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

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Abstract. *Berhanu R, Feyissa T. 2020. Factors influencing micropropagation and somatic embryogenesis of two cassava varieties, Kello and Qulle. Cell Biol Dev 4: 71-81.* Cassava (*Manihot esculenta* Crantz) is a perennial Euphorbiaceae shrub cultivated in the tropics, Africa, and Asia for its tuberous starchy roots. Lack of good-quality seed, low productivity and profit, virus and insect pests, high heterozygosity, low natural fertility, poor seed set, and seed germination limit its cultivation. The conventional breeding system is ill-equipped to address these issues. Therefore, additional biotechnological solutions are required to address the abovementioned issues. This research aimed to devise a method for obtaining a maximum number of shoots and morphologically sound in vitro materials by micropropagation, as well as to investigate the various parameters related to the micropropagation of the two cassava varieties, 'Kello' and 'Qulle.' The research also contains a somatic embryogenesis technique that is an optimal source of in vitro materials for large-scale multiplication. In this experiment, a Solid MS medium with different salt concentrations, sucrose concentrations, TDZ, a two-step procedure involving pre-soak of nodal explants in 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 $CuSO_4$ were utilized. At a concentration of 0.2 mg/L TDZ, both 'Kello' and 'Qulle' exhibited the highest average number of shoots per explant and improved morphological properties of in vitro material. At a TDZ concentration of 0.2 mg/L, the two-step cultivation strategy produced the greatest mean number of shoots per plant for both kinds. The maximum mean number of shoots per explant was produced by 'Kello' at a medium salt strength of a quarter, but 'Qulle' required a medium salt strength of full. 'Kello' and 'Qulle' produced the greatest average number of shoots per explant at pH values of 5.6 and 6.6, respectively. At 0.15% sucrose, 'Kello' and 'Qulle' attained their highest mean values for all parameters. During somatic embryo induction, treatments with varying doses of 2,4-D generated FEC and somatic embryos, but the somatic embryos failed to progress through the globular stage. The micropropagation property of the two types through sequential subculturing suggested that repeated subculturing causes the in vitro materials to lose their multiplication property.

Keywords: 2,4-D, FEC, *Manihot esculenta*, salt tolerance, shoot multiplication, sucrose, TDZ

INTRODUCTION

Manihot esculenta Crantz is a perennial shrub of the Euphorbiaceae family. It is mostly cultivated in the tropics for its starchy tuberous roots, consumed by humans, fed to animals, and used as raw material in the starch industry. Among the most important tropical crops in calorie supply, which include rice, sugarcane, maize, and cassava, it is more inexpensive to plant cassava (Mathews et al. 1993; Raemakers et al. 1993; Nweke 2004). Cassava ranks five as a world food crop but two for tuber crops after potatoes (Lestari and Apriyadi 2017). In addition, due to its vast adaptation to a variety of soil, climate, drought tolerance, and capacity to grow on marginal soil, it is vital to the agricultural economies of several tropical nations (Mathews et al. 1993; Raemakers et al. 1993; Le et al. 2007).

The centers of diversity include central and north-eastern Brazil, south-western Mexico, and eastern Bolivia. Cassava is also cultivated on the continents of Africa and Asia. However, the global average yield is merely a small fraction of the maximum yield. One of the reasons for this low yield is the use of diseased and insect-infested cuttings as the starting material (Raemakers et al. 1993).

Cassava, sometimes referred to as manioc, mandioc, tapioca, and yucca is Africa's second most important crop regarding calories consumed. Its starchy tuberous roots generate 25-35% starch, which provides food for about 500 million people in developing nations through small-scale and subsistence agriculture (Smith et al. 1986; Li et al. 1998).

Cassava is the third largest source of calories in the tropics and has the potential to become a major feed and industrial crop in the coming decades. With more than one billion people suffering from hunger and malnutrition, it is evident that staple crops such as cassava must play a significant role in addressing these issues. Since 1970, the output of cassava has increased by 75%, with the majority of this increase attributable to greater acreage devoted to the crop rather than yield enhancements. In contrast to the major cereals, scientific and technical advancements over the previous decades have not significantly impacted cassava yield (Fauquet 2001).

In the tropics, cassava is an inexpensive source of carbohydrates. However, low protein and high cyanogen concentrations drastically reduce the nutritional value of cassava (Konan et al. 1994). With the traditional breeding technique, addressing these issues, such as the sharply

declining output caused by viruses, insects, and pests, is difficult. In addition, allopolyploid cassava plants demonstrate significant heterozygosity, low natural fertility, poor seed set, and germination. Therefore, additional strategies are required to address the issues mentioned above. Cassava biotechnology provides potent tools to supplement conventional breeding techniques and can expand the useful gene source pool outside the species (Raemakers et al. 1993; Hankoua et al. 2006; Saelim et al. 2006; Danso and Ford-Llyod 2008).

Cassava is propagated mostly through stem cuttings, a slower method than cereal crops. It is mostly cultivated on small farms, and cuttings are typically planted at the beginning of the rainy season. Depending on whether early or late cultivars are planted, the crop cycle is either eight or eighteen months after planting (Santana et al. 2009).

According to Santana et al. (2009), despite its significance, the average global yield over the past 30 years has been only 12 - 13 tonne/hectare, which is considerably below its potential yield of 80 tonne/hectare. Furthermore, it is difficult to cultivate on a wide scale due to the scarcity of high-quality seeds and the low yield and profit. However, these obstacles can be solved by tissue culture techniques that provide high-quality vegetative planting material, resulting in a productivity improvement of more than 30 tonnes per hectare (Santana et al. 2009). Furthermore, utilizing genetic variants resistant to pests, diseases, and environmental restrictions may also increase crop yield.

