Ethiop. J. Biol. Sci., 9(2): 127-142, 2010
 ISSN: 1819-8678

MICROPROPAGATION OF SELECTED CASSAVA (*MANIHOT ESCULENTA* CRANTZ) VARIETIES THROUGH MERISTEM CULTURE

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ABSTRACT: Cassava, (*Manihot esculenta* Crantz) is a perennial woody herb which is cultivated in sub-Saharan Africa, Latin America and Asia for its edible starchy roots and its ability to withstand marginal environmental conditions. Due to high seed dormancy and sluggish germination rate, farmers normally practice propagation of cassava by stem cuttings which have led to accumulation of viral and bacterial diseases that reduce productivity and causes loss of superior genotypes. To develop a protocol for rapid *in vitro* micropropagation, two cassava varieties ('Qulle' and 'Kello') were selected. Solid MS medium supplemented with different hormonal combinations was used for shoot induction, shoot multiplication and rooting. Among different treatments used for shoot multiplication, 0.5 mg/l BAP in combination with 1 mg/l GA_3 and 0.01 mg/l NAA was found to be the best; with a mean number of 12.23 and 7.22 shoots per explant for cultivars 'Qulle' and 'Kello', respectively. Root induction potential of the two varieties increased by supplementing the medium with 1mg/l of IBA. Of those shoots which were acclimatized in the greenhouse, 89.1% of 'Qulle' and 75% of 'Kello' survived. The protocol could be used for the multiplication of cassava for large-scale production.

Key words/phrases: *Manihot esculenta*, Meristem, Micropropagation, Tissue culture.

INTRODUCTION

Cassava is a perennial woody herb that belongs to the family Euphorbiaceae with 2n=2x=36 (Gilbert, 1995; Alves, 2002). It is cultivated throughout the lowland tropics, typically between 30°N and 30°S of the equator, in areas where the annual mean temperature is greater than 18°C (Bolhuis, 1966). Cassava is considered as an ideal food crop for tropical Africa where drought, poverty, and malnutrition are prevalent. This is due to the fact that the crop presents easy propagation systems, high drought tolerance, satisfactory yields even in low-fertile soils, low exigency for sophisticated cultural requirements, potential resistance and tolerance to pests and diseases, high root starch contents and flexible harvesting dates, but it has

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less fat and protein content (0.2 % and 1 %), respectively, from a total of edible fleshy portion of the tuber (Negeve, 1999; FAO, 2001; Ricardo *et al*., 2007; Nassar *et al*., 2009).

In Ethiopia, there is high cultivation of cassava in the south and southwest parts of the country; like Kefa, Bench, Maji, Sheko and Gambella region mainly for the edible tubers consumed in the form of bread or injera by mixing its flour with different cereal crops (Amsalu Nebiyu *et al.*, 2000; Amsalu Nebiyu and Elfinesh Firidisa, 2006).

In the year 2000, 172 million tones of cassava were produced worldwide, and 226 million tons in 2006 (IITA, 2007; Bamidele *et al*., 2008; FAO, 2008).

The production of cassava is hampered by several constraints. The propagation method used is one of the major bottlenecks for cassava production; this is basically due to the reasons that cassava's seeds are normally dormant and germinate very slowly, hence farmers practice the conventional stem cutting propagation method. This propagation method, however, leads to the accumulation of viral and bacterial diseases that significantly reduce productivity of the crop, and even causes loss of superior genotypes (Nassar and Ortiz, 2007).

Through harnessing of plant tissue culture techniques, several crop production constraints that cannot be addressed by classical breeding approach have been overcome (Rao, 1996). Meristem culture involves culturing of a very small shoot apex (0.2-1.0 mm in length) consisting of the apical meristem with or without one or two leaf primordial (George, 2008). The major advantages of meristem culture are its potential to eliminate pathogenic organisms that may have been present in the donor plants, rapid propagation rate and maintenance of stability in genetic inheritance (Hu and Wang, 1984; Grout, 1990; Brian, 1999). In cassava somatic embryogenesis from leaf, meristem culture and nodal culture research activities have been conducted by many researchers (Konan, *et al*., 1997; Atehnkeng, *et al*., 2006).

