained maximum number of nodes at a full strength salt. But with regard to mean number of shoots for „Qulle", the results of this particular study is similar to the work of Mantell and Hugo (1989) which are obtained at a full strength mineral salt concentration of the medium.

6.4. Response to medium with different pH

Kozai *et al.* (1997) recommended controlling environmental factors which include pH in the mass production of plants through *in vitro* techniques and the result of this study showed certain ranges of pH that can be tolerated in the micropropagation of cassava. All explants on the different pH ranges responded well and gave rise to *in vitro* material by the end of the month. Maximum mean number of shoots, number of leaves, number of nodes, and length of shoots were attained at a pH level of 5.6 in case of „Kello". While in „Qulle" maximum mean number of shoots, number of leaves, number of nodes and length of shoots were attained at a pH level of 6.6. The explants with pH levels of 5.0, 5.6 and 6.0 and that of 5.8 and 6.6 displayed no significant difference depending on the number of shoots produced per explants in case of „Kello". While in „Qulle" pH levels of 5.8 (control), 5.0 and 5.6 and that of 6.0 with 5.0, 5.6 and the control were not significantly different. Despite, the fact that medium is usually prepared within the pH range of 5.0 to 6.0 the performance and pH fluctuation tolerance of „Qulle" is quite impressive.

6.5. Response to different sucrose concentrations

Mantell and Hugo (1989) in their study of the effects of different factors on root, shoot and microtuber development in shoot cultures of *Dioscorea alata* L. and D. *bulbifera* L. yams. Described, maximum number of shoots and nodes per explant at a lower concentration of sucrose which is similar to the result of this particular study.

The results of Nhut *et al.* (2001) on plant and shoot regeneration of *Lilium longiflorum* is quite different from the results of this experiment in which their work indicated that, the use of 3% or 4% sucrose resulted in a higher frequency of shoot formation and at 2% sucrose the frequency of shoot formation was slightly less. While, in this particular study both „Kello" and „Qulle" varieties displayed the highest number of means based on number of shoots, number of leaves, number of nodes and length of shoot per explants at a sucrose concentration of 0.15 %. This result has an enormous advantage and promise in practicing cassava tissue culture in developing countries in which the reduction in the quantity of carbon source used is a reduction in cost that might have been used in buying the expensive plant tissue culture grade sucrose.

Kozai *et al.* (1997) even recommended the use of sugar-free (photoautotrophic) for large scale micropropagation of plants as it is advantageous over the heterotrophic photomixotrophic micropropagation in such a way that it supports a faster and more uniform growth and development of *in vitro* plantlets , *in vitro* plantlets exhibiting less physiological and morphological disorders and less biological contamination *in vitro*.

6.6. Somatic embryo induction

Apart from the result of Stamp (1987) who used a consistent procedure in inducing and maturing somatic embryos, none of the somatic embryos obtained from the induced callus on 2,4 D medium showed embryo maturation and shoot induction on a medium containing different concentrations of BAP alone as well as along with a drastically lowered concentration of 2,4- D. The result also did not agree with that of (Groll *et al.,* 2002) which used a full and half strength MS medium in inducing somatic embryos as well as shoot germi nation.

Unlike the result of Saelim *et al.* (2006) which used 2 µM $CuSO_4$ as a supplement along with different concentrations of 2,4- D in order to have the best embryo induction frequency, the result of the present experiment on both „Kello" and „Qulle" resulted in browning of the callus subsequently resulting in no formation of somatic embryos.

The work of Konan *et al.* (1994) is in agreement with the present result in which 2,4-D induced globular somatic embryos.

The work of Atehnkeng *et al.* (2006) showed that pro-embryos formed by two genotypes of cassava did not survive beyond the globular developmental stage and the level of pro-embryo formation was not adequate as an indicator of embryogenic competence which is similar to the present work.

The result of Sudarmonowati and Henshaw on their unpublished work indicated that, among 16 cultivars of cassava all failed to induce somatic embryos except six cultivars on a medium supplemented with 2,4-D. These authors indicated the possible cause being strong influence of genotype towards somatic embryogenesis and the different response of genotypes towards 2,4-D. However Sudarmonowati and Henshaw (1996) indicated that they were able to induce somatic embryos on those cultivars that previously failed to do so on a 2,4-D containing medium, on a medium containing picloram as well as dicamba. Hankoua *et al.* (2005) reported the genotypic differences in the ability of immature leaf lobes and apical shoot meristems of cassava to form primary somatic embryos.

Danso *et al.* (2010) reported that even though both 2,4-D and picloram induced embryogenic calli and matured primary embryos, picloram has an enhanced early calli development than 2,4-D which is due to picloram‟s ability to acidify and loosen the cell wall earlier than 2,4-D which is important in weakening cell-cell interaction gradient which coordinates normal bipolar development of the embryo.

6.7. Micropropagation scheme

Dawit Beyene (2009) described that the multiplication data of his 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. Hence, recommended more subculturing.

Cultured shoot tips responded very well at the early subcultures in case of „Qulle‟ and as the subculturing became subsequently increased; the mean number of shoots starts to drop. In contrast „Kello‟ displayed quite a different trend; the mean number of shoots linearly increased up to the 3rd month and suddenly reached an exponential peak at 4th month and again showed a drastic decline in the fifth subculture. These might be due to loss of the multiplication property of the plantlets because of repeated subculturing.