As one of the key components of biotechnology, plant tissue culture plays a significant role in developing crop varieties and complementing traditional breeding techniques. It is always involved in improving the quality of the crops we consume. Plant tissue culture with the notion of cellular totipotency, media formulation, and cell, tissue, organ, and protoplast culture led to the formation of haploids, somatic hybrids, and pathogen-free plants in numerous resistant plant species. Tissue culture techniques have also been used to investigate the fundamental features of plant development, metabolism, differentiation, and morphogenesis, and they provide the ideal opportunity to control these processes (Gupta and Ibaraki 2006).

This study aimed to determine the optimal method for obtaining a maximum number of shoots and suitable in vitro materials through micropropagation, as well as the various factors associated with the micropropagation of the two cassava varieties "Qulle and "Kello to overcome the obstacles as mentioned above. Furthermore, to develop a method with fewer limitations for producing micropropagated cassava plantlets, and because in vitro propagation may be influenced by several environmental factors, it is necessary to understand the effects of various factors associated with cassava micropropagation.

MATERIALS AND METHODS

Stock plant preparation

Hawassa Agricultural Research Center's Root Crops Research Division provided the fresh stem cuttings of two types of cassava, "Kello" and "Qulle," which were planted in containers with a 1:2:1 mixture of sand, soil, and compost. The glasshouse at Addis Ababa University's College of Natural Sciences, Ethiopia, was kept at a steady 25°C ± 2°C with natural light shining on the plants all day.

Stock Solutions and medium preparations

MS medium and growth regulators stock solution preparation

Plant growth regulators of the right type and concentration were applied alongside the Murashige and Skoog (1962) MS nutrition. The mixture of macronutrients, micronutrients, a Fe-Na-EDTA/FeSO4 mixture, and vitamins was used at maximum dosage. Stock solutions of the MS components (Appendix 1) were prepared by dissolving the correct amount of each component into double-distilled water and then chilling the mixture to -20°C. For example, to prepare a 1 mg/mL concentration, the plant growth regulators were weighed, dissolved in 1 N NaOH using drops, and dissolved in double-distilled water while swirling on a magnetic stirrer. The stock solutions were kept at +4°C for quick access.

The making of a culture medium

MS Basal Medium Preparation

With 100 mL/L of MS macronutrients, 10 mL/L of MS micronutrients, 10 mL/L of MS vitamin, 10 mL/L of Fe-Na-EDTA and $FeSO_4$ mixture, and 2% sucrose, a full-strength MS basal medium was created. First, a pH of 5.8 was reached by adding 1 N HCl and 1 N NaOH, and then 7.0 g of agar was added and melted on a swirling hot plate or in the microwave. As soon as the agar solution was clear, 25 mL was dispensed into baby food jars for starting the shoot to culture, and 50 mL was put onto Magenta GA7 culture vessels for multiplying the shoots before being autoclaved at 121°C with 0.15 Kpa pressure for 15 minutes.

Culture medium for the effect of TDZ on solid medium

TDZ was given to the MS basal medium at 0.1 mg/L, 0.15 mg/L, 0.2 mg/L, and 0.25 mg/L. There were four TDZ treatment groups, and each group was replicated five times.

Liquid media for growing cultures at various salt concentrations

Full strength culture medium was made with 100 mL/L of MS macronutrient, 10 mL/L of MS micronutrient, 10 mL/L of MS vitamin, 10 mL/L of Fe-Na-EDTA and $FeSO_4$ mixture, and 2% sucrose. In the case of half and quarter salt strengths, the amount of macronutrient, micronutrient, Fe-EDTA, and $FeSO_4$ mixture, and vitamin stock solutions utilized was reduced by half and quarter, respectively, and supplemented with 0.5 mg/L BAP + 1 mg/L GA_3 + 0.01 mg/L NAA. A pH of 5.8 was reached by adding 1 N HCl and 1 N NaOH, and then 7.0 g of agar was added and melted on a swirling hot plate or in the microwave. When the agar solution was clear, 25 mL was dispensed into baby food jars to start the shoot to culture, and 50 mL was put onto Magenta GA7 culture containers for multiplying the shoots before being autoclaved at 121°C with 0.15 Kpa

pressure for 15 minutes. Three salt concentrations were utilized in the experiments (full, half, and quarter), and each was replicated five times.

Media preparation for the different types of pH

The MS medium (preparation instructions may be found in section 4.3.1 of this publication) was used, and the pH was changed to 5.0, 5.6, 5.8, 6.0, and 6.6 with 1 N HCl and 1 N NaOH, respectively. After the pH was corrected, different amounts of agar were added: 1.3%, 1.1%, 0.8%, 0.7%, and 0.6%. Five replicates were performed using pH 5.0, 5.6, 5.8, 6.0, and 6.6 as the treatments.

Culture medium for different sucrose concentrations

2%, 1.5%, and 1% sucrose were added to MS baseline medium that had been supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 1 mg/L GA_3. Five replicates were performed on three treatments (2%, 1.5%, and 1% sugar concentrations).