The aim of this study was to develop an efficient *in vitro* protocol for micropropagation of selected cassava varieties ('Qulle' and 'Kello') through meristem culture and thereby to optimize culture initiation, shoot multiplication and rooting for further routine cassava tissue culture application and ensure acclimatization of *in vitro* derived plantlets in the green house. The two varieties were selected because they are the only

nationally released varieties with less cyanide content (4.62±0.01 mg cyanide per 100 g of cassava flour for 'Qulle' and 6.04±0.02 mg cyanide per 100 g of cassava flour for Kello) (Tilahun Abera, 2009).

MATERIALS AND METHODS

Donor plant preparation

Freshly harvested stem cuttings of two cassava varieties namely 'Qulle' and 'Kello' were collected from Hawassa Agricultural Research Center, Root Crops Research Division. The cuttings were planted in plastic pots (polyethylene bags) containing red soil, compost and sand at 1:1:2 ratio of respectively and allowed to sprout in glasshouse, at Holetta Agricultural Research Center (HARC). Normal agronomic practices such as watering, weed control and others were applied to the developed mother plants throughout the study period.

Stock solutions and medium Preparation

The Murashige and Skoog (1962), (MS) stock slution with its full macro nutrient, micro nutrient and vitamin compositions were used as the basic components of the medium. The growth regulators used for this study are 6-benzyl aminopurine (BAP), gibberrelic acid (GA_3), α-naphthalene acetic acid (NAA) and indol-3-butyric acid (IBA). Stock solutions of the hormones were prepared by weighing and dissolving the powder using few drops of the appropriate solvents (double distilled water, 1N NaOH, 1N HCl or absolute ethanol) and finally the volume adjusted using double distilled water at the ratio of 1mg/ml. The prepared stock solutions were stored at $+4^0$C.

The culture media were prepared by mixing MS stock solutions (100 ml/l macro nutrient, 10 ml/l micro nutrient and 10 ml/l vitamin) and 3% sucrose (w/v). Then the volume was adjusted after adding growth regulators and pH was adjusted to 5.8 using 1N NaOH or 1N HCl. Finally, 7.5 g of agar was added and the medium was sterilized by autoclaving.

Explants collection and surface sterilization

Shoot tips (10-15mm) of both varieties were collected from two-month-old glasshouse grown mother plants. The explants were washed twice with tap water and further rinsed once with double distilled water. The explants were sterilized in 70% alcohol for 1 min and rinsed three times with sterile double distilled water and further disinfected with 1% sodium hypochlorite solution containing one drop of tween-20 for 7 min followed by washing five times

with sterile distilled water.

Meristem isolation and culture initiation

Apical meristems consisting of the meristematic dome with one or two leaf primordia were isolated using dissecting microscope, sterile scalpel and hypodermic needle. Five meristems were cultured per Petri dish (90 mm) containing 20 ml basal medium supplemented with five different BAP concentrations (0.1, 0.5, 1, 2, and 5mg/l) in combinations with 0.01mg/l NAA and 1mg/l GA_3. Petri dishes were sealed with Para film. Each treatment was replicated six times and MS medium without growth regulators was used as a control. On every five days interval, data was collected for the number of callus induced, shoot induced and dead meristems.

Shoot multiplication

Meristem-derived *in vitro* shoots were used as explants and cultured on MS basal medium supplemented with different concentrations and combinations of growth regulators (Table 1) in Magenta® culture vessels. Growth regulators free MS basal medium was used as a control. Three explants were cultured per culture vessels and each treatment was replicated five times. After one month of culture, data were collected for the number of shoots multiplied per explant.

Table 1. Hormonal combination used for observing shoot multiplication.

