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In Vitro propagation of sweet potato (*Ipomoea batatas* (L.) Lam) through apical meristem culture

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Abstract
Sweet potato (*Ipomoea batatas* L.) is an important warm season crop that grows best in long, hot growing seasons, cultivated in over all developing countries as a valuable source of human food, animal feed and industrial raw material. Its yield is highly decreased due to disease in Eastern African Countries. Production of virus free sweet potato is of great potential to increase yield and improve the crop. In the present study, different treatments, each with a combination of (BAP and Kn) in a ratio 0.25, 0.5, 0.75, 1.0 and 0.25, 0.5, 0.5, 0.75 and 0.0 mg/l (control) and auxin (IBA and NAA) in a ratio 0, 0.5, 0.5, 1.0, 1.0mg/l and 0, 0.25, 0.5, 0.5 and 1.0mg/l in combination respectively were used for three varieties (Beletech, Awassa-83 and Belela) to optimize the concentrations of BAP, Kn, IBA and NAA in MS basal media for meristem culture. The experiment was laid out in CRD with three replications in factorial arrangement. Among the growth regulator combinations tested 0.5mg/l BAP with 0.5mg/l Kn followed by 0.75mg/l BAP with 0.5mg/l Kn were best for all shoot induction and growth parameters for the three varieties. The minimum days to root induction was recorded for Beletech (3.167days) shoots cultured on media supplemented with 0.5mg/l IBA with 0.5mg/l NAA and Awassa-83 (3.83days) shoots cultured on media supplemented with 1.0mg/l IBA and 0.5mg/l NAA. Whereas, the variety Belela shoots have induced in (3.83 days) on media with 1.0mg/l IBA with 1.0mg/l NAA. Maximum number of roots per shoot were recorded on MS media supplemented with a combination of 1.0mg/l IBA with 0.5mg/l NAA (11.7) followed by (9.3) on media with 0.75mg/l IBA with 0.5mg/l NAA respectively. The maximum root length was observed for Beletech (3.4cm) followed by Awassa-83 (3.43cm) cultured on the media with a combination of IBA 0.75mg/l with NAA 0.5mg/l. The results indicated that better response was obtained for root related parameters of all variety shoots transferred for rooting on media supplemented with 0.5mg/l IBA with 0.5mg/l NAA and above up to 1.0mg/l IBA with 0.5mg/l NAA. During acclimatization, 90%, for Beletech and 80% for Awassa-83 and Belela varieties survival was obtained.

Keywords: BAP, IBA Kn, MS media meristem culture NAA

Introduction
Sweet potato (*Ipomea batatas*) is a delicious root crop with high in vitamin and sugar content. It is a dicotyledonous plant that belongs to the family *Convolvulaceae*. It is tuberous root crop important for food security in tropical, subtropical and temperate regions. It is a warm season crop cultivated in over all developing countries as a valuable source of human food, animal feed and industrial raw material (Jarret and Florkowski, 1990) [12] and ranks among the five most important food crops in over 50 countries (FAOSTAT, 2012) [6]. Sweet potatoes are among of the most widely grown root crops. The roots mature in 4 to 5 months. Sweet potatoes grow in all soil types provided they are fertile, moist, well drained and nutrient rich widely grown in countries surrounding the Great Lakes in Eastern, Central Southern and in Nigeria in West Africa. China is being the largest producer worldwide with 80-85% sweet potato production, mainly as starch industries feed stock or animal feed (International Starch Institute, 2003) [11]. Sweet potato is among well-known and established crops in Southern, Eastern and South western parts of Ethiopia. It is produced annually on over 53 thousand hectares of land with total production over 4,240 tons and average productivity of 8.0 tons per hectare.

