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Micro-Propagation of Cassava (*Manihot Esculenta* Crantz) Using Bulla Flour (*Ensete Ventricosum* (Welw.), Cheesman) as an Alternative Source of Agar in Plant Tissue Culture Media

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Abstract: *Cassava is perennial, drought resistant crop with tuberous edible root. Low cost propagation of such crop is important. Agar is the most frequently used solidifying agent and expensive component in plant tissue culture media. Cost efficient tissue culture protocol design is prerequisite in the low cost tissue culture adoption technology in developing countries. This study aim to evaluate efficiency and cost effectiveness of enset starch (bulla) as an alternative source of solidifying agent in the micro-propagation of cassava meristem. Micro-propagation of cassava using enset starch as an alternative gelling agent revealed that the concentration 80gm/l bulla alone reduces 86% of cost, while composite of bulla 60gm/l and 70gm/l with agar 2gm/l and 1gm/l respectively saved 65-75% cost of gelling agent in plant tissue culture media. Besides substituting conventional agar, bulla enhances root length compare to conventional agar which might be due to carbon ingredient found within it. However bulla shown poor clarity that causes difficulty of detecting contamination. Detailed study would be needed in future to assess impact and characterization of bulla ingredient on in vitro plantlets of different plant varieties.*

Keywords: *Cassava, Enset starch, Micro-propagation, Cost effective, Gelling agent*

1. INTRODUCTION

Ethiopia is one of the most populated countries with its population currently estimated at 90 million in Africa. The country has agrarian economy with agriculture contributing to about 50% of the growth and development program (GDP), providing employment for about 85% of the population and accounting for about 90% of total foreign exchange earnings. People of Ethiopia mostly rely on locally available starchy foods for their food security. Cassava (*Manihot esculenta* Crantz) is a perennial with starchy tuberous edible root and is one of the most important and highly exploited starchy foods in the world. It is cultivated throughout tropical world. It is the fourth most important energy source crop for farmers in tropics after rice, wheat and sugar, consumed by up to a billion people globally (FAOSTAT, 2010). It is a major source of calories to more than 250 million people in the sub-Saharan Africa and 600 million people globally (Obiero *et al*., 2007). In Ethiopia cassava used as both food security crop and source of income. Currently the use of cassava and its by-products is not mainly restricted to human and animal consumption, but also to serve various industrial needs, including bio fuel production, waxy starch, bio plastics, glue, textile and paper (Saelim *et al*., 2009). The average total coverage and production of cassava per annum in southern Ethiopia is 4942 hectares with the yield of 53036.2 tones which implies the average productivity of cassava in Ethiopia is not more than 10 tons per hectare (SNNPR, BOA, 2000). It can be transformed into different forms and stored for several years. It is the third most sources of calories to tropical area. Cassava multiplied mainly by stem cutting (vegetatively) that show slow process, affected by season compared to grain crops (Santan *et al*,.2009). This crop affected by diseases like cassava mosaic disease (Pheneas and James, 2007). Thus infections transmit from one generation to other through cutting, which contributes to the spread of diseases that leading to poor yields in successive seasons (Roca and Mroginski, 1991). This is also a major limitation in germplasm maintenance and exchange of materials across borders. The multiplication rate of cuttings is also very low compared to grain crops, which are propagated by true seeds. Other challenges with the cuttings include high perishing ability since they dry up within a few days, high handling and transport costs and inconvenient weight and bulk of the material (Escobar *et al*., 2006).

In vitro propagation, a common method for vegetative reproduction, is a form of biotechnology which uses plant tissue culture and has a number of advantages over traditional methods of plant propagation. One advantage is that exceptionally large numbers of disease free seedlings can be

produced in a very limited space (Hopkins, 2007). Major limitation in tissue culture is its' expensive technologies including cost of media which is not affordable for a developing country. This drawback of high cost of tissue culture media can be reduced by replacing expensive chemicals with locally available cheaper organic sources, one such attempt is a search for alternative source of solidifying agent to replace expensive agar which is the prime need for tissue culture media preparation.

Enset (*Ensete ventricosum* Cheesman) is perennial monocarpic crop belongs to the family Musaceae and one of the widely cultivated edible crops in Ethiopian and is indigenous crop that produce different starchy food products rich in carbohydrate such as kocho, bulla and amicho (Tsegaye and Struik, 2002). Bulla is a water-insoluble starchy from enset product, which is processed by squeezing and decanting the liquid followed by drying. Quality and starchy nature of the bulla is exploited to substitute agar, also it seems to be a critical alternative gelling agent. This flour substituted conventional agar and save cost in the *in vitro* propagation of pineapple (Ayenew *et al*. 2012) and vanilla.