Hormone	Concentration (mg/l)						
BAP	0	0.1	0.5	1	2	5	10
GA_3	0	0.1	0.5	1	2	5	10
BAP + GA_3	0 + 0	0.1 + 1	0.5 + 1	1 + 1	2 + 1	5 + 1	
BAP + GA_3 + NAA	0 + 0 + 0	0.5 + 1 + 0.01	1 + 1 + 0.01				

Rooting

Produced shoots were cultured on half and full strength MS supplemented with 0, 0.01, 0.1, 0.5 and 1 mg/l IBA concentrations for root induction. A single shoot with 1cm length and more was cultured in a test tube and each treatment was replicated 20 times. After two weeks, data were collected for the numbers of main roots per shoot.

Acclimatization

In vitro produced plantlets were transferred to a plastic pot containing red soil, compost and sand in the ratio of 1:1:2, respectively. Each pot was

covered with modified plastic bags for aeration by making a hole and plantlets grown in glasshouse. The plastic bag was gradually removed after a week. Finally, data were collected on the number of survived plantlets in the glasshouse after three weeks of transplanting.

Culture conditions

All cultures were kept in a growth room under 16h light (2700lux light intensity) and 8h dark cycle at 25^{o}C ± 2^{o}C. Sub-culturing was carried out every four weeks. Contamination and dead cultures were regularly monitored and removed from the cultures.

Experimental design and data analysis

The experiments were performed using completely randomized design (CRD). Statistical analysis of quantitative data was carried out by JMP/SAS computer software of version 8.0.1.

RESULTS

Shoot induction

Shoot induction from cultured meristems were started after 2-3 weeks of culturing (Fig.1). Likewise, calli were induced from cultures that failed to induce shoot. The control for both varieties remained at callus stage even after two months. An average of 84.0 % of 'Kello' and 82.76% of 'Qulle' cultured meristems were induced shoot on the media supplemented with 2mg/l BAP, 1mg/l GA_3 and 0.01mg/l NAA for both varieties (Fig.2).

Fig. 1. Shoot induction of cassava meristems in MS medium with 0.5 mg/l BAP, 1mg/l GA_3 and 0.01 mg/l NAA. (A): Meristem after one day of culture (B): Shoots after four weeks (C): Shoots after eight weeks.

In general, rate of shoot induction increased with increment of BAP concentration (Fig 2), nonetheless, more bushy shoots developed in medium with 2 and 5 mg/l BAP. The proliferations of morphologically best shoots were observed on MS medium supplemented with 0.5 and 1 mg/l BAP in combination with 1 mg/l GA_3 and 0.01 mg/l NAA. The percentage of callus formation varied with variation in BAP concentration. At 0.1 mg/l of BAP, all cultures of 'Kello' and 96.7% of 'Qulle' induced callus.

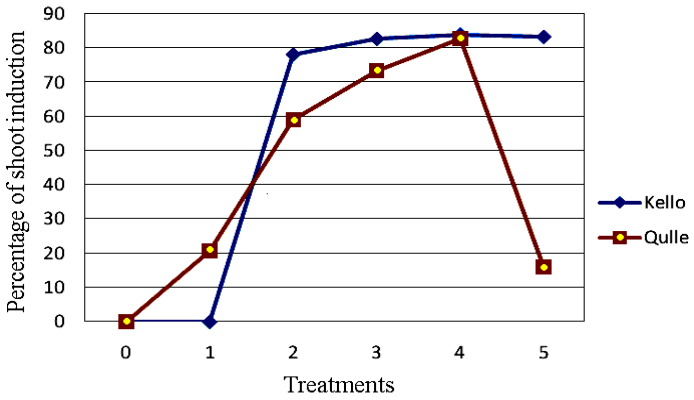


Fig. 2. Shoot induction percentage of Kello and Qulle cassava varieties from meristem cultures on various growth hormone concentrations on MS medium.(0): growth regulators free; (1): 0.1 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA; (2): 0.5 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA (3): 1 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA; (4): 2 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA; (5): 5 mg/l BAP, 1 mg/l GA_3, 0.01 mg/l NAA

Shoot multiplication

The regenerated shoots which were cultured on multiplication media responded differently to various concentrations and combinations of growth regulators. The maximum mean number shoots were obtained per explant (12.2 and 7.2) for cultivars 'Qulle' and 'Kello', respectively, on medium supplemented with 0.5 mg/l BAP in combination with 1mg/l GA3 and 0.01 mg/l NAA, (Tables 2, 3, 4 and 5; Fig.3).

