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## PROTOCOL OPTIMISATION FOR MICROPROPAGATION OF ETHIOPIAN YAM

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### ABSTRACT

Yam (*Dioscorea* spp.) is a monocotyledonous tuber forming tropical vine, which belongs to the family Dioscoreaceae, and genus *Dioscorea*. Yam is well known for medicinal properties, as well as nutritional values. Conventional propagation of yam is limited by low propagation rates; hence, *in vitro* propagation provides the best alternative to overcome such limitations. The objective of this study was to optimise a protocol for *in vitro* micro-propagation of Ethiopian yam (*D. alata*, bulcha variety). Explants were obtained from young leaves of bulcha variety, and sterilised using different concentrations of NaOCl, for different time exposures. Sodium hypochlorite (1%) at 15 minutes exposure time, showed 100% survival of explants. The combination of 0.5 BAP with 1mg l<sup>-1</sup> NAA was the best concentration for shoot induction. For shoot multiplication, BAP at 1.5 mg l<sup>-1</sup> gave the highest shoots per explants (7.28±0.07), with shoot length of 8.72±0.43 cm. MS medium with 2.0 mg l<sup>-1</sup> NAA and 1.5 mg l<sup>-1</sup> IBA gave the highest rooting percentage and root number (10.6±0.44), with a root length of 13.80±0.44. *In vitro* acclimatised plantlets, which were transferred to a greenhouse for hardening, had 90% survival rate on soil medium. Thus, for micropropagation of bulcha yam variety, MS + 1.5 mg l<sup>-1</sup> and MS plus 2.0 mg l<sup>-1</sup> NAA + 1.5 mg l<sup>-1</sup> IBA is the best phytohormonal combination for shoot multiplication and invitro rooting, respectively.

*Key Words:* *Dioscorea*, MS Medium, nodal vine

### RÉSUMÉ

L'igname (*Dioscorea* spp.) est un tubercule monocotylédone qui forme la vigne tropicale, qui appartient à la famille des *Dioscoreaceae* et au genre des *Dioscorea*. L'igname est bien connue pour ses propriétés médicinales, ainsi que pour ses valeurs nutritionnelles. La propagation conventionnelle de l'igname est limitée par de faibles taux de propagation; par conséquent, la propagation *in vitro* offre la meilleure alternative pour surmonter ces limitations. L'objectif de cette étude était d'optimiser un protocole de micro-propagation *in vitro* de l'igname Ethiopienne (*D. alata*, variété bulcha). Des explants ont été

obtenus à partir de jeunes feuilles de la variété bulcha et stérilisés en utilisant différentes concentrations de NaOCl, pour des expositions temporelles différentes. L'hypochlorite de sodium (1%) à un temps d'exposition de 15 minutes a montré une survie de 100% des explants. La combinaison de 0,5 BAP avec  $1\text{mg l}^{-1}$  NAA s'est avérée être la meilleure concentration pour l'induction des pousses. Pour la multiplication des pousses, le BAP à  $1,5\text{ mg l}^{-1}$  a donné les pousses les plus élevées par explants ( $7,28 \pm 0,07$ ), avec une longueur de pousses de  $8,72 \pm 0,43\text{ cm}$ . Le milieu MS avec  $2,0\text{ mg l}^{-1}$  NAA et  $1,5\text{ mg l}^{-1}$  IBA a donné le pourcentage d'enracinement et le nombre de racines les plus élevés ( $10,6 \pm 0,44$ ), avec une longueur de racine de  $13,80 \pm 0,44$ . Les plantules acclimatées *in vitro*, qui ont été transférées dans une serre pour durcissement, avaient un taux de survie de 90% sur un milieu de sol. Ainsi, pour la micropropagation de la variété bulcha yam, MS +  $1,5\text{ mg l}^{-1}$  et MS plus  $2,0\text{ mg l}^{-1}$  NAA +  $1,5\text{ mg l}^{-1}$  IBA est la meilleure combinaison phytohormonale pour la multiplication des pousses et l'enracinement *in vitro*, respectivement.

*Mots Clés:* *Dioscorea*, Milieu MS, vigne nodale

## INTRODUCTION

Yam (*Dioscorea* spp.) is a monocotyledonous tuber, which belongs to the order *Liliflorae*, family *Dioscoreaceae*, and genus *Dioscorea* (Course, 1976). There are about 600 species of *Dioscorea*, among which white yam (*Dioscorea rotundata*), water yam (*Dioscorea alata*), aerial yam (*Dioscorea bulbifera*), yellow yam (*Dioscorea cayenensis*), trifoliate yam (*Dioscorea dumetorum*) and Chinese yam (*Dioscorea sculentata*) are economically the most important species (Onwueme, 1978). It is reported that Ethiopia is partly the centre of origin for one of *Dioscorea* species (*D. abyssinica*) (Zeven and Wet, 1982; Edossa, 1996).