Tissue Culture (TC) is *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions. This technology can be used to produce high quality seedlings instead of the traditionally used cuttings, has a high ability of producing thousands of propagules and is important to overcome problems of using stem cuttings rather than conventional techniques. It is effective in elimination of viruses and other systemic diseases from cassava vegetative materials allowing exchange and conservation of rejuvenated propagation materials with higher yields (Jorge *et al*., 2000). I s the best way to increase the production for farmers, but the cost of *in vitro* plant production is an obstacle for its success. Thus efforts which develop low cost technologies are necessary. Agar constitutes the most expensive components of the culture media. . Because of the high price of agar, attempts have been made to identify suitable alternatives like cassava starch (Moses and Grace2004), Gum Katira (Jain and Babbar, 2002), Guar gum (Jain *et al*,.2011), Ensete starch (Ayenew *et al*.,2012) used as alternative source of agar on different plants.

Micro-propagation is the practice of rapidly multiplying explant to produce a large number of progeny plants, using modern plant tissue culture methods. The need for low-cost plant tissue culture systems, applicable for micro-propagation and *in vitro* conservation of plant genetic resources has been emphasized to allow the large-scale application of such technology in developing countries. *In vitro* propagation of cassava in low cost tried by using nutrient like Hydro Agri´s fertilizer substitute for Murashige and Skoog macro and micronutrients(Santana *et al*,.2009). However cassava not propagated using ensete flour substituting agar. Therefore micro-propagation of cassava using ensete flour as alternative source of agar expected to substitute conventional agar. Accordingly, this study was initiated with objective of evaluate the effectiveness of Ensete flour as an alternative source or in substituting conventional solidifying agent in micro-propagation of cassava and to evaluate cost efficiency of micro-propagation of cassava by enset flour (bulla).

2. MATERIALS AND METHODS

2.1. Experiment Area

This laboratory work was conducted in Areka Agricultural and research institute tissue culture laboratory. This institute found in south nation nationalities people under southern agricultural and research institute.

2.2. Chemicals

Table1. *Chemicals used in the experiment*

Name of the nutrient	Chemicals	Concentration in a liter of media without stock	Amount of stock solution used liter of medium(ml/l)
Macronutrients			10
Ammonium nitrate	NH_4NO_3	1.65	
Potassium nitrate	KNO_3	1.9	
Magnesium sulphate	$MgSO_4.7H_2O$	0.37	
Potassium	KH_2PO_4	0.17	

dihydrogenphosphate			
Calciumchloride	CaCl2	0.44	
Micronutreint			5
Boric acid	H_3BO_3	0.0031	
Manganese sulphate	$MnSO_4$	0085	
Zinc sulphate	$ZnSO_4.7H_2O$	0.0043	
Potassium iodide	KI	0.83	1
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.0025	1
Copper sulphate	$CuSO_4.5H_2O$	0.0025	1
Cobalt chloride	$COCl_2.6H_2O$	0.0025	1
Sodium ethylendiamine tetra acetic acid	$C_{10}H_{12}N_2Na_4O_8.2H_2O$	0.0372	5
Ferreoous sulphate	$FeSO_4.7H_2O$	0.0278	
Vitamines			
D.biotin		0.0002	1
Glycine		0.004	
Myo.inositol		0.01	
Nicotinic acid		0.0001	
Pyridoxine hydrochloric acid		0.0001	
Thiamine hydrochloric acid		0.0001	
Agar			8gm
Sucrose			3ogm

In vitro plant tissue and organ grown on artificial media supply the nutrients necessary for growth and development. Propagation of plants through tissue culture technique influenced by media used type. Plants require some inorganic elements in large quantity so they called major plant nutrients. Thus major elements include ions of Nitrogen (N), Potassium (K), Calcium (Ca), Phosphorus (P), Magnesium (Mg), and Sulfur (S). On the other hand elements that required in small amount called micro-nutrients which contain ions of Iron (Fe), Copper (Cu), Zinc (Zn), Boron (B), Molybdenum (Mo), Manganese (Mn) and Chlorine (Cl).