Table 2. Mean number of shoots per explant in different BAP concentration MS medium.

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

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 produced per explant in different GA_3 concentration MS medium.

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

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

Table 4. Mean number of shoots produced per explant in different BAP and GA_3 combinations MS medium.

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

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

Table 5. Mean number of shoots produced per explant in different BAP, GA_3 and NAA combinations MS medium.

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

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

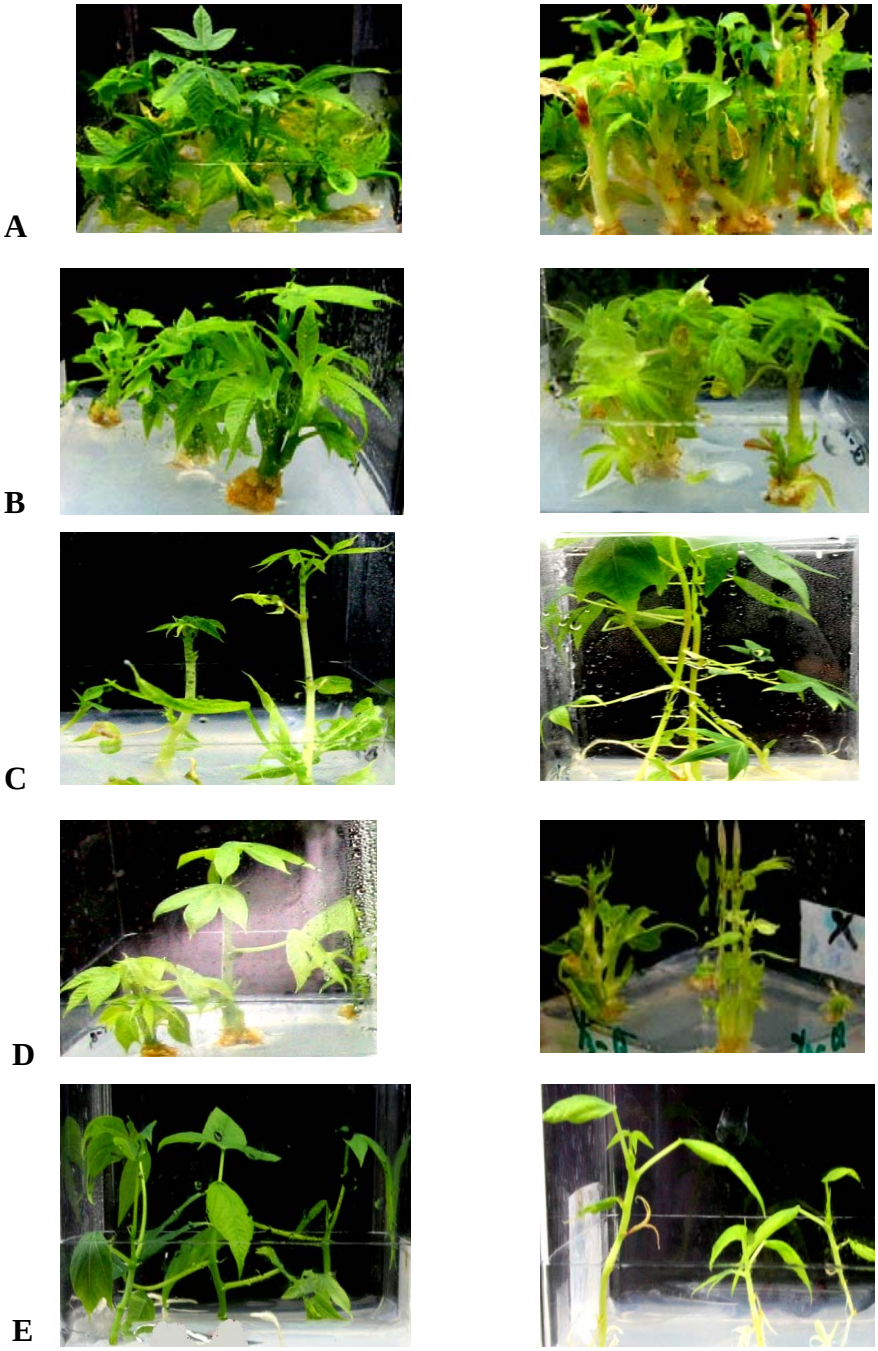


Fig. 3. Shoot multiplication on MS medium supplemented with various concentrations of plant growth hormones: Figures at the left (Kello) and right (Qulle). (A): 0.5 mg/l BAP, 1 mg/l GA_3 and 0.01 mg/l NAA (B): 0.5 mg/l, BAP (C): 1 mg/l, GA_3 (D): 2 mg/l BAP and 1 mg/l GA_3 and (E): Control (without growth regulators).