Media preparation for somatic embryo induction

A final volume of 1000 mL of CIM (Cassava induction medium) was made by combining MS basal medium with various 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 $CuSO_4$. Then, 1 N HCl and 1 N NaOH were used to bring the pH to 5.8. To the pH-balanced mixture, 8.0 g of agar was added and melted using either a stirring hot plate or a microwave. Autoclaving at 121°C with 0.15 Kpa pressure for 15 minutes, sterilized the agar medium before being distributed into baby food jars (holding 25 mL) and Magenta GA7 culture pots (holding 50 mL).

Somatic embryo induction was performed using ten different 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 with 2 µM $CuSO_4$ and six replicates.

Cassava maturation medium (CMM) was made 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. When the medium's pH was at 5.8, it was placed into a 100 mL Erlenmeyer flask and a Magenta GA7 culture vessel fitted with a membrane raft and autoclaved for 15 minutes at 121ºC, 0.15 Kpa pressure. In this case, six replicates were employed across six treatments (0.1 mg/L, 0.2 mg/L, and 0.3 mg/L BAP alone or in combination with 0.01 mg/L 2,4-D).

Explant collection and surface sterilization

Every four to seven days, shoot tips measuring 5-10 cm in length were picked from "Qulle" and "Kello" varieties in the greenhouse to trim them down to 2-3 cm of explants. The cuttings were given the first wash in tap water to remove any loose debris and mud. The second wash was in OMO powder detergent and a thorough rinsing until all traces of detergent were gone, followed by two rinses in double-distilled water. After a minute of rubbing with 70% ethanol, it was disinfected and rinsed three times with double-distilled sterile water. After soaking in sterile double-distilled water for 10 minutes for "Qulle" and 11 minutes for "Kello," the explants were cleaned with 1% Clorox bleach containing 5.25% active chlorine and 1 drop of tween 20. The explants were gently shaken by hand while being treated with Clorox, and the entire disinfection process, beginning with 70% ethanol, was carried out inside a laminar airflow cabinet.

Culture initiation

After being sterilized, shoot explants were cut to 2-3 cm before being utilized for shoot culture, whereas explants with at least two nodes were used for node cultures. Next, all leaves except the two youngest leaf lobes at the tip and the scales were removed using sterile forceps and a scalpel. Following parafilming of the vessels, the explants were grown in the various shoot initiation media employed to investigate the factors. A total of four explants were utilized in each culture vessel, and the experiment was repeated five times for each factor except for the liquid medium treatment, which was conducted with a total of twenty test tubes.

Initially, nodal explants were immersed in liquid MS media supplemented with various doses of TDZ. Then, the cultures were kept on an orbital shaker at 110 rpm for a week. For the next four weeks, the cells were maintained in a solid MS medium containing 0.5 mg/L BAP, 1 mg/L GA_3, and 0.01 mg/L NAA.

Shoot multiplication

Explants for shoot multiplication were grown from cultures obtained from both shoots and nodes. Each Magenta GA7 culture vessel contained six replicates of five explants (five shoots or five nodes).

Culture conditions

The cultures were maintained in the culture chamber at 29±2°C with 16 h/day light and 8 h/day dark cycles (light intensity of 2000 lux) and subcultured every four weeks.

Somatic embryogenesis

Induction of somatic embryos

Young leaf lobes were employed to induce somatic embryogenesis from in vitro propagated cassava shoots of the "Kello" and "Qulle" types. There were a wide range of hormone concentrations utilized, including 4 mg/L, 6 mg/L, 8 mg/L, 10 mg/L, and 12 mg/L 2,4-D, as well as 7 mg/L, 10 mg/L, 12 mg/L, and 16 mg/ L 2,4-D with 2 µM $CuSO_4$. There were six copies of each therapy with five explants, and the results were averaged. After sterile scalpels were used to create small incisions in the leaf lobe explants, the adaxial side of the leaves was placed in contact with the culture medium for a total of 60 days (4, 6, and 8 milligrams per liter of 2,4-D) or 40 days (10 and 12 milligrams per liter of 2,4-D). The cells were moved to a 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 create cyclic somatic embryos (Saelim et al. 2006).

Culture conditions

Baby food jars were used for inducing somatic embryos. The explants were kept in dim light for a week before being moved to a condition with 16 h/day light and

8 h/day dark cycles at a light intensity of 2000 lux and a temperature of 29±2°C.

Micropropagation scheme of the two cassava varieties

Cassava "Qulle" and "Kello" were subcultured, and their micropropagation schemes were analyzed. Subcultured and multiplied shoots were recorded at the start of each subculture to examine the pattern of shoot multiplication. Full-strength MS media containing 0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3 was employed in this experiment.

Rooting and acclimatization

They were put in a hormone-free MS medium to burgeon roots in the propagated shoots. After the cuttings had established roots, the agar was washed away, and they were moved to a greenhouse. Putting the containers in plastic bags allowed them to sit for a week or two.

Study design

The research was performed in the Plant Propagation and Tissue Culture Laboratory at Addis Abeba University, Ethiopia. CRD (Completely Randomized Design) was employed as the statistical approach to this problem, and the data were analyzed with SPSS 17.0 and then loaded into Sigma plot 10.0 for graphic illustration. Mean separation at the 5% probability level was performed using one-way ANOVA.

RESULTS AND DISCUSSION

Shoot induction using solid MS medium with different concentrations of TDZ

Five to seven days after being cultured, shoot explants began reacting across all treatments, and by the end of the second week, shoot elongation and leaf development were obvious. Both types showed mature in vitro development after four weeks (Figure 1). In the case of "Kello," statistical analysis of the number of shoots produced by explants showed that all treatments were substantially different except for those containing 0.1 mg/L and 0.2 mg/L TDZ. In the instance of "Qulle," there is a statistically significant difference between the three treatments (0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3), but not between the treatments and the control (1 mg/L GA_3).

