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FACTORS AFFECTING *IN VITRO* PROPAGATION OF CASSAVA (*MANIHOT ESCULENTA* CRANTZ.) EUPHORBIACEAE, VARIETIES OF 'KELLO' AND 'QULLE'

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ABSTRACT: Cassava (*Manihot esculenta* Crantz.) is a perennial shrub of the Euphorbiaceae family native of eastern tropical South America, grown in the tropics of Africa and Asia for its tuberous roots. Its cultivation is constrained by several problems including the use of cuttings infected with diseases and pests, the lack of high quality seed, low productivity, high heterozygosity, low fertility, poor seed set and seed germination. The objective of this study was to find the optimal conditions for micropropagation of two varieties of cassava released for farmers for the production of high quality planting materials. These include determination of the effect of temperature on bud-break of mother plants, differences in concentrations of salt, sucrose, and thidiazuron (TDZ) in a semi-solid MS medium, pH, two-step MS medium and repeated subcultures. The mean number of shoots per plant for axillary bud-break was highest (10.8) at 26°C for that from 'Kello' and 9.8 at 30°C for that from 'Qulle'. The highest mean number of shoots per explant was obtained on MS medium containing 0.2 mg/L TDZ for both varieties on both semi-solid and two-step MS medium culture system. Maximum mean shoot number was obtained on MS medium of a quarter and full salt strength for 'Kello' and 'Qulle', respectively. The highest mean number of shoots per explant for 'Kello' (4.10) and 'Qulle' (2.40) was obtained at pH 5.6 and 6.6, respectively. 'Kello' produced 3.70 shoots per explant on MS medium containing 1.5% sucrose. Repeated subculturing of 'Qulle' resulted in gradual loss of multiplication rate from the third subculture onwards. The present study contributes to optimization of micropropagation of cassava.

Key words/phrases: Liquid medium, Salt strength, Shoot multiplication, Sucrose, TDZ.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz.) is a perennial shrub that belongs to the family Euphorbiaceae. It is mainly grown in the tropics for its starchy tuberous root, which is used for human consumption, animal feed, and as raw material for the starch industry. It has broad adaptation to a variety of soil, climate, drought 'tolerance', and ability to grow on marginal soil

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(Mathews *et al*., 1993; Raemakers *et al*., 1993; Le *et al*., 2007). It is the most important tuber crop in Africa. Its starchy tuberous root yields 25-35% starch which provides food for over 500 million people living on small-scale and subsistence farming in developing countries (Smith *et al*., 1986; Li *et al*., 1998).

In Ethiopia, cassava is primarily grown as food crop in southern and south-western parts (Dejene Makonnen, 2006). Cassava has its centre of diversity in north-eastern Brazil, south-western Mexico, and eastern Bolivia. The average yield in the world today is only a small fraction of its potential. According to Santana *et al*. (2009), the average worldwide productivity over the past 30 years has been limited to only 12-13 tonnes/hectare when compared to its potential crop productivity of 80 tonnes/hectare. Total average coverage and production of cassava per annum in southern region of Ethiopia is 4,942 hectares with a yield of 53,036.2 tonnes (SNNPR, BoA, 2000). One of the reasons for yield reduction is the use of cuttings infected with diseases and pests as the planting material (Raemakers *et al*., 1993).

Cassava is propagated mainly by stem cuttings and mostly grown on small farms. Compared to the scientific and technical progress made on the major cereals, there has not been substantial impact on cassava production (Fauquet, 2001). The problems include the severe reduction of yield due to virus and insect pests that are difficult to deal with using traditional breeding systems. Its allopolyploid nature, low natural fertility, poor seed set and seed germination has hampered the breeding efforts of cassava. In addition, the rate of bud-break and growth of different cassava varieties is different under different environmental factors such as temperature. Therefore, application of advanced techniques such as biotechnological tools are required to complement traditional breeding methods (Raemakers *et al*., 1993; Hankoua *et al*., 2006; Saelim *et al*., 2006; Danso and Ford-Llyod, 2008).

Plant tissue culture, as one of the major components of biotechnology, plays a key role in improving different varieties of crops complementing the traditional breeding method. It is used in ensuring better qualities of crops such as producing disease-free planting materials and genetic engineering. Although different studies were carried out on cassava tissue culture (Mathews *et al*., 1993; Bhagwat *et al*., 1996; Danso and Ford-Llyod, 2008), there are very few works that have been done on factors that affect micropropagation of cassava. Moreover, there is only one report on micropropagation of the two released varieties (‘Kello’ and ‘Qulle’) in Ethiopia (Dawit Beyene *et al*., 2010). Therefore, the objectives of this study

were to evaluate the bud-break of the cuttings of the two varieties at different temperatures in glasshouse and to investigate the different factors that affect their *in vitro* propagation.