The second maximum mean number of shoot per explants for 'Qulle' and 'Kello' was 6.42 and 4.75, respectively, in MS medium with 0.5 mg/l BAP (Table 2). However, when the concentration of BAP increased, shoots become very dwarf and bushy. The lowest mean number of shoots per explant for 'Qulle' (1.00) and for 'Kello' (1.12) was obtained in the control and in a medium supplemented with 0.1 mg/l GA_3, respectively (Table 3).

Rooting

As shown in Figs. 4 and 5, mean number of main roots proliferated per shoots in full and half strength MS medium with the same IBA concentration didn't show significant statistical difference at probability level of 0.05. However, there was a variation in the number of main roots per explant with variation in IBA concentration (Figs. 6 and 7). For 'Qulle', the highest (9.35) mean number of main roots per shoot was recorded in half MS medium with 1 mg/l IBA and for 'Kello' a maximum (7.00) of mean number of main roots per shoot was observed in full MS medium with IBA concentration of 1 mg/l.

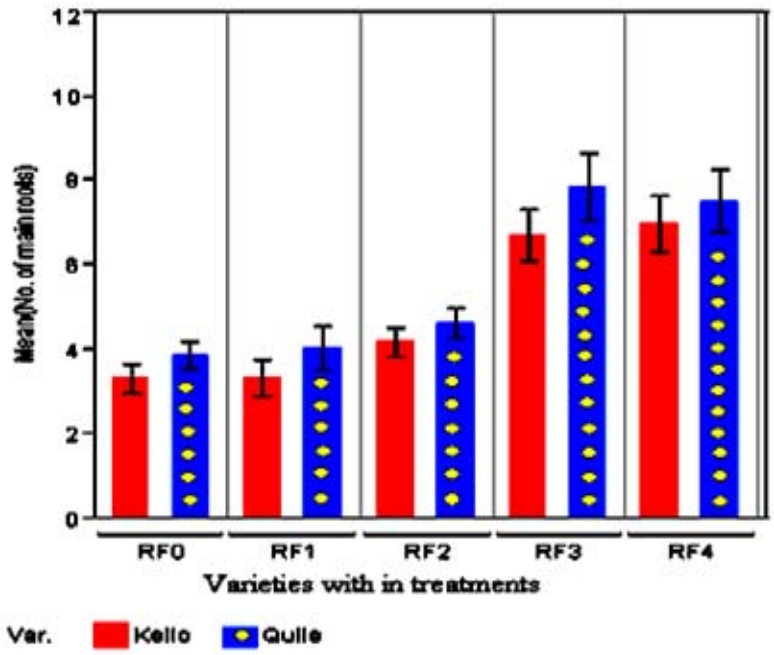


Fig. 4. Mean number of main roots produced per explant in full strength MS with various IBA concentration. (RF0): 0mg/l IBA (RF1): 0.01mg/l IBA (RF2): 0.1mg/l IBA (RF3): 0.5mg/l IBA (RF4): 1mg/l IBA. Each error bar was constructed using 1 standard error from the mean.