In the instance of "Kello," the optimal concentration of TDZ was 0.25 mg/L, where the average number of shoots and leaves per explant were 2.05 and 5.7, respectively, and the optimal length of shoot per explant was 4.55 cm (Table 1, Figure 1). In contrast, in the instance of "Qulle," the maximum mean the number of shoots, leaf, and shoot length per explant were 6.1, 9.45, and 1.90 cm at a TDZ dosage of 0.2 mg/L, 0.1, and that of the control, respectively (Table 2, Figure 2).

Table 1. The average number of shoots, leaves, nodes, and shoot length of the "Kello" variety after 4 weeks on medium supplemented with different concentrations of TDZ

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 a 5% probability level

Table 2. The average number of shoots, leaves, nodes, and shoot length after 4 weeks per explant of the variety "Qulle" on medium supplemented with different concentrations of TDZ

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 a 5% probability level

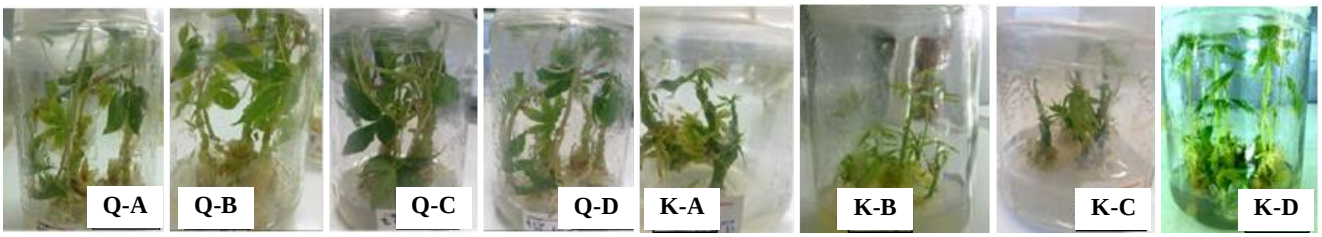

Figure 1. Culture initiation of the two cassava varieties "Qulle" (Q) and "Kello" (K) on MS medium supplemented with different concentrations of TDZ 0.1, 0.15, 0.2, 0.25 mg/L TDZ for A, B, C, and D, respectively

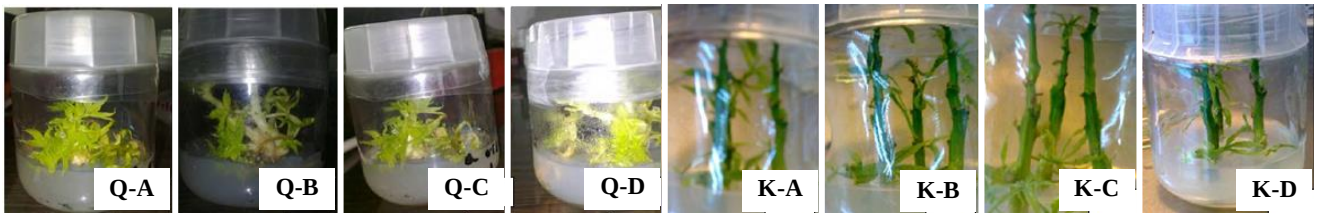

Figure 3. Prior to shoot induction, nodal cells grown in liquid MS media containing varying doses of TDZ A = 0.1 mg/L, B = 0.15 mg/L, C = 0.2 mg/L D = 0.25 mg/L of TDZ

Table 3. The average number of shoots per explant of "Kello" and "Qulle" varieties pre-immersed in liquid medium supplemented with various doses of TZ, followed by initiation of culture on medium supplemented with 0.5 mg/L BAP, 1 mg/L GA_3, and 0.01 mg/L NAA after four 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 a 5% probability level

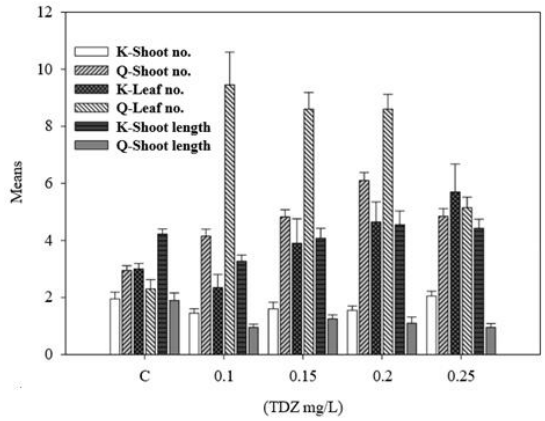

Figure 2. Mean shoot length and number of shoots, leaves, and nodes of "Kello" (K) and "Qulle" (Q) varieties based on various TDZ doses

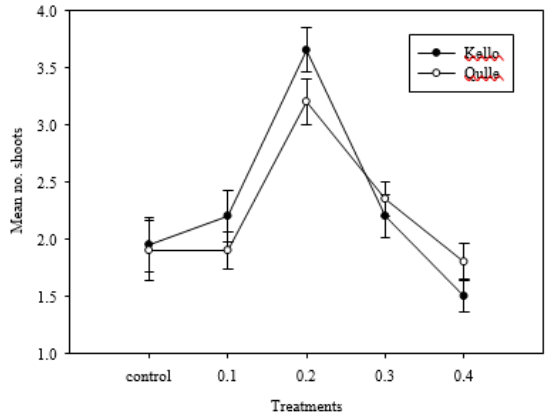

Figure 4. The average amount of shoots induced per explant on a liquid MS media supplemented with various dosages of TDZ before transfer to a solid medium containing 0.5 mg/L BAP, 1 mg/L GA_3, and 0.01 mg/L NAA