Stock Solutions

Stock solution of macro, micro, growth regulators and iron source was prepared with weighing the necessary amount of chemicals at desired composition in distilled water. Since stock solution comprises several chemicals, a liter of media was prepared by aliqouting from small amount from it.

Table2. *Concentration of macro nutrients stock solution*

Chemicals concentration	**10X g/L**
MS Macronutrients ml/L	**100 ml/L**
Ammonium nitrate	16.5
Potassium nitrate	19
Magnesium sulfate heptahydrate	3.7
Potassium phosphate monobasic	1.7
Calcium chloride dehydrate	4.4

Table3. *Concentration of micro nutrients in stock solution*

MS Micronutrients (10 mL/L)	**100X mg/L**
Boric acid	620
Manganese sulfate monohydrate	1690
Zinc sulfate heptahydrate	860
Potassium iodide	83
Sodium molybdate dihydrate	25(250 mg/ 10 ml) and1 mL of stock
Copper sulfate pentahydrate	2.5(25 mg/10 ml) and 1 ml of stock
Cobalt chloride hexahydrate	2.5(25 mg/10 ml) and 1 ml of stock

Table4. *Concentration of iron sources stock solution*

Chemical concentration	**100X g/L**
MS Fe-EDTA ml/L	**10 ml/L**
Disodium EDTA 2H2O (dissolve first in dark bottle)	3.72
Ferrous sulfate heptahydrate	2.78

2.3. Plant Materials

Meristem of cassava (*Manihot esculenta* Crantz) variety called kello with another name Nigeria white was used in the study with the size of 3-5 millimeter. This explant collected from garden field of Areka agricultural research institute. Surface sterilization of explants was done in laminar airflow hood. The explants resized before inoculation because they are more than one centimeter with leaves and nodes while collecting from field.

2.4. Surface Sterilization

The meristem collected from field were washed with tape water 3 times while shaking to removes mainly dusts and then by local detergent (largo) by shaking vigorously for 4 minutes and rinsed thus activities performed with in washing room. Then washed with distilled water 4 times until detergents remove from explants, within sterilization room. Lastly within inoculation room explants sterilized by 70% ethanol for 10seconds, then after washed and rinsed 3times with distilled water it immersed within 2%sodium hyphochloride and two drops of tween20 for 10 minutes and then after washed with distilled water 3times and rinsed to get ready for inoculation. The size of the explants resized and its damaged part removed by help of sterilized forceps and blade within laminar air flow hood.

2.5. Preparation of MS Nutrient Medium

Murasheig and skoog (1962) media was selected for this study due to its suitability to many types of plants. Stock solution of macro nutrients 10X, micro nutrients 100X, Iron sources 100X, vitamins, growth regulators were prepared. Media of one liter prepared by aliqouting 100ml from macro nutrient, 10ml from micro nutrient ,10ml from iron source,1ml from BAP and 1ml from kinetin stock solution with 40gm, 50gm,60gm,70gm and 80gm of bulla alone and with combination of 1gm and 2gm agar respectively. MS media with only 8gm/l agar was used as control group. Thus solidifying agents bulla, agar or their combination were added to the media after pH of the media adjusted to 5.8 with 1N NaOH for increasing PH value and1N HCl to decrease pH value. The media prepared for cassava micro-propagation was according to protocol for micro-propagation of cassava by Genene Gezahign unpublished one. After P^H adjustment agar, bulla and their combination added in media and heated to boiling point of 100% intensity for 7 minutes to dissolve medium and then dispensed in to 24 jars. The dispensed medium was autoclaved for 20 minutes at 121^{o}C with pressure of 105kpa.

2.6. Inoculation and Incubation

Inoculation was carried out in laminar airflow hood after sterilized it with 70%ethanol, 2% sodium hyphochloride with tween20 double distilled water and rinsed. Meristem resized to 5mm with help of sterilized ruler, forceps and blade. Two explants of the same variety inoculated into each single jar containing agar, bulla as well as within their combination. Incubation carried out at 25±2^{o}C under16 hour light and 8 hour dark with photon flux density of 60 $\mu molm^{-2}s^{-1}$ over headed cool fluorescent lamps at light intensity for 45 days.

2.7. Data Collection

Number of leaves, length of shoot in cm, number of shoot, number of nodes, length of roots, number of roots and fresh weight of each explants of each treatments were used as parameters collected starting from 5days of incubation to a period of one month.