MATERIALS AND METHODS

Plant material

About 30 cm long fresh stem cuttings of two varieties of cassava namely 'Kello' and 'Qulle' were obtained from Hawassa Agricultural Research Centre, Root Crops Research Division. The cuttings were planted in pots containing 1:2:1 ratio of sand, soil and compost, respectively. The plants were maintained in glasshouse at an average temperature of 25±2°C under natural light condition.

Effect of temperature on axillary bud-break

Twenty three cuttings from each of the two varieties were planted in separate glasshouse chambers at 20°C, 26°C and 30°C and a relative humidity of 50%. The newly sprouted lateral buds that gave rise to shoots were counted every week and their monthly growth measured for two months.

Explant collection and surface sterilization

About 5.0 cm long newly sprouted shoot tips were collected from 'Kello' and 'Qulle' varieties and washed with tap water using powder detergent (OMO) and rinsed in double distilled water. This was followed by disinfection with 70% ethanol for one minute followed by rinsing with sterile double distilled water three times. The explants were further disinfected with 1% Clorox containing 5.25% active chlorine and one drop of tween-20 for 10 minutes followed by rinsing with sterile double distilled water five times.

Culture initiation

The sterilized shoot explants were trimmed to 2-3 cm long and used for shoot and node culture. The explants for node culture contained two nodes. The explants were cultured on 25 ml full strength MS revised medium (Murashige and Skoog, 1962) in baby food jars at 0.0, 0.1, 0.15, 0.2 or 0.25 mg/L thidiazuron (TDZ), 2% sucrose and 0.7% agar. The pH was adjusted to 5.8 before the addition of agar and autoclaved at 121°C and 0.15 kPa pressure for 15 minutes. The cultures were maintained at a temperature of 29±2°C and light intensity of 23 µmol $m^{-2}s^{-1}$ under 16 h photoperiod. Unless indicated, all cultures were maintained under these growth conditions. Four

explants per culture vessel in five replications were used.

In another experiment, nodal explants were cultured in 15 ml liquid MS medium in 25 mm × 100 mm test tubes at 0.0, 0.1, 0.2, 0.3 or 0.4 mg/L TDZ and maintained on an orbital shaker at 110 rpm for a week.

Shoot multiplication

The shoots that were initiated on semi-solid MS medium were transferred to shoot multiplication medium after four weeks of initiation while the shoots from liquid medium were transferred to the same multiplication medium after one week of initiation. Different salt strengths of MS medium (full, half and quarter), different pH (5.0, 5.6, 5.8, 6.0 or 6.6), and different concentrations of sucrose (2%, 1.5% or 1%) were evaluated for their effect on shoot multiplication. All of these shoot multiplication media were supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA and 1.0 mg/L GA_3. A treatment consisting of five explants in each Magenta GA-7 culture vessels were used in six replications.

Rooting and acclimatization

About 8.0 cm long shoots were transferred to growth regulators-free MS medium for root induction. Twenty shoots of each variety for each treatment were used. After three weeks on rooting medium, agar was removed from the roots of the plantlets by washing with sterile double distilled water and the plantlets were planted in pots containing 1:2:1 ratio of sand, soil and compost, respectively. The pots were covered with polyethylene bags and maintained in glasshouse. The plants were watered every day. The polyethylene bags were removed after one week and the number of plants that survived were counted after three weeks. Twenty plantlets of each variety were used for acclimatization.

Statistical analyses

The statistical analyses of quantitative data was carried out by using Statistical Package for the Social Sciences, SPSS 17.0 and the resulting output was fed into Sigma plot 10.0 to obtain graphical interpretation of the results. A difference at probability level of $p \leq 0.05$ was considered significant for all analyses.

RESULTS

Effect of temperature on axillary bud-break

The highest mean number of shoots (10.08) per plant from axillary bud-break was obtained from 'Kello' variety at 26°C and 9.78 shoots were produced by 'Qulle' variety at 30°C on the seventh week. There was significant difference in mean length of shoots produced through bud-breaks at 20°C, 26°C and 30°C. The maximum mean shoot length obtained from 'Kello' was 9.37 cm while 'Qulle' had 11.22 cm at 30°C in the first month. In the second month, maximum mean shoot length of 'Kello' was 55.66 cm at 30°C while for 'Qulle' it was 47.30 cm at 26°C.