Shoot induction using a two-step procedure

By the end of the week, nodal explants that had been soaked in various TDZ pre-culture treatment concentrations had expanded at the nodes and, in some cases, had begun to initiate new shoot growth (Figure 3). In addition, after being transplanted to solid media, shoots were shown to proliferate. The maximum mean number of shoots per explant was 3.6 for "Kello" and 3.2 for "Qulle" when explants were treated with 0.2 mg/L TDZ (Table 3, Figure 4). Compared to a control group grown in MS media containing 0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3, the in vitro materials showed enhanced leaf proliferation. As for leaf proliferation, the in vitro materials surpassed the control, which had been grown in an MS medium containing 0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3.

Salt strength

By the end of the first week in culture, shoot explants grown on MS media with varying salt concentrations showed signs of life. By the end of the month, mature cultures of both the "Kello" and "Qulle" types had been obtained at all three salt concentrations (Table 4).

For "Kello," the optimal growth was achieved at one-fourth, one-half, and full salt strengths, with mean values of 1.60 shoots, 3.50 leaves, 3.30 nodes, and 4.10 cm shoot length per explant. While in the case of "Qulle," the maximum mean number of shoots, leaves, nodes, and shoot length was attained at full (control) and half salt strength (1.80, 5.20, 3.40, and 3.40 cm, respectively) (Table 5, Figure 5).

Response to medium with different pH

Approximately five to seven days after the commencement of culture, the cultures exposed to varying pH levels began to show signs of life. By the end of the month, explants from throughout the pH spectrum had grown into fully formed in vitro structures (Figure 6). In the case of "Kello," the optimal pH was 5.6, where the average number of shoots, leaves, nodes, and shoot length were 4.10, 12.10, 4.30, and 5.00, respectively (Table 6, Figure 7). The optimal pH for "Qulle" is 6.6, where the mean values for shoots, leaves, nodes, and shoot length are 2.40, 9.80, 4.12, and 4.90, respectively (Table 7, Figure 7).

Response to different sucrose concentrations

Explants began responding during the first week of culture initiation with leaf emergence, shoot elongation, and shoot multiplication, all per the outcomes of earlier treatments. The average number of shoots, leaves, nodes, and shoot length per explant was greatest at a sucrose concentration of 0.15% for both the "Kello" and "Qulle" kinds (Tables 8 & 9, Figure 8). The explants looked healthier and showed almost no signs of necrosis, which is common on a medium supplemented with greater sucrose content like the control (0.2% sucrose concentration).

Somatic embryo induction

After 60 days of culture, those with 4.0 mg/L, 6.0 mg/L, and 8.0 mg/L of 2,4-D had a yellowish friable callus. Those with 4.0 mg/L, 6.0 mg/L, and 8.0 mg/L of 2,4-D had a yellowish friable callus after 40 days of culture (10.0 and 12.0). After a month, none of the other treatments, including 2 µM $CuSO_4$, produced an FEC and went brown (Figure 9).

Table 4. 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 GA_3 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}

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

Table 5. 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 GA_3 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}

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

Table 6. 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 GA_3 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}

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

Table 7. 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 GA_3 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}

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

Table 8. 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 GA_3 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}

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

Table 9. 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 GA_3 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}

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

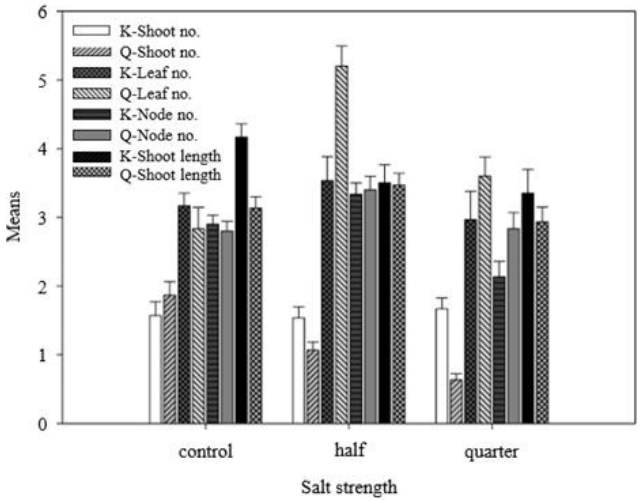

Figure 5. Mean number of shoots, leaves, and nodes and mean shoot length of "Kello" (K) and "Qulle" (Q) varieties based on different salt strengths

Although all leaf lobes were successful in inducing somatic embryos, no somatic embryos obtained from the induced callus on a 2,4 D medium showed embryo maturation and shoot induction on a medium containing different concentrations of BAP alone and along with a drastically lowered concentration of 2,4- D compared to those used while inducing the somatic embryos.

Micropropagation scheme

Subculturing the induced shoot tips every four weeks on a complete MS medium with 2% sucrose and 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA_3 has proven successful. Initially, "Qulle" cultured shoot tips responded very well to subculturing, but as the number of subcultures continued to increase, the average number of shoots began to decrease. In contrast, "Kello" showed a very opposite pattern; the average number of shoots grew linearly up to the third month, then suddenly reached an exponential high in the fourth month, and again showed a drastic decline in the fifth subculture (Table 10, Figure 10).