Current trends indicate an increasing in assets in sweet potato production due to commercialization of the crop. However, there are limiting factors to increased sweet potato production. Among the major biotic constraints for sweet potato production are insect pests, like the stem and root feeders, sweet potato weevils, *Cylas puncticollis (Coleoptera Curculionidae),* sweet potato butterfly, *Acraea acerata*-(*Lepidoptera: Nymphalidae*), sweet potato horn worm, *Agrius convolvuli* (*Lepidoptera:-Sphingidae*), and virus transmitters: *Aphis gossypi* (*Homoptera: Aphididae*) and *Bemisia tabaci* (*Homoptera: Aleyrodidae*). Diseases and environmental factors prevent the crop from reaching its maximum agricultural potential. Virus diseases have been identified as the main cause of low productivity.

Continuous use of varieties despite root yield degeneration, critical shortage of planting materials of superior varieties, and Poor soils (Wambugu, 1991) [21] are the major cause of cultivar decline (Gibson *et al*., 1998) [7]. Moreover frost at high altitude and extreme temperature at very low altitudes are the major natural constraints of sweet potato production (Terefe, 2003) [20].

To overcome these problems, both conventional and biotechnological breeding programs need to be applied. Disease free and genetically uniform plantlets may be produced through tissue culture techniques (Hoque *et al*., 2007) [10]. Plant tissue culture techniques have been employed in a large number of important potato varieties in agriculture. The application of tissue culture and rapid propagation method for potato production continues to become more widely used in both developed and developing countries. Tissue culture techniques can be applied not only to increase propagation rates, but also to modify the germplasm itself while conserving the present resources. (Hashem *et al*., 1990) [9].

In Ethiopia, for the last 10 years no tissue culture methods or biotechnology is practiced to produce diseases free crops even though infrastructure was set up in selected agricultural research centers (Girma, 2003) [8]. But now it is getting attention and being applied in agricultural research centers. One of the most important application of tissue culture as a tool of biotechnology is its application in the production of diseases free plant materials. Moreover; it enables production of large number of plantlets in short period of time as well as maintenance of germplasm under controlled conditions in small spaces and with reduced labor requirement (Rabbani *et al*., 2001) [19]. This study can lead to a greater understanding of the mass propagation of sweet potato species treating with different plant growth regulators for *in vitro* shoot multiplication and *in vitro* rooting. Furthermore significance may be derived in the application of knowledge gained during the process help to improve the quality, and decrease the risk factors on the growth and maturation of plant.

2. Materials and Methods

2.1 Description of the Study Area

The experiment was conducted in tissue culture laboratory of Southern Agricultural Research Institute (SARI) at Areka Agricultural Research Center. It is located 300 km south of Addis Ababa at 7°4'12" north and 37°42'0" east at elevation of 1774 meters above sea level.

2.2 Plant Materials Used

Three sweet potato varieties such as Beletech, Belela and Awassa-83 were selected and used for this study. Beletech, which is orange fleshed variety among the three, is an important source of vitamin A. The others are white fleshed varieties. These varieties were collected from Areka Agricultural Research Centre and were kept in the green house to grow by watering and weeding throughout the duration of the study. These plants were used as stock supply of explants for the repeated meristem cultures. Young and healthy shoots (2.0 to 3.0 mm long), containing axillary buds (third, fourth and fifth nodes; from shoot apex), were excised and collected using sterilized scalpel and forceps and used as explants. Actively growing shoots (juvenile plants) were taken as they are more responsive to shoot regeneration and proliferation than shoot explants from adult forms.

2.3 Experimental Design

The experimental design was based on Completely Randomized Design (CRD) was used for all the treatments (five different combinations of four growth regulators (BAP, Kn, IBA and NAA). The explants samples were collected from three different varieties. The experiment was conducted to identify type and optimum concentration of plant growth regulator for *in vitro* shoot and root multiplication. All the external factors were held constant except the difference in the treatments. Each treatment had three replicates of culture test tubes for all the three varieties.

2.4 Explants Surface Sterilization and Culture Initiation

The explants shoot tip were treated with locally available Gion-berekina (2% chlorox) to use as a substitute for sodium hypochlorite for varying periods of time at different concentrations in order to establish maximum contaminant free cultures.