Culture initiation

The shoot explants of all treatments that were cultured on semi-solid MS medium started responding five to seven days after culture and by the end of the fourth week, both varieties displayed fully developed shoots that were ready for subculturing (Figs. 1A and 1B). Although the percentage of shoots initiated ranged from 85 to 100 at all concentrations of TDZ for both varieties, there was no statistically significant difference (Table 1).

Table 1. Percentage of shoots of 'Kello' and 'Qulle' varieties initiated on MS medium containing different concentrations of thidiazuron (TDZ).

TDZ (mg/L)	Shoot initiation (%)	
	'Kello'	'Qulle'
C	90.0[a]	85.0[a]
0.10	95.0[a]	95.0[a]
0.15	100.0[a]	100.0[a]
0.20	90.0[a]	100.0[a]
0.25	85.0[a]	80.0[a]

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

The nodal explants which were soaked in liquid MS medium containing different concentrations of TDZ displayed nodal expansion and shoot initiation by the end of the first week (Fig. 1C). The percentage of shoots initiated in liquid medium ranged from 80 to 100 and there was no significant difference among all the treatments (Table 2).

Table 2. Percentage of shoots of 'Kello' and 'Qulle' varieties initiated in liquid medium containing different concentrations of thidiazuron (TDZ).

TDZ (mg/L)	Shoot initiation (%)	
	'Kello'	**'Qulle'**
C	90.0[a]	85.0[a]
0.1	95.0[a]	100.0[a]
0.2	100.0[a]	100.0[a]
0.3	100.0[a]	90.0[a]
0.4	80.0[a]	85.0[a]

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

Effect of TDZ on shoot multiplication

The highest mean number of shoots per explant was obtained at 0.25 mg/L (2.05) and on growth regulators-free medium (1.95) (Table 3). The highest number of leaves per explant (5.7) was obtained at 0.25 mg/L TDZ and the maximum mean length of shoots per explant (4.55 cm) was exhibited at 0.2 mg/L TDZ by 'Kello' variety. In variety 'Qulle', the highest mean number of shoots (6.10), leaves (9.45) and shoot length (1.90 cm) per explant was obtained at TDZ concentration of 0.2 mg/L, 0.1 mg/L and the control, respectively.

Table 3. Mean number of shoots, leaves and length of shoots (cm) per explant of cassava varieties 'Kello' and 'Qulle' on medium containing different concentrations of thidiazuron (TDZ).

TDZ (mg/L)	'Kello'			'Qulle'		
	No. of shoots	**No. of leaves**	**Length of shoots (cm)**	**No. of shoots**	**No. of leaves**	**Length of shoots (cm)**
0.00	1.95 ± 1.05[a]	3.00 ± 0.85[c]	4.23 ± 0.78[bc]	2.95 ± 1.16[c]	2.30 ± 1.45[c]	1.90 ± 1.16[a]
0.10	1.45 ± 0.68[b]	2.35 ± 2.03[d]	3.28 ± 0.95[c]	4.15 ± 0.51[b]	9.45 ± 5.13[a]	0.95 ± 0.51[cd]
0.15	1.60 ± 1.04[b]	3.90 ± 3.83b[c]	4.08 ± 1.55[b]	4.83 ± 0.63[b]	8.60 ± 2.60[a]	1.25 ± 0.63[b]
0.20	1.55 ± 0.68[b]	4.65 ± 3.13[b]	4.55 ± 2.15[a]	6.10 ± 0.96[a]	8.60 ± 2.32[a]	1.10 ± 0.96[bc]
0.25	2.05 ± 0.75[a]	5.70 ± 4.36[a]	4.43 ± 1.41[a]	4.85 ± 0.60[b]	5.15 ± 1.63[b]	0.95 ± 0.60[cd]

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

Effect of nodal explants pre-culture in liquid medium on shoot multiplication

Shoot proliferation was observed after the initiated shoots were transferred to the semi-solid MS medium containing 0.5 mg/L BAP, 0.01 mg/L NAA and 1.0 mg/L GA_3 (Fig. 1D). Explants that were cultured in 0.2 mg/L TDZ during initiation in liquid medium produced the highest mean number of shoots (3.65) per explant for 'Kello' and 3.20 for 'Qulle' after transfer to shoot multiplication medium (Table 4). The *in vitro* materials were also

better in leaf proliferation as compared to those that were cultured on MS medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA and 1.0 mg/L GA_3.