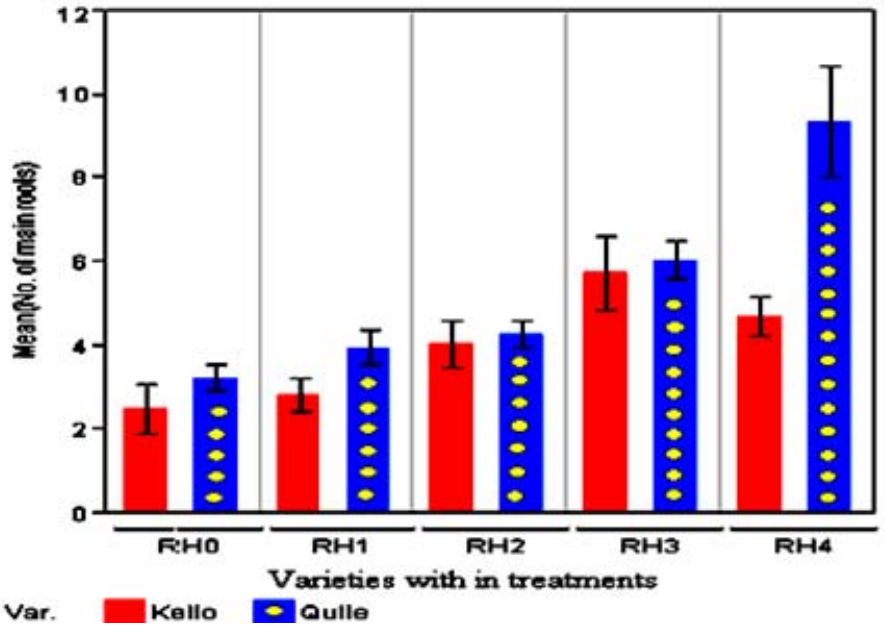


Fig. 5. Mean number of main roots produced per explant in half strength MS with various IBA concentration. (RH0): 0mg/l IBA (RH1): 0.01mg/l IBA (RH2): 0.1mg/l IBA (RH3): 0.5mg/l IBA (RH4): 1mg/l IBA. Each error bar was constructed using 1 standard error from the mean.