Even though yam is an important contributor to food security and hunger reduction in developing countries, its production has been characterised by fluctuation since 2007 (Okwu *et al.*, 2006; FAO, 2012; Price *et al.*, 2016). This is mainly because propagation of this species of yam by seeds, using conventional methods, is slow and inadequate for rapid multiplication (Nweke *et al.*, 1991). Therefore, there is need to develop *in vitro* propagation methods for application in the production of plant materials (Vaillant *et al.*, 2005; Abraham, 2009). Arditti (2009) contended that *in vitro* propagation methods provide large scale proliferation of high quality planting materials. It offers many

advantages over conventional methods, like mass propagation; and produces pathogen free planting materials (Obssi *et al.*, 2015).

Studies have been reported on some economically important *Dioscorea* species and landraces using nodal segments explants, with limited auxin and cytokinin types, concentrations and combinations (Jova *et al.*, 2011; Das *et al.*, 2013; Obsi *et al.*, 2015; Sangar *et al.*, 2018; Hussein *et al.*, 2018). Moreover, each variety requires its own regeneration protocol (Gonzalez *et al.*, 1999). Therefore, the objective of this study was to develop an optimised protocol for *in vitro* micro-propagation of *Ethiopian* yam.

## MATERIALS AND METHODS

**Plant materials.** The material used in this study was the popular variety of yam (*Dioscorea alata*), variety bulcha, which was released by Bako Agricultural Research Centre in 2012. This variety shows superior performance, particularly in terms of productivity and tolerance to disease across the year; and out-yields (46.6 tonnes per hectare) other yam varieties on farmers' fields (Bikela *et al.*, 2015). Mature *Dioscorea* tubers were obtained from Hawasa Agricultural Research Centre, located in regional state of Southern Nations and Nationalities.

About 20-60 grammes per piece of a non-dormant tuber, containing periderm and some

cortex parenchyma, was used (Aighehi *et al.*, 2014). The pieces were grown in plastic pots filled with sterilised soil (Fig. 1) for two months, to be used as mother plant. The host Plant Tissue Culture Laboratory maintained an average temperature of  $25 \pm 2^\circ\text{C}$ . The tuber sets in pots were watered with distilled water, twice a week and allowed to grow for about two months when buds sprouted. Two months later, the apex was differentiated into stems of approximately 10-16 nodes. The plants were staked with sticks and one node size explants were cut from stems for sterilisation.

**Stock solution preparation.** Murashige and Skoog (MS) basal medium was used throughout this study. Initially, full strength stock solutions of macronutrients, micronutrients and vitamins plus other organic supplements were separately prepared (Murashige and Skoog, 1962). To do so, appropriate amounts of each nutrient were dissolved in double distilled water, consecutively in such a way that the next nutrient was added after the first one was completely dissolved. After all the components were completely dissolved using a magnetic stirrer, the solution was poured into plastic