Table 4. Mean number of shoots per explant of the two varieties 'Kello' and 'Qulle' pre-soaked in liquid medium containing different concentrations of thidiazuron followed by culture on medium supplemented with 0.5 mg/L BAP, 1.0 mg/L GA_3 and 0.01 mg/L NAA.

TDZ (mg/L)	Mean no. of shoots/explants	
	'Kello'	**'Qulle'**
0.0	1.95 ± 1.05^{bc}	1.90 ± 1.16^{bc}
0.1	2.20 ± 1.00^{b}	1.90 ± 0.71^{bc}
0.2	3.65 ± 0.87^{a}	3.20 ± 0.89^{a}
0.3	2.20 ± 0.83^{b}	2.35 ± 0.67^{b}
0.4	1.50 ± 0.60^{c}	1.80 ± 0.69^{c}

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

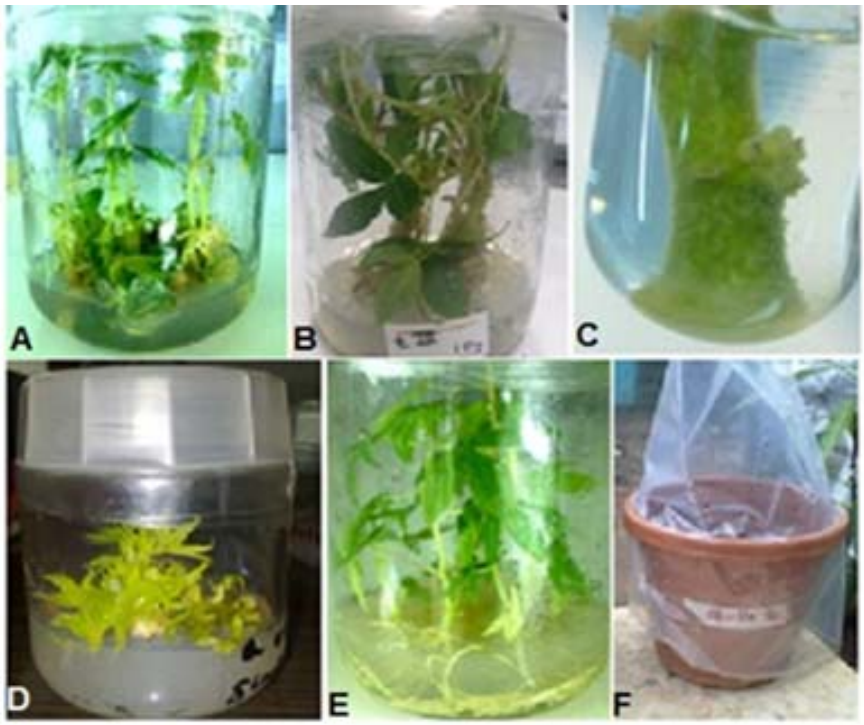

Fig. 1. *In vitro* propagation of cassava: Culture initiation of (A) 'Kello' and (B) 'Qulle'; (C) varieties on MS medium containing 0.2 mg/L TDZ; Node culture of 'Qulle' variety in liquid MS medium containing 0.2 mg/L TDZ and (D) shoot multiplication; (E) rooting and (F) acclimatization.

Effect of MS salt strength on shoot multiplication

In the case of 'Kello', although there was no significant difference among the three MS salt strength in the number of shoots and leaves, the highest mean number of shoots per explant (1.67) was obtained on quarter salt strength MS medium. The highest mean number of leaves (3.53) and nodes (3.33) per explant were obtained on half strength MS medium whereas the longest shoot (4.17 cm) was obtained on full strength MS medium. In the case of 'Qulle', the highest mean number of shoots per explant (1.87) was obtained on full salt strength medium whereas the highest mean number of leaves (5.20), nodes (3.40) and shoot length (3.47 cm) per explant were obtained on half strength MS medium (Table 5).