The costs of agar, bulla as well as other ingredients of medium were collected from Addis Ababa chemical shops and the minimum cost of the medium components used.

2.8. Experimental Design and Data Analysis

Data from each treatment with bulla and its combination with 1and 2gms of agar effect on the development of explants containing MS medium was recorded and analyzed. Completely randomized block design with five treatments replicated three times. The data was analyzed by variance (ANOVA) with SPSS version 20 at significance difference ($p<0.05$). The costs of chemicals were computed using Microsoft excel 2007.Tables and charts were computed for interpretation of experimental results. The photograph illustrations were taken by sonny 14.1 megapixels camera with 4X optical zoom.

3. RESULT AND DISCUSSION

3.1. Media Clarity and Gelling Nature

Clear media is one of the required characteristics of media in plant tissue culture because that helps to detect contamination. In this study utilization of bulla alone showed poor clarity relative to the conventional agar. At concentration of sole bulla of 40, 50, 60 gram per liter don't become stable rather are liquid. But bulla at concentration of 80, 90 gram per liter are stable and solidify the media. The clarity and stability of bulla increase by increasing agar composite. The concentration of sole bulla above 90gm/l become hard and below 60gm/l can't stable gelling rather looks liquid. The poor clarity of sole bulla was reported by Alelign *et al.*,(2012). Bulla alone at 80gm/l and composite of bulla and agar at concentration of 60gm/l and 2gm/l and 70gm bulla with 1gm agar respectively were stable for whole multiplication phase for 45 days which is on par with the reports of Alelign *et al.*,(2012). Almost similar media stability observed between agar and bulla. This indicated that bulla has nature of forming bonding with hydrogen of media.

Table5. *Gelling nature of bulla & agar media*

		Bulla concentration with in liter					
agar		40	50	60	70	80	90
	0	L	L	L	L_S	G	G
	1	L	L	L	G	G	Gh
	2	L	L	G	G	Gh	Gh

L=liquid, L_S =semi liquid, G= gelling , Gh= gelling and become hard

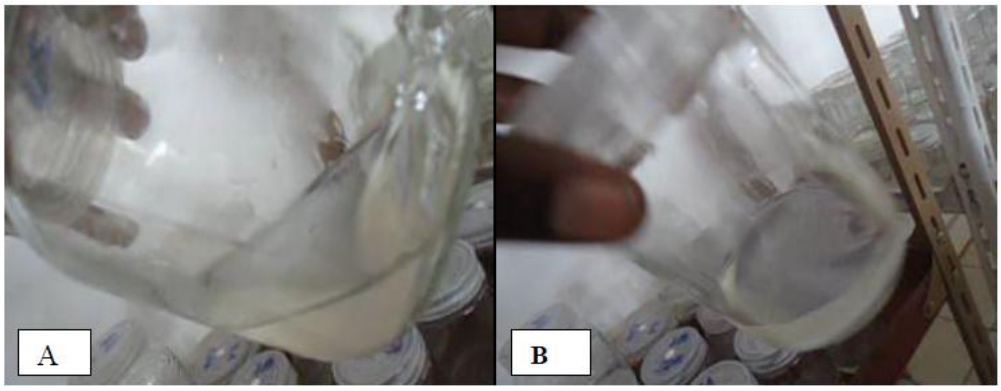

Figure1. *No solidified bulla after five days of preparation A) 60gm/l B) 40gm/l*

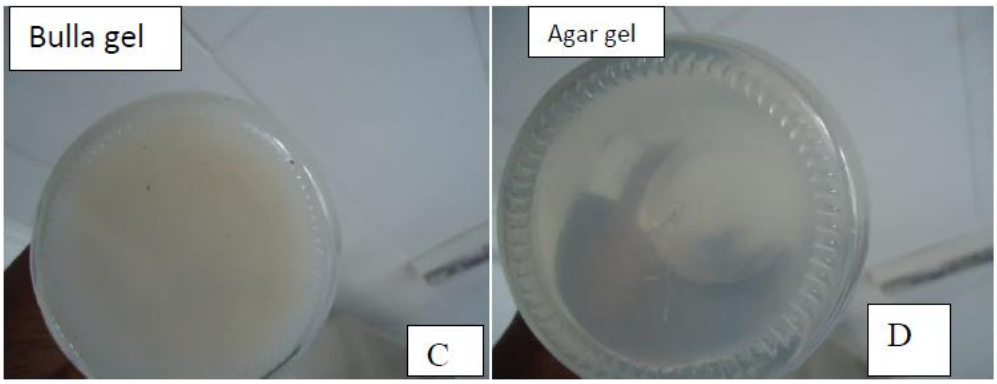

Figure2. *Appearance of bulla and agar solidified after four days of preparation C) clarity of bulla D) Clarity of agar*