Rooting and acclimatization

By the end of the third week, the plantlets had grown roots and were ready to be moved to the field. Half of the "Qulle" plantlets and 66.6% of the "Qello" plantlets that had been acclimated survived (Figure 11).

Table 10. Mean number of shoots per explants on different successive subculturing stages

Monthly subculture	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 a 5% probability level

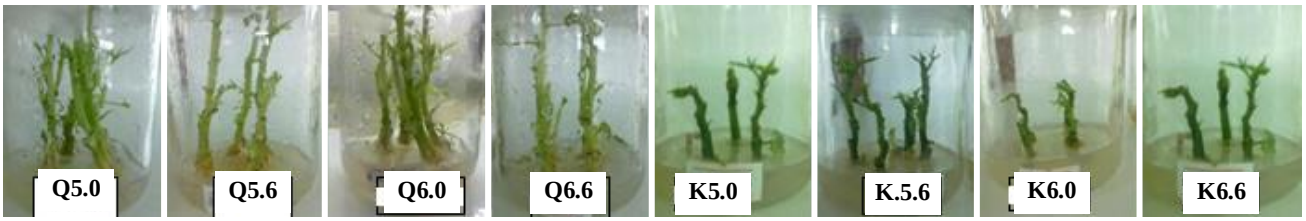

Figure 6. Shoot induction in a medium containing 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA_3 at different pH levels (5.0, 5.6, 6.0 and 6.6 pH). Q = Qulle, K = Kello

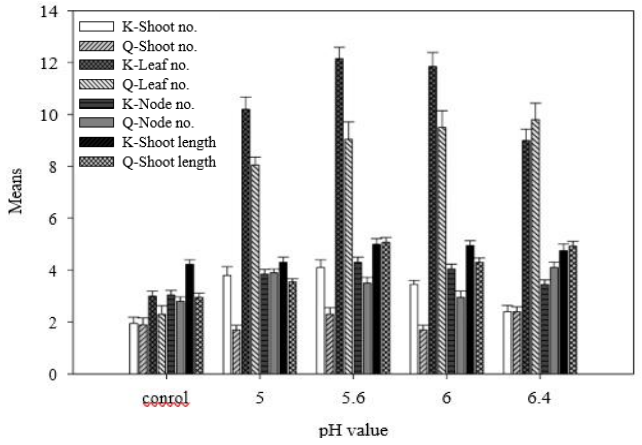

Figure 7. Mean number of shoots, leaves, and nodes and mean shoot length of "Kello" (K) and "Qulle" (Q) varieties based on different pH values

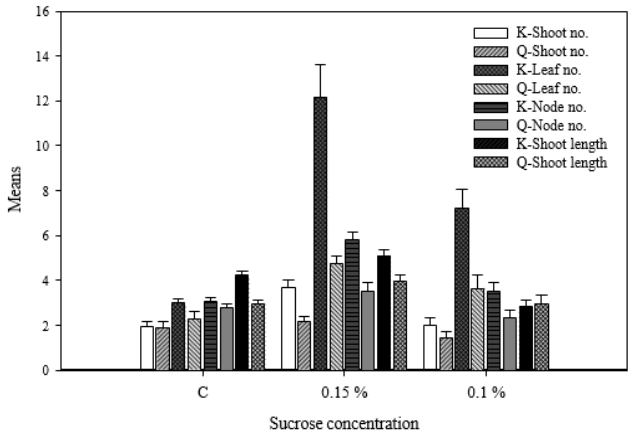

Figure 8. Mean number of shoots, leaves, and nodes and mean shoot length of "Kello" (K) and "Qulle" (Q) varieties based on different sucrose concentrations

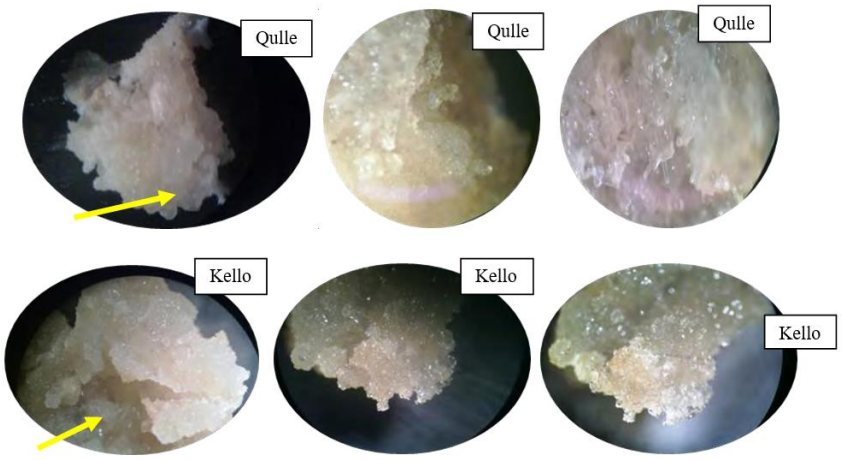

Figure 9. Structure of globular somatic embryos as seen under a dissecting microscope