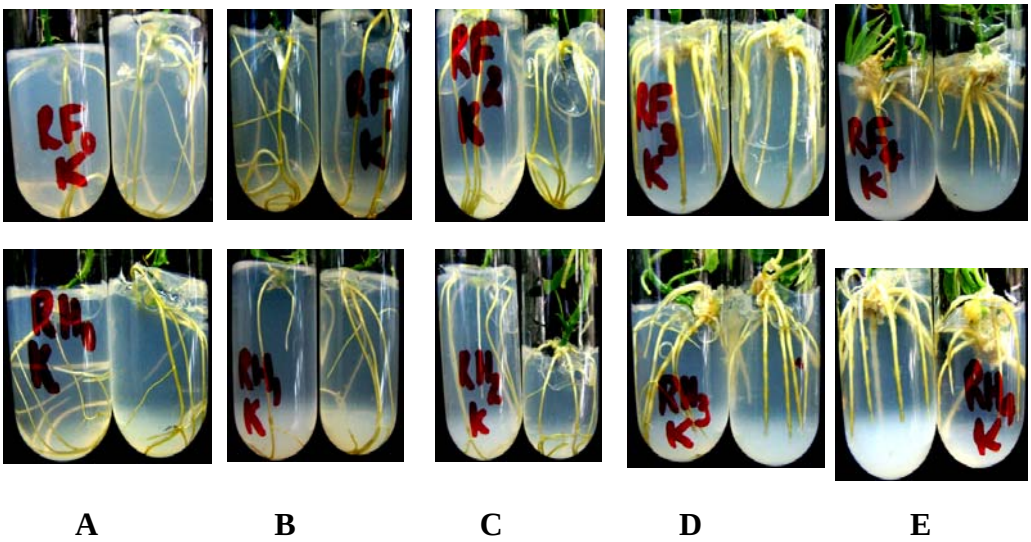
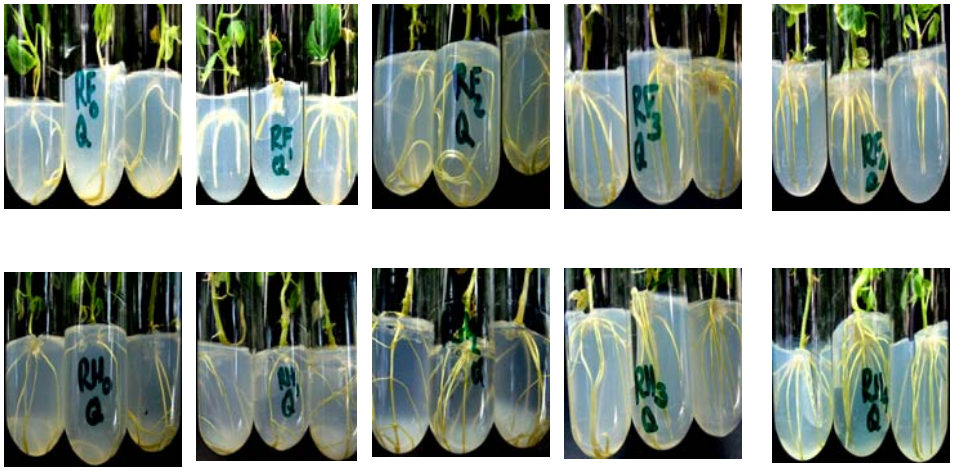


Fig. 6. Rooting of Kello cultivar in full strength MS (RF0-RF4) upper and half strength MS (RH0-RH4) lower with different IBA concentration, (0, 0.01, 0.1, 0.5 and 1 mg/l of IBA in respect to the letters A,B,C,D,E).



A B C D E

Fig. 7. Rooting of Qulle cultivar in full strength MS (RF0-RF4) upper and half strength MS (RH0-RH4) lower with different IBA concentration, (0, 0.01, 0.1, 0.5 and 1 mg/l of IBA in respect to the letters A,B,C,D,E).

Acclimatization

The survival rate of plantlets acclimatized in the green house with sterile soil mix of red soil, humus and sand in a ratio of 1:1:2 was 89.1% and 75% for 'Qulle' and 'Kello' varieties, respectively.

A B C

Fig. 8. Acclimatization of *in vitro* plantlets of cassava and three weeks old plantlets in the greenhouse: (A) Acclimatization stage (B) 'Qulle' plantlets after three weeks (C) 'Kello' plantlets after three weeks.

DISCUSSION

Shoot induction

In the work of Acedo (2006), on *in vitro* propagation of cassava from meristem culture; the highest percentage of shoot induction (80%) from meristem was obtained on MS medium supplemented with 0.25 mg/l GA_3, 0.1 mg/l BAP and 0.2 mg/l NAA. In the present study, MS medium with 2 mg/l BAP, 1 mg/l GA_3 and 0.01 mg/l NAA gave 84.0% shoot induction for 'Kello' and 82.7% for 'Qulle'. In addition, from the present study it was observed that, as BAP concentration increased to 2 mg/l, all survived meristems gave shoot but they become very bushy and short. In contrast, on 0.1mg/l BAP with 1 mg/l GA_3 and 0.01mg/l NAA, except one meristem all the rest gave callus and died after 45 days by turning into dark brown color. On MS medium supplemented with 5 mg/l BAP, 1 mg/l GA_3 and 0.01 mg/l NAA, meristems enlarged and gave undifferentiated shoot like green structure that didn't grow any further. But on 0.5 and 1 mg/l BAP in combination with 1mg/l GA_3 and 0.01mg/l NAA, both varieties gave morphologically differentiated shoots.

