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Protocol Optimization for Rapid Multiplication of Cassava (*Manihot esculenta* Crantz) using the Nodal Segment

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ABSTRACT

Background: Cassava is a vital food security crop for millions of people, especially in sub-Saharan Africa. Since the crop produces a reasonable yield on marginal soils, it could help alleviate world hunger. Consequently, the increase in cassava production and its quality characteristics are significant. However, the low multiplication rate of this main crop has delayed the spread of improved varieties among farmers. As a result, tissue culture techniques can be a viable solution to overcome these challenges.

Methods: The study used a nodal segment as an explant to evaluate different concentrations of BAP and NAA for an efficient, cost-effective *in vitro* mass multiplication of the AWC-1 cassava variety. $CuSO_4$, commercial bleach and ethanol had used to sterilize nodal explants taken from greenhouse-grown plants at various time intervals.

Result: The best medium for micro shoots induction had found to be medium without growth regulators. Among different treatments used for shoot multiplication purposes, the maximum shoot number has been recorded on M.S. medium supplemented with 0.75 mg/l BAP and 0.2 mg/l NAA. Medium with 0.5 mg/l NAA concentration was the best for rooting induction. A survival rate of 86% has obtained in the greenhouse and the plantlets appeared to be morphologically normal.

Key words: 6-benzylaminopurine (BAP), Micropropagation, α-naphthalene acid (NAA).

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial dicotyledonous plant, which belongs to the family Euphorbiaceae. It is diploid with a chromosome number of 2n=36 and the species generally display normal diploid meiosis (Nassar, 1978). It is grown in many countries worldwide and contributes to the food security of millions of people in Sub-Saharan Africa (Nkouaya Mbanjo *et al*., 2020). Cassava is the fourth most important food crop in the developing countries after rice, wheat and maize (Narmilan and Puvanitha, 2020). Its roots are also one of the most important sources of commercial starch. The crop is the second most important source of starch worldwide after maize (Stapleton, 2012).

It is regareded as a food security crop due to its minimal input requirement, resistance to disease and pests and capacity to thrive in bad weather and marginal soil conditions (Nassar *et al*., 2009). In addition, analyses of agricultural sensitivity to the effects of climate change suggest that Cassava may be more suited to surviving climatic fluctuations than other major tropical staple crops, making it a crucial food security crop for the future (Jarvis *et al*., 2012). It is also the only prospective crop capable of producing and storing more carbohydrates than any significant grain or a root crop.

Cassava is grown in Ethiopia by small, resource-limited farmers on smallholding pieces of land. It provides both food security and a cash crop for small-scale producers. The majority of cassava output in the country originates from the country's southern area. It is grown as a reliable food crop in the south region of the country, notably in the Amaro-Kello region (Enidiok *et al*., 2008).

It has proven challenging to enhance Cassava genetically through sexual crosses. Cassava multiplication by seeds is challenging because of its limited blooming capability, self-incompatibility, male sterility, short seed viability and low seed set. As a result, the botanical seed rarely uses in commercial cassava cultivation. Cassava is therefore propagated vegetatively by stem cuttings (Ceballos *et al*., 2004). However, this method also comes with disadvantages such as low rate of propagation, ten cuttings per plant per year (1:10), which is difficult, time-consuming, slow and delayed diffusion of new, improved cultivars, bulky to transport and planting materials are insufficient in number for large-scale plantations (Demeke *et al*., 2014).

Accumulation of diseases over a vegetative cycle, high distribution cost and poor storage quality of the planting material are other limitations (Escobar *et al*., 2006). In general, the scarcity of high-quality, true-to-type planting material for newly released varieties is a significant impediment to their widespread commercialization and improved cassava productivity (Escobar *et al*., 2006).

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As a result, the development of tissue culture technology for rapid multiplication of planting material has been a significant step toward adequate, true-to-type and high-quality seed production in Cassava (Rani and Raina, 2000). Its quickness, capacity to generate a large amount of planting material in a short space by starting with small explants and simplicity of transport make it more appealing.

Plant tissue cultures are initiated from tiny pieces, called explants, taken from any plant part. Practically all parts of a plant have been used successfully as sources of explant. Micropropagation through nodal explant is the commercially feasible protocol for producing genetically uniform plantlets identical to the mother plant in a relatively short period and with a high multiplication rate (Alla, 2013). Different researchers (Bhagwat *et al.,* 1996; Konan *et al.,* 1997; Demeke *et al*., 2014; Shiji *et al*., 2015) published scientific articles on successful micropropagation protocol through nodal culture for different cassava clones. Their results showed the existence of interaction of genotype with hormonal type and concentration. Thus, an efficient protocol is required for every clone under different conditions.