Sterilization procedures were carried out in laminar airflow cabinet bench. For surface sterilization greenhouse grown shoots about 2.0 to 3.0 mm long were cut and put into tap water in 50 ml glass bottle and rinsed three times and then washed with double distilled water followed by dipping in 70% alcohol for one minute and immediately thereafter washed with distilled water and subsequently sterilized in the laminar air flow cabinet with 0.1% w/v 2% chlorox for 4 to 7 minutes. Surface sterilized segment were washed 4 to 5 times with sterilized distilled water. Finally, the size of explants was trimmed to appropriate size (1mm) using sterile scalpels and forceps and inoculated to initiation media as per treatments under laminar hood. Surface sterilization experiment result shows 95% contamination free explants and very less (2%) dead explants for all the varieties until 10 days of inoculation in MS media. The microbial contamination, in particular by apparently endophytic microbes that are resistant to antimicrobial agents was encountered during micro propagation work of enset (*Ensete ventricosum*).

2.5 Preparation of plant growth regulator stock solution

Stock solution of 6-Benzylaminopurine (SIGMA) (BAP), Kinetin (UNI-CHEM) (Kn), a-Naphthalene acetic acid (NAA) and Indole-3-butyric acid (HIMEDIA) (IBA) were used. MS media with 30gm of sucrose for carbon source, 6gm of agar type as solidifying agent, 100mg of mayo-inistol for osmo-regulation and other organic components of recommended concentration were prepared with different levels of growth regulators (BAP and Kn) for initiation & multiplication. For rooting of shoot lets, MS media with different levels of auxin (IBA and NAA) was prepared. Each PGRs were dissolved with 5 drops of specific solvents and the volume was adjusted by adding double distilled water. The stock solutions were stored at ±4 ^{O}C.

The surface sterilized sprout cuttings were transferred into sterile test tubes. About 1mm long sprouted apexes were excised with the help of sterilized scalpel and forceps. The cuttings were inoculated into agar solidified MS medium with sucrose 30gm as a source of carbon and 6gm agar as solidifying agent were used as a basal medium (Murashinge and Skoog, 1962) [14]. The macro nutrients, micro nutrients and organic additives were added and the final volume was adjusted to one litter. Test tubes and flasks be tightly capped and labeled properly and followed by autoclaving at 121°C for 20 minute at15psi. Autoclaved media and materials were allowed to cool in sterile environment after which it is ready for use. In each test tubes a single cutting and in magenta jar three cuttings were placed in the medium respectively keeping the position of the nodes upside (Aggarwal and Barna, 2004) [1].

The inoculated test tubes and magenta jars were incubated under florescent light at 25±2°C temperature. The sprout cuttings give raise to shoot bud within 2 to 3 weeks. The shoot bud was sub-cultured regularly in order to get large scale of shoot apex and nodal segments. Apical meristem were used for mass propagation in MS medium supplemented with different kinds of PGRs (Al-Mazrooei *et al.,* 1997) [2].
Careful surveillance of the initiated cultures was undertaken because of high contamination rate. Contaminated cultures were replaced by fresh cultures whenever possible to avoid the introduction of errors due to unequal replication.

2.6 Subculture of shoot apex and nodal segments
To facilitate growth of the cultures, the cultures were transferred to fresh media of the same combination after about three to four weeks. The cultures were left for at least two months on the same culture medium before shoots were transferred to shoot multiplication media. The nodes and shoots of three varieties (Beletech, Awassa-83 and Belela) were treated by different concentrations of BAP and Kn for shoot multiplication.
The combination of BAP and Kn concentrations in ratio used were 0.25, 0.5, 0.75, 1.0 and 0.25, 0.5, 0.5, 0.75 and 0.0 mg/l. Fifteen shoots were used for multiplication for each of the varieties in each treatment. The days of shoot induction, number of shoots per explants, leaf number, shoot length and other parameters induced was counted after about a month for each of the combination of BAP and Kn concentrations used.