3.2. Cost Analysis of Gelling Agent

In vitro micro-propagation of cassava with bulla as gelling agent is efficient and is proved to be a promising gelling agent. While agar is the most frequently used and expensive component in plant tissue culture media, using enset starch (bulla) as an alternative source saves 65% to 86% (table 6) that is on par with the reports of Daud *et al.*, 2011 who studied alternative source of agar such as

potato starch, rise flour, cassava flour and corn flour and obtained 66%-90% gelling cost reduction. Cost analysis obtained result using the following formula according (Ayelign *et al.*, 2012) Cost saved (%) = [100 − (bulla cost/agar cost) ×100]

Table6. *Cost comparison of gelling agents and their concentration used per a liter media*

Gelling agent	Concentration used in media	Cost of a gram of gelling agent in ETB*	Cost per Kg in ETB*	Cost of gelling agent used in a liter in ETB*	Cost saved per a liter in percent
Agar	8gm/l	3.5	3500	28	-
Bulla + Agar	80gm/l+2gm/l	3.55	-	10	65
Bulla + agar	70gm/l+1gm/gm	3.55	-	7	75
Bulla	80gm/l	0.05	50	4	86
Bulla	90gm/l	0.05	-	4.5	84

(*) 1USA dollar= 21 Ethiopian birr (ETB)

3.3. Root Number

Plants inoculated on composite media of bulla with agar at 60gm and 2gm and also at 70gm bulla and 1gm agar respectively developed significantly high number of roots compared to conventional agar. This might be due to higher Zn concentration, related with biosynthesis of Indoleacetic acid (George *et al*., 2008). This result also agreement with finding of (Kwame *et al*., 2012) substitution of conventional media with low cost modified media. Least number of roots recorded at sole bulla 80gm and 90gm respectively (Table-7).

Figure5. *in vitro rooted cassava (A) 60gm bulla+2gm agar/l (B) 70gm bulla+1gm agar/l (C) agar 8gm/l*

3.4. Shoot Length

Longest mean length of shoot was recorded at composite of bulla and agar 60gm with 2gm agar in a liter of media respectively followed by sole 80gm bulla. The shortest length of shoot recorded at composite of 70gm bulla with 2gm conventional agar (Table-7). Similar results were reported by Lalitha *et al.,* 2014 in shoot length of mulberry grown with composite of agar and cassava flour.

3.5. Number Of Nodes

Highest mean number of node were recorded at composite of 60 gm bulla and 2gm agar whereas the number of node at bulla alone 80gm and 90gm was respectively (Table-7).This result is in consonance with the reports of Kwame *et al*., 2012.

3.6. Leaf Number

High mean number of leaves was recorded with composite of 60gm Bulla + 2gm Agar per liter of media. Whereas least means leaves number was recorded with bulla alone 90gm per liter of media (Table-7). Similar results were reported by (Kuria et al., 2008) potato propagation by cassava starch as gelling agent.

3.7. Fresh Weight

Gelling agent of bulla with agar 60gm/l and 2gm/l resulted high weigh (0.9) then followed by bulla alone 80gm/l mean weight (0.74). This result in line with bulla, similar observation has been found by (Ayenew *et al*., 2012) and Mbanaso (2008) for shoots robustness after repeated subcultures derived

from starch-gelled medium than agar. This could be due to additional supplements of Mg, constituent of chlorophyll, and Mn which activates several enzymes that are involved in the processes of electron transport system in photosynthesis (Atlabachew and Chandravanshi 2007) and also water potential difference

Table7. *Effect of conventional agar and bulla agar on the growth of cassava root, shoot leaf and node and fresh weight under in vitro conditions*