Effect of pH on shoot multiplication

The highest mean number of shoots (4.10), number of leaves (12.15), number of nodes (4.30) and length of shoots (5.00 cm) per explant were obtained at a pH of 5.6 from 'Kello' variety. For 'Qulle' variety, the highest mean number of shoots (2.40), number of leaves (9.80), number of nodes (4.10) and shoot length (4.93 cm) per explant were obtained at pH of 6.6 (Table 6). There was no significant difference among number of shoots and nodes per explant at pH 5.0, 5.6 and 6.0 in 'Kello'.

Effect of different sucrose concentrations on shoot multiplication

With the exception of 'Kello' variety which showed no significant difference in the number of shoots among the three sucrose concentrations, sucrose at 1.5% gave the highest mean number of shoots for 'Kello' variety and highest mean number of leaves, nodes and length of shoots per explant for both varieties (Table 7). These shoots had better appearance as compared to those cultured on medium containing 2% sucrose and there was no sign of necrosis which was frequently observed on the medium supplemented with higher sucrose concentrations.

Table 5. Mean number of new shoots, leaves, nodes and length of shoots per explant of 'Kello' and 'Qulle' varieties of cassava on MS medium with different salt strengths supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA and 1 mg/L GA_3.

MS salt strength	'Kello'				'Qulle'			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots (cm)	No. of shoots	No. of leaves	No. of nodes	Length of shoots (cm)
Full	1.57 ± 1.10^{a}	3.17 ± 1.01^{a}	2.90 ± 0.71^{a}	4.17 ± 4.16^{a}	1.87 ± 1.07^{a}	2.83 ± 1.68^{b}	2.80 ± 0.76^{b}	3.13 ± 0.88^{b}
Half	1.53 ± 0.89^{a}	3.53 ± 1.92^{a}	3.33 ± 0.92^{a}	3.50 ± 3.5^{a}	1.07 ± 0.63^{b}	5.20 ± 1.60^{ab}	3.40 ± 1.06^{a}	3.47 ± 0.93^{a}
Quarter	1.67 ± 0.88^{a}	2.97 ± 2.25^{a}	2.13 ± 1.22^{b}	3.35 ± 3.35^{b}	0.63 ± 0.49^{c}	3.60 ± 1.49^{a}	2.83 ± 1.28^{b}	2.93 ± 1.17^{b}

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

Table 6. Mean number of shoots, leaves, nodes and length of shoots per explant of 'Kello' and 'Qulle' varieties of cassava on MS medium containing 0.5 mg/L BAP, 0.01 mg/L NAA and 1 mg/L GA_3 at different pH.

pH	'Kello'				'Qulle'			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots (cm)	No. of shoots	No. of leaves	No. of nodes	Length of shoots (cm)
5.8*	1.95 ± 1.05^{b}	3.00 ± 0.85^{c}	3.05 ± 0.75^{c}	4.23 ± 0.78^{b}	1.90 ± 1.16^{ab}	2.30 ± 1.45^{c}	2.80 ± 0.76^{d}	2.95 ± 0.74^{d}
5.0	3.80 ± 1.47^{a}	10.20 ± 2.09^{b}	3.85 ± 0.81^{ab}	4.30 ± 0.89^{b}	1.70 ± 0.80^{ab}	8.05 ± 1.35^{b}	3.90 ± 0.64^{ab}	3.55 ± 0.53^{c}
5.6	4.10 ± 1.33^{a}	12.15 ± 1.95^{a}	4.30 ± 0.86^{a}	5.00 ± 0.94^{a}	2.30 ± 1.12^{ab}	9.05 ± 2.98^{ab}	3.50 ± 1.00^{bc}	5.08 ± 0.81^{a}
6.0	3.45 ± 0.68^{a}	11.85 ± 2.41^{a}	4.05 ± 0.82^{a}	4.95 ± 0.80^{a}	1.70 ± 0.80^{b}	9.50 ± 2.87^{ab}	2.95 ± 1.09^{cd}	4.30 ± 0.78^{b}
6.6	2.40 ± 1.09^{b}	9.00 ± 1.94^{b}	3.45 ± 0.75^{bc}	4.75 ± 1.14^{ab}	2.40 ± 0.82^{a}	9.80 ± 2.85^{a}	4.10 ± 0.91^{a}	4.93 ± 0.83^{a}

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

Table 7. Mean number of shoots, leaves, nodes and length of shoots per explant of 'Kello' and 'Qulle' varieties of cassava on MS medium containing 0.5 mg/L BAP, 0.01 mg/L NAA and 1 mg/L GA_3 and supplemented with different concentrations of sucrose.