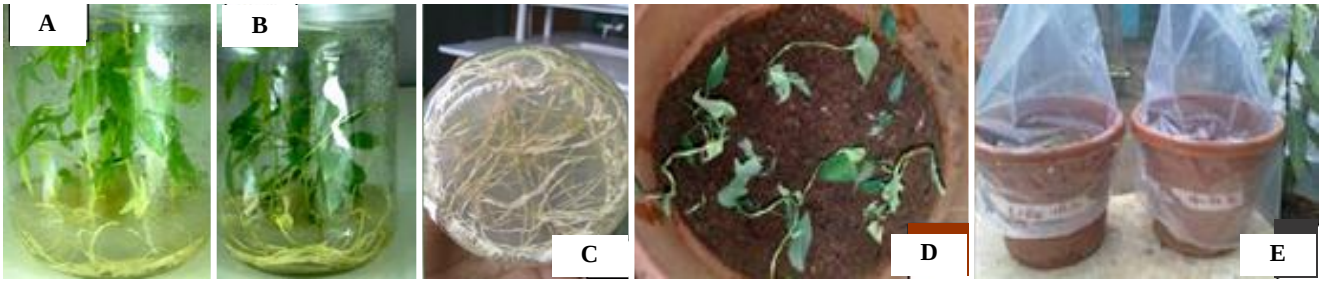

Figure 11. Rooting and acclimatization. Rooting in "Qulle" (A), rooting in "Kello" (B), roots at the bottom of the culture vessels (C), rooted plants after being planted (D), Plants covered in a polyethylene bag (E)

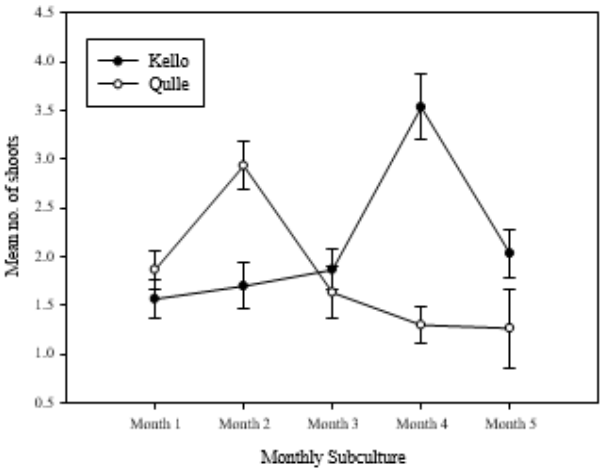

Figure 10. Subculturing schemes of the two cassava varieties in successive subcultures

Discussion

Shoot induction using solid MS medium with different concentrations of TDZ

The results of shoot induction on MS medium with different concentrations of TDZ were the same as those of Escobar et al. (2001) and Siddique and Anis (2006). Their results also showed that using different concentrations of TDZ alone and with IAA from cotyledonary node explants caused multiple shoots to grow. It was shown that multiple shoots grew from the explants as early as 15 days after they were taken. It is very similar to the results of this study, which showed that the shoots were growing well by the end of the first week and that the growth and number of shoots continued as early as the second week. They also said that when there was more than 5.0 µM of TDZ, there was a big drop in the number of shoot buds and the number of times they grew back. They thought this might be because of too much callus growth, while its specific concentration supports the most shoot bud formation. In the same way, this study on "Qulle" showed that at a concentration of 0.25 mg/L, the average number of shoots produced per explant dropped, and the explants showed a mass of callus at the base.

Sajid and Aftab (2009) reported in 2009 that TDZ stimulates growth when added to a tissue culture medium at a low concentration (10-1000 times lower than the concentration of other PGRs). The results of this study agree with those of Sajid and Aftab (2009). The same goes for the results of Huetteman and Preece (1993). In addition, Aasim et al. (2009) said that TDZ is the most active

cytokinin substance that causes more shoot growth in vitro than many other cytokinins in many plant species.

The results of Konan et al. (1997), which involved growing cassava shoots on MS medium with BAP, kinetin, thidiazuron, and zeatin, don't match up with this study. However, their results found that BAP was the most effective at causing shoot growth and development because of the abovementioned cytokinins.

Shoot induction using a two-step procedure

Bhagwat et al. (1996) found that an open-ended shoot proliferation process produced the most shoots per nodal explant after 10 weeks. It was based on a two-step procedure that involved putting the nodal explants in TDZ-containing liquid MS-medium for 6-8 days and then growing them on agar solidified medium with 22.2 µM BAP and 1.6 µM GA_3. Consistent findings were found between the current investigation and a previous one that used a liquid MS-medium pre-treatment for a week followed by culture on agar solidified MS-medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3.

According to Bhagwat et al. (1996), TDZ stimulates the growth of nodal explants, and this growth (expansion) persists into the second stage of culture on agar solidified medium. The enlarged explant gives rise to new shoots by continuously producing new bud clusters and fasciated stems.

For example, in hybrid tea rose cultivars, Singh and Syamal (2001) report that axillary shoot proliferation can be greatly increased by exposing explants to TDZ for a brief time prior to subculturing onto a conventional shoot proliferation medium.

Salt strength

Variety "Kello" generated the highest mean number of shoots, leaves, nodes, and shoot length per explant (1.60, 3.50, 3.30, 4.10, respectively) at quarter-MS, half-MS, half-MS, and full-MS medium salt concentrations. However, when grown in complete (control), half (half), and a half (half) salt strength of the medium, the "Qulle" variety produced the greatest mean number of shoots, leaves, nodes, and shoot length per explant (1.80, 5.20, 3.40, and 3.40, respectively).