The study of Mbanaso (2008) on the effect of multiple subcultures on Musa shoots after four consecutive subcultures indicated that, shoots derived from starch-gelled medium after several subcultures became less robust. This tendency for reduced robustness over time increased with increase in starch concentration in the medium.

7. Conclusion

With regard to the use of thidiazuron as shoot induction and multiplication medium, maximum mean number of shoots per explant at 0.2 mg/L TDZ was attained for „Qulle‟ and at 0.25 for „Kello‟ varieties as well as an excellent morphological property of *in vitro* material was observed. This finding is very important from economic aspect of developing countries in which at times tissue culture methods could get costly so, the use of a single type of PGR yet with a very small quantity could be rewarding.

In both varieties, the employed two-step culture system resulted in a maximum mean number of shoots/explants at a TDZ concentration of 0.2 mg/L. TDZ made the nodal explants to expand and multiply continuously that they forced to develop faciated stems which are sources of multiple shoots that can be used in the large scale micropropagation of cassava plants.

With regard to medium salt strength, „Kello‟ produced maximum mean number of shoots per explant at a quarter strength MS medium while „Qulle‟ at a full strength MS medium and all the treatments in case of „Qulle‟ were significantly different to each other at 5 % probability level. This result, apart from understanding how far cassava micropropagation tolerates mineral strength fluctuation, has an important role in ceasing an opportunity to again employ a low-cost tissue culture mechanisms for developing countries still gaining the best result that the method could come up with.

The pH fluctuation in the medium mostly didn‟t bring a significant difference as such although the highest mean number of shoots and as well as other parameters per explants for „Kello‟ and „Qulle‟ were obtained at pH 5.6 and 6.6, respectively.

For all parameters maximum mean numbers for both „Kello‟ and „Qulle‟ was obtained at 0.15 % sucrose. Even if it was not used to compute the statistical analysis in this experiment at the beginning, 3 % sucrose was used to multiply *in vitro* propagated cassava shoots and there was a frequent sign of necrosis on such medium. But in those of lowered sucrose concentrations necrosis occurred seldom.

During somatic embryo induction, the treatments with different 2,4-D concentrations induced FEC and somatic embryos but the somatic embryos failed to develop beyond the globular stage. Treatments with different 2,4-D concentrations along with 2 µM $CuSO_4$ didn‟t result in FEC hence there were no embryo induction on those treatments.

With regard to the micropropagation property of the two varieties through successive subculturing resulted in an increase in the first round of subculture and slowly lowering in the succeeding ones in case of „Qulle‟. In case of „Kello‟, there was a linear increase up to the 4th month and then a sudden drop in the mean number of shoot took place. This implies that repetitive subculturing of the same material might cause the *in vitro* materials to lose their competence towards multiple shoot formation.

This study recommends the best combinations for micropropagation to get the best result is, the use of a full MS medium supplemented with 0.2 mg/L TDZ, 0.15% sucrose at pH 6.6 in case of „Qulle‟ and a quarter MS medium supplemented with 0.25 mg/L TDZ, 0.15 % sucrose at pH 5.6.

It is also recommendable to use a two-step procedure involving pre-soak of explants on a full MS liquid medium supplemented with 0.2 mg/L TDZ and transfer to a full MS solid medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA and 1 mg/L GA3 for continuous shoot proliferation.

8. Recommendations

- More subculturing should be done with the developed protocol which involves the addition of thidiazuron on the medium in order to understand the multiplication property of the two varieties as well as to compare it with the one that was done in this study which involved the use of MS medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA and 1 mg/L GA_3.
- More investigation is required by using a wide range of pH adjustments so that one can understand how far the two varieties tolerate the pH variation.
- In case of medium salt strength respective subculturing should be done to study the response of induced shoots to further subjection of similar treatments.
- Other concentrations of sucrose like 3 % and 4% should be incorporated to further identify the impact of high concentrations of sucrose towards necrosis in specific and micropropagation of cassava in general.
- Other factors which might impact cassava micropropagation like relative humidity, light intensity, photoperiod and gelling agents should be incorporated to provide a constraint free mass-propagation system.
- Since the present study didn"t come up with a respective maturation of somatic embryogenesis further study in somatic embryogenesis of cassava on these two varieties is important by using other growth regulators such as picloram and dicamba.
- The embryogenic competence should be studied both variety wise as well as between different 2,4-D concentrations.

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Appendix 1

Table 12. Nutrient compositions and concentrations for full strength MS basal medium

Components	**Concentration (g/L)**
Macro nutrients	
NH4NO3	16.5
KNO3	19.0
CaCl2.2H2O	4.4
MgSO4.7H2O	3.7
KH2PO4	1.7
Micro nutrients	
ZnSO4.7H2O	0.86
H3B O3	0.62
MnSO4.4H2O	2.23
CUSO4.5H2O	0.0025
KI	0.083
Na2MoO4.2H2O	0.025
CoCl2.6H2O	0.0025
Vitamins and organic supplements	
Myo-inositol	1.0
Glycin (Glycocoll)	0.2
Nicotinic acic	0.05
Pyridoxine (B6)	0.05
Thiamin (B1)	0.01
Fe-Na-EDTA and $FeSO_4$ mixture	
Fe-Na-EDTA	4.0
FeSO4. 7H2O	13.9

Declaration

I Roza Berhanu, hereby declare that, this thesis is my original work. It has never been presented for a degree in any other institution and that all sources of materials used in it have been duly acknowledged.

Name: ___________________________

Signature: ________________________

Date: _____________________________