2.7 Multiple shoots regeneration media
For the purpose of shoot initiation, MS basal medium (Murashige and Skoog, 1962) [14] was used.
Media was prepared with different concentrations and combination of two plant growth hormones; 6-benzylaminopurine (BAP) and kinetin (Kn). Stock solutions of growth regulators were prepared and stored at ±4°C until used. Stock solutions of macronutrients, micronutrients and vitamins were prepared and stored at -20°C until used. After melting the stock solutions, 100ml/l macronutrients, 10ml/l micro nutrients, 10ml/l vitamins with the addition of 30g/l sucrose were used to prepare the media. And then the required concentrations of growth regulators were added. Before the addition of 6g/l agar, the mixture was homogenized using magnetic stirrer and the pH was set to 5.8 using either 1M NaOH or 1M HCl. Steam oven was used at 120°C for 15 minutes, then after immediately about 25 ml media was poured onto test tubes or jars. Stem autoclave was used at 120ºC for 30 minutes for further sterilization. All the media prepared were put in refrigerator (±4 °C) at least for three days to identify the contaminated media and until required.
A total of fifteen different combinations of growth regulators were prepared with the media. For elongated shoots, about 60 ml media with different concentrations of BAP and Kn was prepared in magenta jars and test tubes for further elongation and multiplication of shoots following the same procedures in front of laminar air flow cabinet bench(Panta *et al*., 2006) [17].
The cultures were kept under 28 ± 2 ºC and 300 foot-candle for 16 h light per day (Aggarwal and Barna, 2004) [1]. Stem segments were transferred onto glass jars of 500ml containing 30ml of propagation MS medium or test tube supplemented with constant concentration of different PGRs were used. All treatments for shoot regeneration were repeated 5 times.

2.6 Rooting
For the induction of roots, the *in vitro* grown shoots were used. Medium preparation for root culture was carried out in the same fashion as that of shoot medium culture except in here NAA and IBA were used. Each treatment was consisting of 10 glass jars, each jar containing three cuttings. Different combinations of auxins (IBA, NAA) were used with this strength of MS medium.

2.7 Acclimatization
The *in vitro* rooted plantlets were taken out from the culture bottles with the help of forceps and dipped in warm water to remove any traces of solidified agar media. Then plantlets were carefully planted in the cell tray containing a mixture of cockpit and sand then transferred to prepared plastic pots containing mixtures of 2:1:1 ratio (sand, red soil and compost, respectively). Potted shoots were allowed to grow under porous inverted plastic (polyethylene) bags (Clark *et al*., 1990) [3]. Then the plants were shifted to green house with less humidity level indirect sun light.

2.8 Data Collection
All necessary data such as mean number of days to shoot and root induction, mean number of node on the shoots, mean number of leaves and roots per explants, length of shoots and roots, as well as biomass data's of shoot fresh and dry weight were recorded.

2.9 Data Analysis
Data were subjected to ANOVA by using SAS software (JMP version 9.2.). The differences between treatment means were compared using Least Significance Difference (LSD) test at probability of 5% and 1% levels.

3. Results and Discussion
3.1 Effects of Growth Regulators on Shoot Induction in Selected Varieties of Sweet Potatoes (Beletech, Awassa-83 and Belela)
Significant variation was observed for days required for shoot initiation among the varieties and hormone combinations. The minimum days required for shoot initiation (3.5 days) was noted in Awassa-83 0.5mg/l BAP and 0.5mg/l Kn in combination, Beletech (4.25 days) on the same hormone combination, as in Belela (4.83 days). Whereas, the maximum days required for shoot initiation for Awassa-83 (7.67) and (6.5) were recorded on hormone free and media with 1.0mg/l BAP and 0.75 Kn respectively followed by Belela (7.67) and Beletech (5.67) on hormone free MS media (Table 1).
The number of days recorded for shoot initiation in this experiment which was cultured on MS media solidified with 6gm/l of agar and supplemented with 0.5mg/l BAP and 0.5mg/l Kn is very much earlier as compared to the previous study reported by Mulugeta and Staden (2004). [15] The most probable reasons for this early initiation in the present experiment were the low amount of solidifying agent used that can reduce nutrient uptake by explants if in large amount since the media becomes very compact and the combined effect of BAP and Kn. Notable variation was observed for length of shoots among varieties and different hormone combinations. The maximum shoot length was recorded for Belela (5.833cm) on media with hormone combination of 0.5mg/l BAP and 0.5mg/l Kn followed by 3.633cm for Beletech on media with hormone combination of 0.75mg/l BAP and 0.5mg/l Kn and 3.567cm for Awassa-83 on media supplemented with 0.25mg/l BAP and 0.25mg/l Kn (Table 2). Whereas, the lowest shoot length (1.80-2.667cm) was