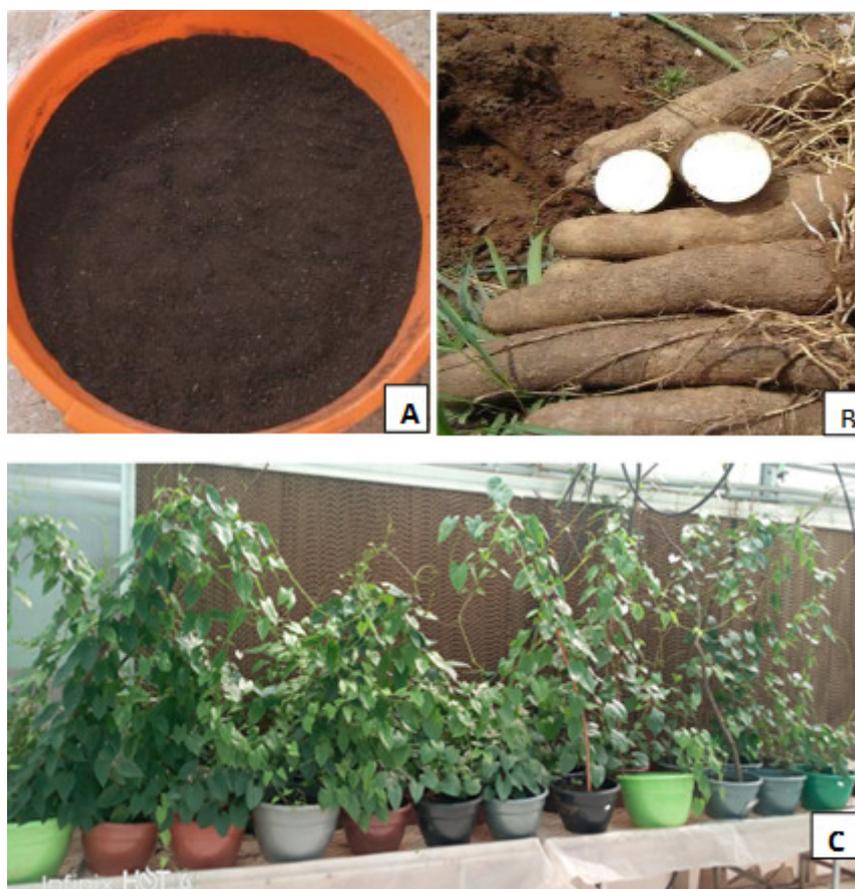


Figure 1. Plant material source and mother stock establishment in a greenhouse: (A), plastic pots already filled with sterilised soil; (B), tubers and fragments of tuber; (C), Mother Stock plant under greenhouse condition.

bottles and stored at +4 °C until it was used, for a maximum of four weeks.

**Plant growth regulators stock solution.**

Plant growth regulators, namely 6-Benzyl Amino-purine (BAP), Indol-Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA) were used in this study. All of the plant growth regulators stock solutions were prepared by dissolving the powder in 2-3 drops of NaOH based on the requirement of the plant growth regulators. Then, the volume was adjusted by adding double distilled water at a ratio of 1:1 (1 mg ml<sup>-1</sup>); and stirred using magnetic stirrer. Upon complete dissolution, the solution of each PGR was poured into labeled 50 ml plastic bottles and stored in a refrigerator at +4 °C for short term use.

**Culture medium preparation.** MS nutrient medium with its full macro- and micro-nutrients, vitamins and iron source was used to prepare the stock solution. Each component was added into one litre beaker and 30 g l<sup>-1</sup> of sucrose and 1 g l<sup>-1</sup> activated charcoal were dissolved in double distilled water in all cases. The shoot initiation medium containing different concentrations of BAP (0.25, 0.5, 0.75 and 1 mg l<sup>-1</sup>) and different concentrations of NAA (0.5, 1 and 1.5 mg l<sup>-1</sup>) in combination, were used. The shoot multiplication medium prepared using different concentrations of BAP (0.5, 1.0, 1.5, 2.0 and 2.5 mg l<sup>-1</sup>), was also used. On the other hand, the rooting media containing IBA (0.5, 1.0, 1.5 and 2 mg l<sup>-1</sup>) in combination with NAA (0.5, 1.0, 1.5, 2 and 2.5 mg l<sup>-1</sup>) was used in full MS medium.

After the appropriate amount of medium component was added and stirred using a magnetic stirrer, the volume was adjusted to full level using double distilled water. The pH of the medium was adjusted with HCl and NaOH solution, before 2.3 g l<sup>-1</sup> agar powder was added. Then, the gently mixed medium was boiled in a microwave oven (85 °C), until the agar was melted. Then, 30 ml of the prepared medium was dispensed into culture jars, which were covered with caps

immediately after dispensing the medium. They were then autoclaved at a temperature of 121 °C and pressure of 105 KPa for 15 minutes. After autoclaving, the medium was kept in the culture room until use.

**Experiments and their designs.** A total of four experiments were conducted (Effective sterilisation of explants using NaOCl, effects of BAP in combination with NAA for shoot induction, Effect of different concentrations of BAP on shoot multiplications and effect of NAA and IBA combinations on *in vitro* rooting). All experiments were laid in a completely randomised design (CRD), in factorial treatment combinations, five replications per treatment and three explants per jar under each replicate.

Before laying out the multiplication and rooting experiments, sufficient explants were multiplied, until the desired numbers of explants were obtained. Explants were cultured on a PGR-free medium, before their use to avoid carry-over effects from the previous culture media. Controls were set for each experiment with zero concentration of growth regulators.

**Explants preparation.** Nodal segments collected from actively growing shoots from 1-2 months old established mother plant in a greenhouse, were used as a sources of explants. Nodal vine cuttings of 6-10 cm were washed with a detergent solution (liquid soap) and rinsed several times with running tap water. Then, the nodal vine cuttings/explants were transferred to the laminar flow hood cabinet, and surface sterilised in 70% ethanol for 3 minutes. Thereafter, the treated explants were rinsed four times with sterilised distilled water and surface sterilised using 0.5, 1 and 1.5% of sodium hypo-chlorite (NaOCl) disinfectant solutions, plus a few drops of tween-20 (2-3 drops) for varying exposure times (5, 10 and 15 minutes).