SC (%)	'Kello'				'Qulle'			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots (cm)	No. of shoots	No. of leaves	No. of nodes	Length of shoots (cm)
2.0	1.95 ± 1.05^{b}	3.00 ± 0.85^{c}	3.05 ± 0.75^{b}	4.23 ± 0.78^{b}	1.90 ± 1.16^{a}	2.30 ± 1.45^{b}	2.80 ± 0.76^{ab}	2.95 ± 0.74^{b}
1.5	3.70 ± 1.55^{a}	12.15 ± 6.57^{a}	5.80 ± 1.57^{a}	5.10 ± 1.22^{a}	2.15 ± 1.03^{a}	4.75 ± 1.48^{a}	3.50 ± 1.76^{a}	3.95 ± 1.29^{a}
1.0	2.00 ± 1.55^{b}	7.20 ± 3.86^{b}	3.50 ± 1.76^{b}	2.85 ± 1.33^{c}	1.45 ± 1.27^{a}	3.65 ± 2.58^{a}	2.35 ± 1.38^{b}	2.96 ± 1.69^{b}

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

Effect of subcultures on shoot multiplication

Cultured shoot tips responded well at the early subcultures in case of 'Qulle' and as the number of subcultures increased, the shoot number started to drop beginning from the second (2.93) subculture to the third (1.63), the fourth (1.30) and the fifth (1.26) subculture. In contrast, 'Kello' displayed quite a different trend where the mean number of shoots linearly increased up to the third subculture (from 1.56 to 1.70 and 1.86) and reached a maximum (3.53) at the fourth subculture and declined thereafter to 2.03 at the fifth subculture (Fig. 2).

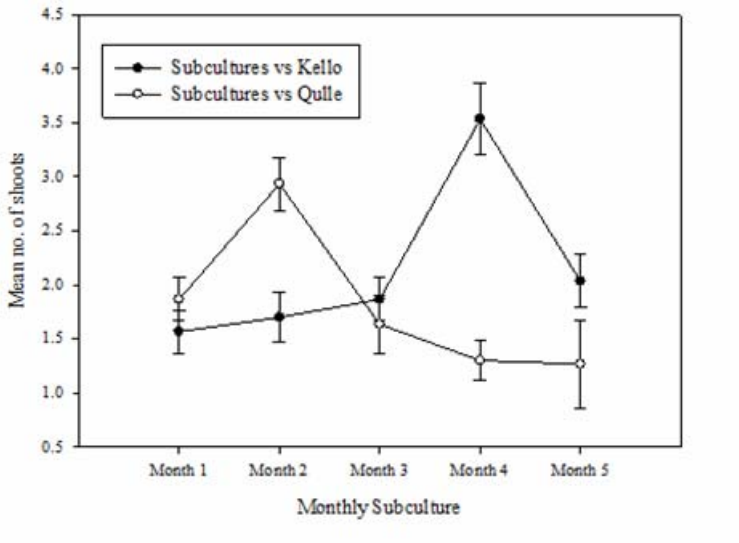

Fig. 2. Subculturing schemes of the two cassava varieties in successive subcultures.

Rooting and acclimatization

All shoots that were cultured on growth regulators-free MS medium for root induction produced well proliferated fibrous roots (Fig. 1E). The mean number of roots per shoot for 'Kello' was 3.20 while that of 'Qulle' was 2.05. Among the acclimatized plants (Fig. 1F), 66.7% of 'Kello' and 50% of 'Qulle' survived and no aberrant plants were observed. The plantlets were hardened and ready to be transferred to the field by the end of the third week.

DISCUSSION

Effect of temperature on axillary bud-break

The mean numbers of axillary bud-breaks showed a linear increase every week for both varieties. Time and temperature at which the highest number of bud-break was recorded varied between the two varieties. This shows that the rate of bud-break is affected by both temperature and genotypes. De Vries *et al*. (1986) also reported that temperature has significant effect on axillary bud-break of F1-seedling populations of glasshouse-grown rose cultivars.

Effect of TDZ on shoot initiation and multiplication

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 of cassava using different concentrations of TDZ alone or in combination with IAA from cotyledonary node explants. The results of this study are in agreement with those previous reports where an excellent shoot elongation occurred at the end of the first week of culture with continued growth that was followed by shoot multiplication at the second week of culture. They also reported that at TDZ concentrations higher than 5.0 μM, there was a considerable reduction of number of shoot buds. 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 consistent with the present study. Aasim *et al*. (2009) described TDZ as the most active cytokinin which induced more *in vitro* shoot proliferation than many other cytokinins in many plant species.