recorded for all the three variety shoots developed on hormone free MS media (Table 1).

According to the result of this experiment knowing the appropriate concentration of BAP and Kn for shoot length is vital for all the three varieties. Because further increase in concentration does have negative effect and ends with reduced shoot length for all the three varieties. So far there is no sufficient study report on sweet potato concerning effect of growth regulators on the length of shoots per plantlets. However, Mulugeta and Stedany, (2004)[15] reported that the effect of growth regulators on plantlet regeneration from shoot tip explants and reported 3-5 cm shoot length on media with 2.5mg/l BAP which is less than shoot length reported in this experiment.

3.2 Effects of Growth Regulators on Root Induction in Selected Varieties of Sweet Potatoes (Beletech, Awassa-83 and Belela)

There is highly significance difference at (P≤0.01) in hormone combination and variety on root induction was observed and indicated in the table-2, but the interaction effect of the two factors is non-significant. Negash *et al*. (2000) [15] reported that root formation occurred in less than two weeks after transfer to root induction medium supplemented with 5 µM IBA, 1 µM IAA and 1 µM BAP in combination for all three varieties on which they have conducted their experiment.

The highest number (10.667) is found on MS media supplemented with a combination of 1.0mg/l IBA with 0.5mg/l NAA followed by (10.66) on media with 0.5mg/l IBA with 0.5mg/l NAA (Table 2). Whereas, the lowest mean number (3.0) of roots per shoot was recorded for roots induced on hormone free MS media for Beletech.

Better survival and acclimatization for longer shoots is in accordance with the recommendations of (El Far *et a*l., 2009) [5] and (Ozturk and Atar, 2004) [16]. Moreover, the significance of appropriate root development *in vitro* for successful establishment of sweet potato shoots during acclimatization agrees with (Zobayed *et al*., 1999)[22].

The maximum mean root length was obtained for Awassa-83 (3.433cm) cultured on the media with a combination of IBA 1.0mg/l with NAA 0.5mg/l. Whereas 3.3cm, for Belela shoots transferred to media with a combination 0f 1.0 with 0.5mg/l of IBA and NAA respectively. (Table 2 and Figure 2A-D). On the other hand, the lowest root length (1.767cm) was recorded for Beletech on media with a combination of 1.0mg/l IBA and 1.0mg/l NAA followed by (2.23cm) on media with a combination of 1.0mg/l IBA and 0.5mg/l NAA (Table 2).

Awassa-83 has responded better for root length as compared to the two varieties. The optimum level of IBA concentration is 1.0mg/l for all the three varieties and it was observed that the morphology of main and branch roots was very uniform and better on this concentration of IBA

2.3 Acclimatization

The acclimatization results (Table 3) were revealed that, 90% survival rate was recorded for Beletech plantlets and 80% for both Awassa-83 and Belela after 15 days of acclimatization whereas, 60% survival was recorded for Beletech plantlets acclimatized to soil mix potted to plastic bags without sterilization followed by 50% for Awassa-83 and 40% for Belela. Soil sterilization has high effect on survival of plantlets and Beletech variety has high performance in terms of survival in acclimatization even in non-sterile acclimatization media. Rabbani *et al*. (2001) [19] reported eighty percent survival rate of ginger *in vitro* plantlets acclimatized to solar sterilized peat soil mix after 15 days of acclimatization which is in agreement to the present finding. Deepa *et al*. (2011) [4] also reported that the well rooted plantlets transferred to pots containing a mixture of sterilized sand, soil and vermiculate (2:1:1), looks healthy and vigorous growth with 70% survival rate after two weeks