So far, there is no report available on *in vitro* rapid multiplication of AWC-1 cassava variety in Ethiopia. Therefore, the present study was initiated to optimize the protocol for in vitro propagation of AWC-1 cassava variety using nodal segments.

MATERIALS AND METHODS

The study had conducted at the Plant Tissue Culture Laboratory, Department of Horticulture and Plant Science, College of Agriculture and Veterinary Medicine, Jimma University (JUCAVM) from November 2016 to July 2017. Stem cuttings of AWC-1(MM96/5280) cassava variety were used as plant material evaluation on its response to in vitro regeneration. Stems of this clone were cut and planted in the greenhouse where the study has carried out. Three months old actively growing shoot tips were collected and prepared as initial explants. Explants were cleaned with tap water and double distilled water immediately after being cut from the mother plant. Sterilization techniques were followed by 30g/l of $CuSO_4$ for 30 minutes and flashed three times with distilled water for 5 min. The explants were then placed in a sterilized laminar airflow cabinet, rinsed three times with distilled water for five minutes, sanitized with 70% ethanol for 30 seconds and washed three times with distilled water. They were then rinsed for 15 minutes in a 25 per cent commercial bleach solution before being washed three times for 5 minutes with gentle shaking to remove the chemical residue. The sterilized nodal explants were put in a sterile petri dish or plate and cut to 1.0 cm in length on both ends.

Shoot initiation

A full-strength M.S. medium had used throughout experiments. Five variable concentrations of BAP (0.5, 1, 1.5, 2.0 and 2.5mg/l) were evaluated for shoot initiation. Each treatment is composed of three shoots/jars with five replications. The cultures had placed in white florescent lightroom adjusted at 16/8 hrs light/dark regimes at room temperature. The stock solution composition, agar concentration and other physical conditions were the same for all the treatments. After a month of growth, every change in growth had carefully observed and recorded. A plant growth regulator-free medium had used as a control.

Shoot multiplication

For the shoot multiplication experiment, the initiated shoots were taken out from the culture medium after four weeks of the culture and then cultured on a hormone-free M.S. basal medium for two weeks to avoid carry-over effects of growth hormones. Then shoot explants of about 3 cm length were cultured on fresh MS medium containing BAP at 0.0, 0.25, 0.5, 0.75 and 1 mg/l and in combination with 0.0, 0.1, 0.2 and 0.3 mg/l NAA. The experiment had arranged in a completely randomized design in a factorial arrangement (5×4) with three shoots per jar and three replications for each treatment. The cultures had placed in white florescent lightroom adjusted at 16/8 hrs light/dark regimes at room temperature. Data on the number of shoots, shoot length and numbers of leaves had record after one month.

Rooting

The growing shoots were aseptically removed from the culture jars and inoculated on a full-length M.S. medium supplemented with various amounts of NAA (0.25, 0.5, 0.75 and 1 mg/l). M.S. medium without PGRs had used as a control. Three shoots had placed in each culture jar and each treatment had replicated three times.

Data analysis

The data collected from the three experiments were subject to analysis of variance (ANOVA) using the SAS software package (version 9.3). The least significant difference (LSD) was used to compare significant differences between means at $p< 0.01$.

RESULTS AND DISCUSSION

Effect of BAP on establishment of culture shoots

Explants were able to initiate in both controls as well as BAP supplemented M.S. medium. However, shoot initiation %, shoot length and leaf number had found to differ across treatments. The highest shoot initiation percentage had obtained on the control medium (Table 1). Shoot induction showed a decreasing trend with increased BAP concentrations (0–2.5 mg/l). With increasing BAP concentration, the shoot organogenesis frequency had gradually increased until it reached the maximum in a medium containing one mg/l BAP. Above 1mg/l concentration of BAP shoot organogenesis frequency reduced and shoots became thick and very short. The decrease in the percentage of initiation at the higher level is due to callus formation (Fig 1D). The current study's findings are consistent with Beyene (2009), who showed a similar

relationship between BAP concentrations and shoot growth. A similar result had reported by Onuoch and Onwubiku (2007), who observed in vitro recalcitrance at BAP (1.25mg/l). In addition, a similar result had also presented by Fan *et al*. (2011), who obtained that shoot induction frequency decreased as BAP concentration increased. Saelim *et al*. (2009) recommend BAP concentration up to 1 mg/l for nodal culture. Above 1mg/l concentration of BAP, shoot induction frequency reduced and shoots became thick and very short. Also, Acedo (2006) observed that higher levels of Cytokinin induced meristem cultures to form callus. Callus induction, however, was known to inhibit shoot development.