Gelling agent	Concentration used in a liter	Root number	Root length	Number of shoot	Length of shoot in cm	Number of leaf	Number of node	Fresh weight (gm)
Agar	8gm	9±1.32a	2.67±0.21a	2.33±0.31a	3.33±0.04a	3.67±0.13a	2.67±0.41a	0.63±0.05a
Bulla + Agar	60gm + 2gm	14±3.6a	2.77±0.27a	2.0±0.11a	4.67±0.34a	4±1.34a	3.33±0.73a	0.9±0.12a
Bulla + agar	70gm + 1gm	15±4.68a	3.1±0.23b	1.67±±0.3a	2.67±0.64a	3.33±0.31a	2.33±0.32a	0.45±0.03a
Bulla	80gm	6.33±±0.43b	3.63±0.57a	3.0±1.2a	3.17±0.26a	3.33±0.53a	2.0±0.24a	0.74±0.06a
Bulla	90m	7.33±0.28a	2.23±0.65a	1.0±0.08a	2.83±0.5a	2.33±1.03a	2.0±0.47a	0.42±0.02a
Coefficient of variance		15.521	0.272	0.554	0.627	0.391	0.289	0.432
P value		0.114	0.986	0.987	0.193	0.697	0.084	0.677

Legend: *means with the same letter in column are not significantly different*

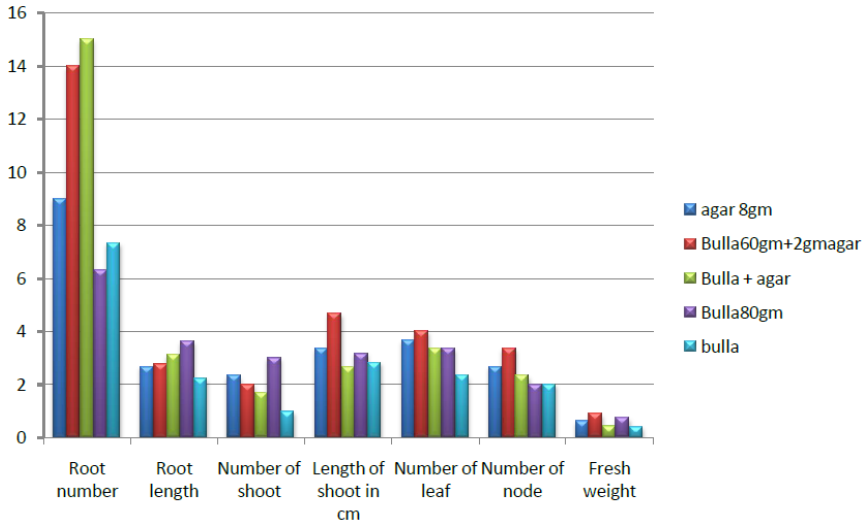

Figure3. *Effects of gelling agents on in vitro propagation of cassava*

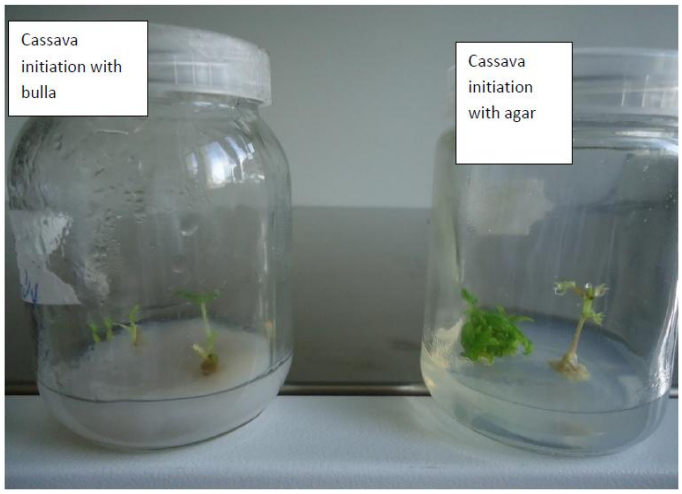

Figure4. *Cassava at initiation stage with bulla and agar gelling agent*

4. CONCLUSION AND RECOMMENDATION

Cassava *in vitro* propagation was efficient using enset flour (bulla starch) that conveniently serves as a substitute for conventional agar as gelling agent. Bulla starch is locally available from 65-86% cost

saving gelling agent when using different concentration of agar alone and composite with agar in cassava micro-propagation in tissue culture. Apart from gelling agent bulla starch improve *in vitro* cassava plantlets and growth performance which might be the result of additional carbon found within bulla starch. Further study need to be conducted up on effect of different elements found within bulla to use bulla as gelling agent commercially and different plants than studied. This research shown that it is possible to reduce the cost of plant production during tissue culture by using bulla as an alternative source of agar.