Table 1: Effect of plant growth regulators (BAP and Kn) on shoot induction and growth related parameters of

Hormone Concentration		Variety	Days to shoot initiation	Node number	Leaf number	Shoot length(cm)
BAP	Kn					
0	0	Beletech	5.67	2.33^{def}	3.67^{cd}	1.80^{j}
0	0	Awassa-83	7.67^{a}	2.33^{def}	3.0^{ed}	2.53^{h}
0	0	Belela	7.67^{a}	3.0^{dbc}	2.0^{f}	2.700^{h}
0.25	0.25	Beletech	4.42^{g}	2.33^{def}	2.67^{ef}	2.18^{i}
0.25	0.25	Awassa-83	5.25^{fe}	2.00^{gef}	4.00^{c}	3.567^{ed}
0.25	0.25	Belela	6.083^{dc}	2.33^{def}	5.00^{b}	2.700^{h}
0.5	0.5	Beletech	4.25^{g}	2.33^{def}	3.667^{cd}	2.75^{gh}
0.5	0.5	Awassa-83	3.5^{h}	3.33^{bc}	5.667^{b}	2.933^{g}
0.5	0.5	Belela	4.83^{fg}	2.667^{cde}	6.667^{a}	5.833^{a}
0.75	0.5	Beletech	5.583^{de}	6.00^{a}	3.0^{ed}	3.633^{cd}
0.75	0.5	Awassa-83	5.5^{fde}	1.667^{gf}	3.33^{cde}	3.367^{ef}
0	0	Beletech	5.67	2.33^{def}	3.67^{cd}	1.80^{j}
0	0	Awassa-83	7.67^{a}	2.33^{def}	3.0^{ed}	2.53^{h}
0	0	Belela	7.67^{a}	3.0^{dbc}	2.0^{f}	2.700^{h}
0.25	0.25	Beletech	4.42^{g}	2.33^{def}	2.67^{ef}	2.18^{i}
		CV (%)	7.43	19.86	13.67	4.14
		LSD (5%)	0.7086	0.8611	0.8611	0.2265

Beletech, Awass-83 and Belela varieties

Means with the same letter in the same column are non-significant at 5% significance level.

Table 2: Effect of plant growth regulators (IBA and NAA) on root induction and growth related parameters of Beletech, Awass-83 and Belela varieties.

Hormone combination		Variety	Root number	Root length (cm)
IBA	NAA			
0	0	Beletech	3.0^{g}	3.3^{bc}
0	0	Awassa-83	5.0^{f}	3.1^{d}
0	0	Belela	3.33^{g}	2.72^{f}

0.5	0.25	Beletech	9.0^{cb}	2.567^{g}
0.5	0.25	Awassa-83	9.0^{cb}	3.213^{dc}
0.5	0.25	Belela	9.0^{cb}	2.833^{e}
0.5	0.5	Beletech	8.33^{cd}	2.867^{e}
0.5	0.5	Awassa-83	10.66^{a}	3.333^{ba}
0.5	0.5	Belela	7.667^{ed}	2.633^{fg}
1.0	0.5	Beletech	10.667^{a}	2.333^{h}
1.0	0.5	Awassa-83	9.677^{ba}	3.433^{a}
1.0	0.5	Belela	8.33^{cd}	3.3^{bc}
1.0	1.0	Beletech	6.667^{e}	1.767^{i}
1.0	1.0	Awassa-83	9.333^{cb}	3.267^{bc}
1.0	1.0	Belela	7.667^{ed}	2.933^{e}
		CV (%)	8.93	2.35
		LSD (5%)	1.1659	0.1137

Means with the same letter in the same column are non significant at 5% significance level.