Shoot multiplication

In this work, for both varieties, the highest (12.23 and 7.22) mean number of shoots per explant for cultivars 'Qulle' and 'Kello', respectively, was obtained in MS medium with 0.5 mg/l BAP in combination with 1 mg/l GA_3 and 0.01 mg/l NAA, where 27 shoots for 'Qulle' and 21 shoots for 'Kello' were counted; this might be due to the combination effect of the three growth regulators, Staden *et al*. (2008), reported that plant growth regulators might promote or inhibit *in vitro* plant growth when they are in combination.

As Konan *et al*. (2006), reported in the nodal culture of cassava with axillary meristems; MS medium supplemented with 0.1 mg/1 NAA, 1 mg/1 BAP and 0.1 mg/1 GA_3 was best to produce multiple shoots. Besides, Smith *et al*. (1986) also reported multiple-shoot formation from MS medium supplemented with 1.0 mg/l BAP and 0.25mg/l NAA for *in vitro* propagation of cassava using nodal culture. In medium with GA_3 only and in the control, the lowest mean number of shoots per explant was obtained. In medium with GA_3 only, shoots become very thin and long rather than multiplying as the concentration of the hormone increased. Similar result was also obtained in the work of Acedo (2006). This might be due to the physiological effect of the hormone, that GA_3 promotes stem elongation and inhibits formation of adventitious root and shoot (Moshkvo *et al*., 2008).

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

As BAP concentration increased to 5mg/l and 10mg/l, in a medium with BAP only, shoots became very dwarf. This might be from the supra-optimal amount of the hormone. This observation is consistent with the physiological effect of BAP on plants. Also, Berrie (1984), reported that synthetic cytokinins are inhibitory to shoot growth at high concentration.

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

Rooting

In this study, the effect of full and half strength MS medium for rooting and the relationship between different IBA concentrations and root induction potential of the two varieties were observed. Statistically no significant difference was observed in full and half strength MS medium of the same IBA concentration at a probability level of 0.05. However, rooting frequency of plantlets varied with variation in the concentration of IBA. In both half and full strength MS in combination with 0, 0.01 and 0.1mg/l of IBA; less rooting frequency with fragile roots and few number of secondary roots were observed for both cultivars. Nevertheless, at higher concentration of IBA (0.5 and 1mg/l of IBA), roots become short and thick without secondary root formations. The work of Smith *et al*. (1986) also showed that the use of 2.5 mg/l of IBA was good to improve root initiation of cassava plantlets which agrees with the present finding that rooting increased with increment in IBA concentration. Mycock *et al*. (2002), tested half, full and double strength MS for somatic embryogenesis of cassava and recommended use of both half and full MS for somatic emryogenesis work. In addition Fotopoulos and Sotiropoulos (2005) reported that reducing the mineral concentration of MS medium by half potentially increased the rooting percentage of *Prunus persica* and it is better in regard to cost for the chemicals.

Acclimatization

The survival rate of the two cassava varieties in the green house was found

to be 89.1% and 75% for 'Qulle' and 'Kello', respectively, which is inconsistent with the report of Zimmerman *et al*. (2007) where 85-95% survival rate was achieved in the acclimitization of *in vitro* derived cassavas plantlets in vermiculite.

ACKNOWLEDGEMENT

The authors acknowledge the Department of Biology, Addis Ababa University, School of Graduate Studies; Holetta Agricultural Research Center, Ethiopian Institute of Agricultural Research and Canadian Educational and Training Award Africa (CETAA) for financing the research and also Saba Abdulsemed* and Kirubel Terefe for the logistic support of the research.

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