5. BIOGRAPHICAL SKETCH

The author was born in southern region South Omo Zone Debub Ari Wereda in 1978E.C He attended his primary school at Gorgocha primary school, secondary school at Jinka secondary school. Then he joined diploma in Arbaminch college of teachers education in field of Natural science since 1997 E.C and his B.Sc. in Dilla University by chemistry from 2008-2012. After getting his B.Sc. he was employed in Debub Ari wereda education office. Until his joining school of graduate school he worked by being as leader to employer in debub ari woreda.

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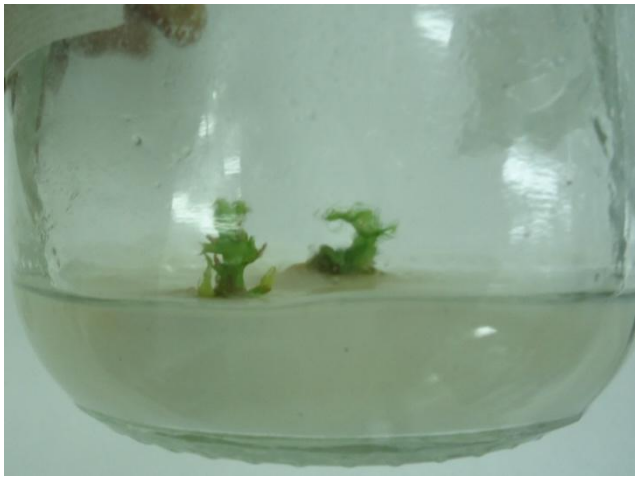
APPEDIX

Appendix A. *amount of chemicals in a liter of MS media with costs in ETB*

Chemical	Amount of chemicals used per a liter	Cost of chemical used in a liter by ETB*	Cost of one gram of the chemical ETB*	% of one gram cost in liter	%of cost of chemicals used in a liter	rank
NH4NO3	1.65	8.25	5	12.48439451	18.0049	2
KNO3	1.9	2.87	1.5	3.745318352	6.263524	4
MgSO4.7H2O	0.37	0.481	1.3	3.245942572	1.04974	7
KH2PO4	0.17	0.221	1.3	3.245942572	0.482313	8
CaCl2.2H2O	0.44	0.616	1.4	3.495630462	1.344366	6
H3BO3	0.0031	0.0062	2	4.993757803	0.013531	14
MnSO4	0.0085	0.017	2	4.993757803	0.037101	11
ZnSO4.7H2H2O	0.0043	0.0086	2	4.993757803	0.018769	13
KI	0.83	1.245	1.5	3.745318352	2.717104	5
Na2MoO4.2H2O	0.0025	0.005	2	4.993757803	0.010912	15
CuSO4.5H2O	0.0025	0.005	2	4.993757803	0.010912	15
CoCl2.6H2O	0.0025	0.003125	1.25	3.121098627	0.00682	19
EDTA	0.0372	0.0372	1	2.496878901	0.081186	9
FeSO4.7H2O	0.0378	0.0372	1	2.496878901	0.081186	9
Gly	0.004	0.004	1	2.496878901	0.00873	17
myoinstol	0.01	0.01	1	2.496878901	0.021824	12
nicotinic acid	0.0001	0.000125	1.25	3.121098627	0.000273	21
Pyridoxine	0.0001	0.0004	4	9.987515605	0.000873	20
thiamine	0.001	0.004	4	9.987515605	0.00873	17
agar	8	28	3.5	8.739076155	61.10755	1
bulla	80	4	0.05	0.124843945	8.72965	3
		45.82085	40.05	100	100	

Appendix B. Contamination of bulla by fungus

Appendix C. Initiation of cassava using bulla



Appendix B - Abbreviations and Acronyms

ABA- Abscisic Acid

BAP- Benzyl Aiminopurine

DNA- Deoxyribo NuclicAcid

EDTA- Etylene Diamine Teteraaceticacid

EIAR- Ethiopian Institute of Agricultural Research

GA_{3-} Gebberellic Acid

GDP- Goal of Development Program

IAA- Indole-3-Acetic Acid

PGR- Plant Growth Regulators

PTC- Plant Tissue Culture

2,4-D- 2,4-Dichlorophenoxyacetic acid

2 ip- 2-isopentyl Adenine

NAA- Naphthaline Acetic Acid

TC - Tissue Culture