Table 3: Survival rate of plantlets on acclimatization

Variety	Number of plants acclimatized		No. of plants survived after 15 days		Surviving Plants (%)	
	Sterile soil mix	Unsterile soil mix	Sterile	Unsterile	Sterile	Unsterile
Beletech	10	10	9	6	90	60
Awassa-83	10	10	8	5	80	50
Belela	10	10	8	4	80	40

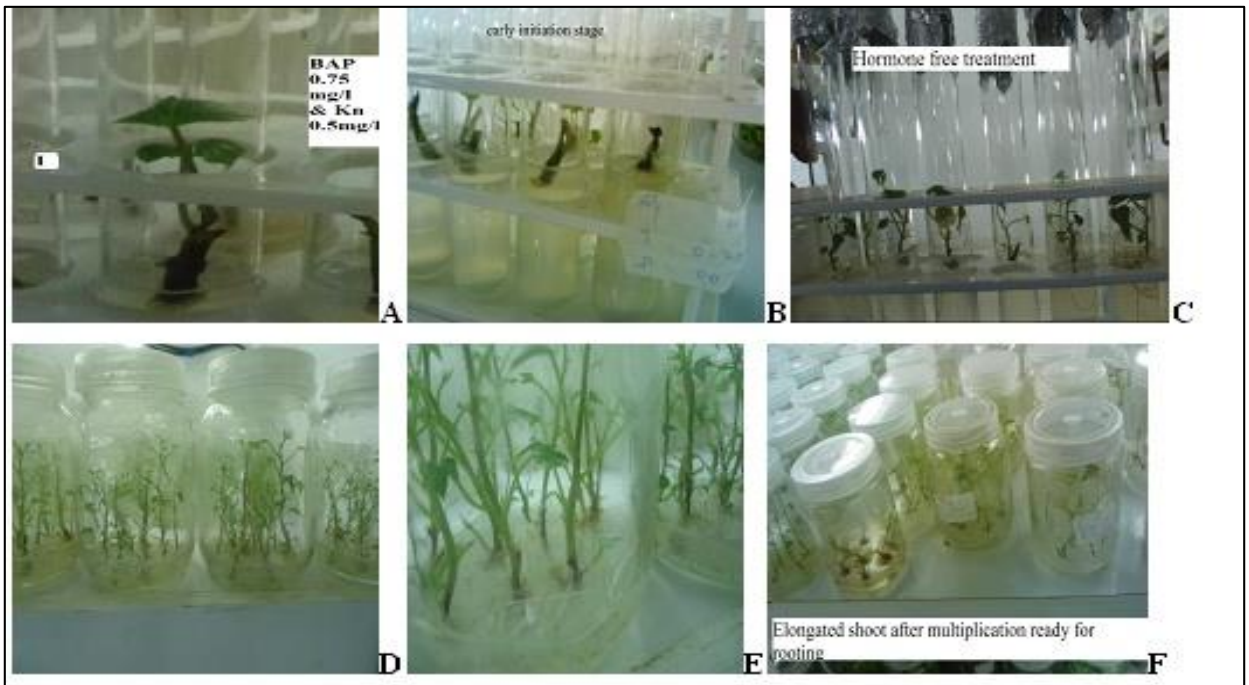

Figure 1 Shoot induction and growth at different combination of hormones A. Initiated Awassa-83 explants, B. Initiated shoots from Belela explants, C. Hormone free initiated shoots from all variety explants D. Initiated shoots from Beletech explants. E and, F. elongated shoots ready for rooting.