Effects of BAP and NAA on shoot multiplication

After two weeks of culture in medium supplemented with all concentrations of BAP and NAA, the shoot had proliferated. Maximum shoot proliferation was recorded on M.S. media supplemented with 0.75 mg/l BAP with 0.2 mg/l NAA (Table 2, Fig 2C). This result agrees with Alla (2013), who found the best shoot multiplication on M.S. medium supplemented with 1.0 mg /l BAP 0.05 mg/l NAA. The combined impact of NAA and BAP shows to vary with NAA and BAP concentration in two shoot growth parameters (mean the number of the shoot and shoot length). High concentration of (0.75 mg/l) BAP with low concentration of 0.1 and 0.2 mg/l NAA, relatively better result was recorded. This multiplication difference might be due to the high concentration of BAP, reduced apical dominance and promoted axillary branching or axillary bud proliferation. Although cytokinins are known for stimulating cell division, they do not induce DNA synthesis (Pan, 2001). However, auxin at low concentration is important to promote cell division and elongation and can induce DNA synthesis (Vieitez and Vieitez, 1980). As a result, combining auxins and cytokinin promotes cell division, which impacts adventitious shoot development. Similar results had been reported by Smith *et al*. (1986), where M.S. medium supplemented with 0.2 mg/l BAP and 0.05 mg/l NAA provided the maximum shoot multiplication.

Table 1: Effect of BAP on shoot initiation and shoot establishment of AWC-1 cassava clone.

BAP in mg/l	% initiation Means±S.D.	Number of Leaves Means±S.D.	Shoot length Means±S.D.
0	100.00^{a}±0.00	5.31^{a}±0.32	5.83^{a}±0.29
0.5	90.00^{b}±8.81	3.80^{d}±0.20	3.63^{bc}±0.30
1	83.33^{b}±0.00	5.09^{a}±0.48	3.93^{b}±0.25
1.5	73.33^{c}±5.78	3.13^{e}±0.32	3.23^{c}±0.35
2	70.00^{c}±3.33	4.62^{bc}±0.12	2.33^{d}±0.06
2.5	68.33^{c}±2.89	4.44^{c}±0.14	2.02^{d}±0.07
CV	5.77	6.67	7.12
LSD (P= 0.05)	8.29	0.52	0.44

Note: Means not connected by the same superscript in the same column are significantly different at a 5% probability level. The values represent mean ± standard error (S.E).

Effect of α-Naphthalene acetic acid (NAA) on rooting

The well-grown shoots of about 1 to 1.5 cm in height were taken and cultured on a full-strength MS medium supplemented with various doses of NAA. At all concentrations examined, all treatments exhibited statistically significant results for root formation. The number of roots/shoots recorded and the length of roots/shoots observed substantially differed across all treatments evaluated (Table 3; Fig 3). The M.S. medium supplemented with 0.5 mg/l NAA produced a significantly higher rooting percentage and more roots/shoot and root length/ shoot (Table 3; Fig 3). The current study's findings are consistent with those of Demeke *et al*. (2014), who obtained the maximum number of roots (6.14) on 0.5 mg/l NAA. The results also largely support the findings of Shiji *et al*. (2015), who observed the maximum number of roots (4.80±0.44) at