However, the highest number of nodes was found at full-strength salt, which contradicts the mean number of nodes for "Kello" found by Mantell and Hugo (1989). However, the results of this study are consistent with the work of Mantell and Hugo (1989) regarding the mean number of shoots for "Qulle," which is reached at a full-strength mineral salt concentration of the medium.

Response to medium with different pH

The results of this investigation demonstrated that specific pH ranges are tolerable in the micropropagation of cassava, supporting the advice of Kozai et al. (1997) that pH is controlled in the mass production of plants by in vitro procedures. By the end of the month, all of the explants across all pH ranges had successfully given rise to in vitro material. Optimal growth for "Kello" occurred at a pH of 5.6, as measured by the mean number of shoots, leaves, nodes, and shoot length. Maximum averages for shoot number, leaf count, node count, and shoot length were all reached at a pH of 6.6 in "Qulle." In "Kello," there was no discernible difference in the average number of shoots produced by explants when the pH was either 5.0, 5.6, 6.0, or 5.8 or 6.6. A comparison of the pH levels 6.0 with the control, 5.0 and 5.6, and 6.0 with 5.0 and 5.6, showed no significant differences. The performance and pH variation tolerance of "Qulle" is particularly impressive, especially given that the medium is often created within the pH range of 5.0 to 6.0.

Response to different sucrose concentrations

The maximum number of shoots and nodes per explant was found to be attained at a lower concentration of sucrose, which is consistent with the findings of Mantell and Hugo (1989), who investigated the effects of various factors on the root, shoot, and microtuber development in shoot cultures of *Dioscorea alata* L. and *D. bulbifera* L. yams.

Nhut et al. (2001) studied plant and shoot regeneration in *Lilium longiflorum* Thunb. and found that using 3% or 4% sucrose resulted in a higher frequency of shoot development. In contrast, using 2% sucrose resulted in a slightly lower frequency of shoot creation. While at 0.15% sucrose concentration, the "Kello" and "Qulle" varieties showed the highest mean values for shoots, leaves, nodes, and shoot length per explant. This finding holds great potential for the practice of cassava tissue culture in developing nations. The significant benefit is the savings in purchasing costly plant tissue culture grade sucrose from a reduced need for that carbon source.

Sugar-free (photoautotrophic) micropropagation was recommended for use on a large scale by Kozai et al. (1997) due to its many benefits over heterotrophic photomixotrophic micropropagation, including the in vitro plantlets' faster growth and development, fewer physiological and morphological disorders, and lower risk of biological contamination.

Somatic embryo induction

None of the somatic embryos obtained from the induced callus on a 2,4 D medium showed embryo maturation and shoot induction on a medium containing different concentrations of BAP alone as well as together with a drastically lowered concentration of 2,4-D, in contrast to the result of Stamp (1987), who used a consistent procedure in inducing and maturing somatic embryos. The outcome was also at odds with the work of (Groll et al. 2002), which induced somatic embryos and shot germination using full and reduced-strength MS medium.

Browning of the callus and the absence of somatic embryo formation were the results of the present experiment on both "Kello" and "Qulle," in contrast to the result of Saelim et al. (2006). The latter used 2 µM $CuSO_4$ as a supplement along with different concentrations of 2,4-D to have the best embryo induction frequency. Consistent

with the present observation, Konan et al. (1994) found that 2,4-D generated globular somatic embryos.

Similar results were found by Atehnkeng et al. (2006). They demonstrated that pro-embryos created by two cassava genotypes did not survive past the globular developmental stage and that the pro-embryo formation level was insufficient to indicate embryogenic competence.

According to Sudarmonowati and Henshaw's unpublished research, only six of sixteen cassava cultivars tested successfully inducing somatic embryogenesis when grown in a medium enriched with 2,4-D. The authors hypothesized that this was because various genotypes react differently to 2,4-D and that genotypes substantially influence somatic embryogenesis. However, when given a media with picloram and dicamba, Sudarmonowati and Henshaw (1996) reported success in inducing somatic embryogenesis in cultivars that had failed to do so when given a medium with 2,4-D. In addition, cassava's immature leaf lobes and apical shoot meristems have been found to differ in their genotype's propensity to generate primary somatic embryos, as reported by Hankoua et al. (2005).

Although both 2,4-D and picloram induced embryogenic calli and matured primary embryos, Danso et al. (2010) reported that picloram enhanced early calli development more than 2,4-D. It is because picloram can acidify and loosen the cell wall earlier than 2,4-D, which is critical in weakening the cell-cell interaction gradient that coordinates the normal bipolar development of the embryo.

Micropropagation scheme

According to Beyene (2009), the study's multiplication data was gathered from the first subculture of plantlets after four weeks in the multiplication medium. For this reason, the second and third subcultures may significantly increase their numbers. Thus, it was suggested that other subculturing take place.

In the instance of "Qulle," the apex of cultured shoots responded well during the initial subcultures, but the mean number of shoots began to decline as the frequency of subculturing increased. While "Kello" revealed a completely different pattern, with a linear increase in the mean number of shoots through the third month, an exponential peak in the fourth month, and a subsequent sharp fall in the fifth subculture, the two trends were in stark contrast to one another. These may have arisen because frequent subcultures caused the plantlets to lose their ability to reproduce.

Mbanaso (2008) found that after four successive subcultures, shoots obtained from starch-gelled medium were significantly less strong than those grown in regular soil. The higher content of starch in the medium exacerbated this tendency toward diminished resilience over time.

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