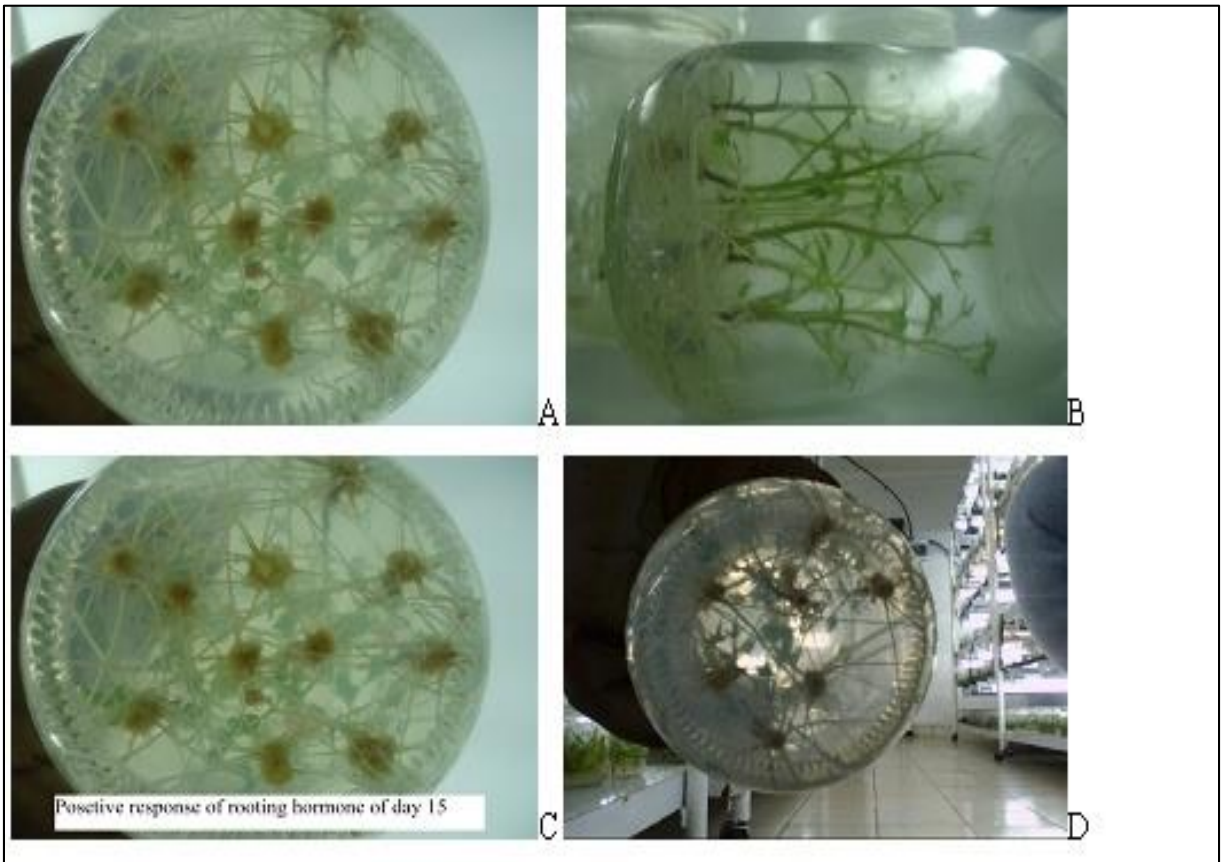

Figure 2 Plantlets in rooting MS media with a combination of IBA and NAA. A. Regenerated plantlets of Belela shoot with roots. B. Roots of Beletech variety. C. Roots of Awassa-83

4. Conclusion

The *in vitro* meristem culture protocol developed for mass propagation of sweet potato for three selected varieties (Beletech, Awass-83 and Belela) is efficient. Where surface sterilizing explants with 70% of ethanol for one minute followed by 2% of chlorox for four to seven minute found as optimum. MS media with 0.5mg/l BAP with 0.5 Kn mg/l followed by 0.75mg/l BAP with 0.5mg/l Kn in combination is sufficient for shoot induction and growth parameters for the three varieties, whereas, 0.5mg/l IBA with 0.5mg/l NAA and above up to 1.0mg/l IBA with 0.5mg/l NAA is obtained to be optimum for root induction and growth. The maximum acclimatized plantlets (90%) were achieved under controlled atmosphere and humidity. Though the results obtained in the present research can be used as guidelines for improving *in vitro* mass propagation of sweat potato.

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