Effect of liquid medium on shoot initiation and multiplication

The results of Bhagwat *et al*. (1996) indicated an open-ended shoot proliferation process which yielded a maximum number of shoots per nodal explant of 31.5 after 10 weeks. This was based on a protocol which involved a two-step procedure by 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. According to these authors, TDZ caused the nodal explants to expand and this expansion continued during the second stage of the culture on agar solidified medium. From the expanded explant, clusters of buds and stems developed continuously and these buds gave rise to shoots. This 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 in combination with 0.01 mg/L NAA and 1.0 mg/L GA_3 is in agreement with the above reports.

Effect of MS salt strength on shoot multiplication

Mean number of shoots per explants obtained from 'Qulle' variety were consistent with the results of Mantell and Hugo (1989) who obtained similar number of shoots per explant using *Dioscorea alata* L. and *D. bulbifera* L. yams in full strength MS medium. However, contrary to the results of the present study where the highest number of nodes per explant from 'Kello' variety was obtained on half strength MS medium, these authors obtained highest number of nodes per explant on full strength MS medium. This difference could be due to the fact that different plant species respond differently to particular media.

Effect of pH on shoot multiplication

The result of this study showed certain ranges of pH that can be tolerated in the micropropagation of cassava. All explants at the different pH values of the culture medium responded well. Maximum mean number of shoots, number of leaves, number of nodes, and length of shoots per explant were obtained at pH of 5.6 in case of 'Kello' and at pH of 6.6 in 'Qulle'. 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' was quite impressive indicating that different genotypes have different potential for pH tolerance. Kozai *et al*. (1997) recommended controlling environmental factors which include pH in the mass production of plants through *in vitro* techniques.

Effect of different sucrose concentrations on shoot multiplication

In the present study, both 'Kello' and 'Qulle' varieties displayed the best shoot length, highest number of shoots, leaves, and nodes per explant at a sucrose concentration of 1.5%. Mantell and Hugo (1989) obtained maximum number of shoots and nodes per explant at 2% sucrose during culturing of *Dioscorea alata* and *D. bulbifera* yams. The results of Nhut *et al*. (2001) on plant and shoot regeneration of *Lilium longiflorum* were 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. The result of the present study has an enormous advantage for reduced

cost of cassava tissue culture in developing countries by using low concentration of sucrose for mass propagation.

Effect of number of subcultures on shoot multiplication

The decline in mean number of shoots per explant after a certain period of subculturing (after two consecutive subcultures in 'Qulle' and after four consecutive subcultures in 'Kello') could be due to loss of the multiplication potential of the shoots. This result is supported by the work of Vujović *et al*. (2012) in which they observed a sharp decrease in multiplication index in the second and third subcultures as well as in the fifth subculture of *in vitro* shoots of vegetative root stocks of contemporary fruit, which they explained might be due to cytokinin habituation.

Rooting and acclimatization

All the shoots cultured on growth regulators-free MS medium rooted well, indicating that there was no need of adding auxins to a medium for cassava rooting. However, the results obtained during acclimatization of the two cassava varieties were lower than the results obtained by Dawit Beyene *et al*. (2010) for the same varieties of cassava. This may be explained by the fact that success in acclimatization might be related to the use of auxin for root induction. According to these authors, with increasing concentration of indole-butyric acid (IBA), the number of roots increased. As plantlets with higher number of roots survive more during acclimatization, those shoots rooted in growth regulators-free medium produced less number of roots and survival rate was lowered indicating that number of roots induced *in vitro* have strong impact on acclimatization capacity of the plants. The result of the present study showed better percentage of survival (66.7%) for 'Kello' than that of Ogero *et al*. (2012) who obtained 53.3 and 55.2% survival of two different cassava varieties after acclimatization on soil consisting of rice husks and red soil. However, according to these authors, 80 and 81% survival were obtained for the two cassava varieties studied after the plantlets were acclimatized on vermiculite. Adaptation of plantlets to natural conditions is crucial for any protocol to be successful. Cassava is a delicate plant to harden, and a large amount of loss occurs while transferring from *in vitro* to *ex vitro*. This low percentage of survival could be probably due to the effect of the type of soil used during acclimatization. Therefore, there is a need for careful handling and identification of a proper soil type and composition for its acclimatization.

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