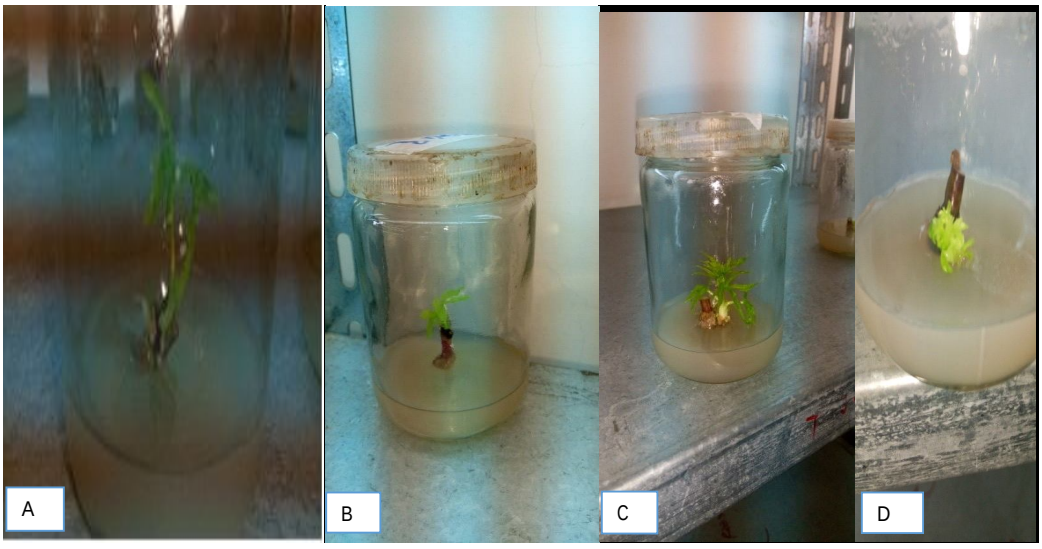

Fig 1: Effect of BAP on initiation and shoot formation from the nodal segment of AWC-1 cassava clone after four weeks.
Note: A= PGRs free (control); B= MS + 1.0 mg/l BAP; C= MS + 2.0 mg/l BAP; D= MS + 2.5 mg/l BAP.

the same concentration of NAA. Moreover, these findings support Sesay *et al*. (2018), who found that M.S. with NAA (1.0 mg/l) was the optimum rooting medium. The use of NAA (0.25 to 0.75 mg/L) produced higher roots than NAA (1 mg/l). These findings support Fan *et al*. (2011), who found that NAA (0 to 2.0 mg/L) was beneficial in stimulating root growth in Cassava. However, increasing the concentration of NAA to 1.0 mg/l resulted in a decrease in the mean root number per shoot, consistent with Sukmadjaja and Widhiastuti (2011), who found the lowest number of roots (3.80±1.92)

Table 2: Effect of BAP and NAA on shoot multiplication of AWC-1 cassava clone.

PGRs				
BAP	NAA	No. of shoots/explant (Means±SD)	Shoot length/shoot (Means±SD)	No. of leaves/shoot (Means±SD)
0	0	1.63^{l}±0.06	3.86^{a}±0.05	4.67^{bc}±0.58
0	0.1	2.10^{k}±0.10	2.42^{h}±0.03	2.80^{h}±0.26
0	0.2	2.40^{jk}±0.10	2.13^{jk}±0.15	2.33^{i}±0.58
0	0.3	2.15^{k}±0.26	2.17^{jk}±0.15	2.20^{j}±0.35
0.25	0	2.80^{hi}±0.20	2.54^{g}±0.04	3.93^{d}±0.12
0.25	0.1	3.17^{fg}±0.15	2.30^{i}±0.01	5.22^{a}±0.02
0.25	0.2	3.45^{ef}±0.05	2.45^{gh}±0.04	5.00^{ab}±0.20
0.25	0.3	2.20^{k}±0.20	2.18^{j}±.08	4.43^{c}±0.03
0.5	0	3.10^{gh}±0.10	3.04^{e}±0.06	5.00^{ab}±0.20
0.5	0.1	4.20^{d}±0.20	3.48^{b}±0.03	3.92^{d}±0.01
0.5	0.2	4.47^{cd}±0.15	3.28^{c}±0.03	3.78^{de}±0.10
0.5	0.3	3.50^{ef}±0.50	3.03^{e}±0.12	3.47^{ef}±0.02
0.75	0	5.00^{b}±0.00	3.20^{cd}±0.00	3.34^{fg}±0.04
0.75	0.1	6.60^{a}±0.10	3.10^{de}±0.00	4.62^{c}±0.02
0.75	0.2	6.82^{a}±0.07	2.83^{f}±0.06	3.55^{ef}±0.05
0.75	0.3	4.58^{c}±0.08	2.75^{f}±0.05	3.28^{fg}±0.08
1	0	3.52^{e}±0.03	3.09^{de}±0.09	3.36^{fg}±0.01
1	0.1	4.18^{d}±0.16	2.75^{f}±0.04	3.90^{d}±0.04
1	0.2	4.51^{cd}±0.10	2.53^{g}±0.01	3.32^{fg}±0.03
1	0.3	2.67^{ij}±0.60	2.07^{k}±0.04	3.03^{gh}±0.15
CV		5.74	2.48	5.88
LSD (**P= 0.05**)		0.34	0.11	0.36

Note: Values are given as mean ± SD. Means with different letters within the same column (s) are significantly at p≤0.05. In contrast, Means with the same letter in the same column are not significantly different at p≤0.05.

Fig 2: Effect of BAP and NAA on shoot multiplication of AWC-1 cassava clone.
Note: A=PGRs free (control); B = 0.75 mg/l BAP + 0.1 mg/l NAA; C = 0.75 mg/l BAP + 0.2 NAA.

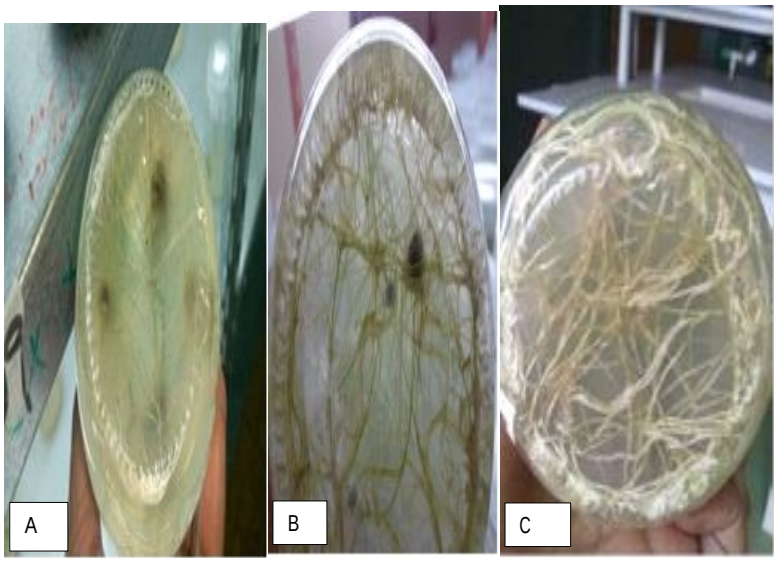

Fig 3: Effect of NAA on *in vitro* rooting of AWC-1 cassava clone.
Note: A = PGRs free medium (control); B = MS + 0.5 mg/l NAA; C = MS + 1.0 mg/l NAA.

Table 3: Effect of NAA on *in vitro* rooting of AWC-1 cassava clone.

NAA	% of rooted shoots (Mean±SD)	No. of roots (Mean±SD)	Root length (Mean±SD)
0	83.33^{b}±0.00	1.93^{d}±0.15	3.63^{e}±0.15
0.25	90.00^{b}±8.82	3.67^{bc}±0.15	4.30^{c}±0.20
0.5	100^{a}±0.00	5.57^{a}±0.15	7.20^{a}±0.10
0.75	73.34^{c}±5.77	3.90^{b}±0.10	5.07^{b}±0.12
1	70.00^{c}±0.00	3.60^{c}±0.10	3.90^{d}±0.10
CV	5.65	3.59	2.88
LSD (P= 0.05)	8.57	0.24	0.25

Note: Means not connected by the same superscript in the same column are significantly different at a 5% probability level. The values represent mean ± standard error (S.E).

on one mg/l of NAA. The current experiment's findings corroborated Weiler's (1984) findings that a high dose of auxins hindered root growth because auxins stimulate ethylene production on plant cells when used in larger quantities.

CONCLUSION

A viable micropropagation approach for the Ethiopian cassava variety AWC-1 had developed. The best shoot initiation was obtained in a PGR-free medium. However, 0.75 mg/l BAP and 0.02 mg/l NAA produced more shoots in the AWC-1 cassava cultivar. The MS medium with 0.5 mg/l NAA, on the other hand, was shown to be effective for root regeneration in the clone examined. A total of 86% of plantlets had acclimatized under field conditions. As a result, this procedure will serve as the foundation for mass production of the examined variety using in vitro methods. Finally, this study shows that plant tissue culture using a nodal segment as an explant is successful for *in vitro* cassava micropropagation.

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