The nodal cut vines were transferred into double distilled water and washed several times (at least 3 changes) till all traces of the sterilant

were washed off; and then soaked with sterile blotting paper. Sterilised nodal explants were further reduced to 1 cm size by removing all the dead and affected tissue prior to culture. All steps were performed in a laminar flow cabinet to maintain aseptic condition. Three explants were cultured per glass jar with five replicates.

#### **Initiation and culturing of explants.**

Aseptic nodal cultures were transferred onto MS media, fortified with different concentrations of BAP in combination with NAA for two weeks to determine best medium for shoot induction. The media used contained macro- and micro-nutrient salts according to Murashige and Skoog. In addition, NAA (0.5, 1 and 1.5 mg l<sup>-1</sup>) in 3 x 4 combination with BAP (0.25, 0.5, 0.75 and 1 mg l<sup>-1</sup>), were added to the MS medium with 1 g l<sup>-1</sup> activated charcoal (AC). The sterilised explants which were dissected and reduced into 1 cm single nodal segments were implanted onto the Murashige and Skoogs agar gelled medium. It was then sealed, labeled and kept inside the growth room with 16 hours photoperiod (8 hours dark) and 2500 lux light intensity from cool white florescent 60 watt bulbs, at average temperature of 25 ± 2 °C. The growth of the nodal cultures were observed daily. The subculture was done on the 15<sup>th</sup> days of culture. Data on explants showing shoot initiation were collected after two weeks of culture.

**BAP and shoot multiplication.** Bud initiation was enhanced by the addition of BAP and NAA. After bud initiation, for further growth, cultured plants were transferred to the media, supplemented with five concentrations of BAP (0.5, 1.0, 1.5, 2.0 and 2.5 mg l<sup>-1</sup>) to investigate its effect on shoot multiplication. The mean of shoot length, shoot number and leaf number were recorded after 30 days of culture.

**NAA and IBA combinations on *in vitro* rooting.** The effect of two auxin types and their concentrations on rooting of *in vitro*

shoots were studied using MS medium, supplemented with five concentrations of NAA (0.5, 1, 1.5, 2 and 2.5 mg l<sup>-1</sup>) and four concentration of IBA (0.5, 1, 1.5 and 2 mg l<sup>-1</sup>) in 5 x 4 combination was tested with 1 g l<sup>-1</sup> AC. Root number and root length were determined after 40 days of culture.

**Acclimatisation.** For acclimatisation, plantlets of about 50 mm tall and 4 to 5 leaves; and well-developed root systems were washed with tap water to remove adhering media and agar on the roots of plantlets. Rooted micro-propagates were transplanted into plastic pots filled with top soil, sand soil and compost in the ratio of 1:2:1.

The plastic pots were placed in a greenhouse where pot trays were covered with a white plastic bag to maintain uniform humidity. The plantlets were transferred to greenhouse for gradual acclimatisation until they established themselves in the soil. After 10 days, the transparent plastic bags were removed, and the data on survival were recorded.

**Data analysis.** Data collected were analysed using Analysis of Variance of SAS software version 9.3. The Least Significance Difference (LSD) was used for mean separation at P < 0.05.

## **RESULTS AND DISCUSSION**

**Effective sterilisation of explants using NaOCl.** Surface sterilisation with 1% of NaOCl for 15 minutes caused minimal contamination; while the same reagent caused maximum survival response *in vitro* cultures. The percentages of contamination and survival rates in cultures were recorded as, 0 and 100%, respectively. After 7 days of culture, 0% tissue death was recorded with unaffected growth rate, when explants were sterilised for 15 minutes with increased concentration of NaOCl (1.5%), high efficiency of sterilisation was achieved, but there were negative effects on survival rate, as well as on growth and development of explants. Sodium hypochlorite

treatment for 10 minutes at 1% was also an effective surface steriliser, with 86.66% survival rate and 60.00% of contamination (Table 1). The contamination was dramatically decreased as the exposure time increased within the same level of sodium hypochlorite solution (Table 1). Disinfection with 1.5% sodium hypochlorite solution for 15 minute also resulted in the least contamination (0%) and highest clean explants (100%), but no live tissues.

Comparing the interaction effect posed by the chemical and the duration of disinfection against the aim of sterilisation, disinfection of Bulch variety with 1% sodium hypochlorite solution, for 15 minutes was the most effective sterilisation treatment. This resulted in the highest survival (100%) without any contamination.

The increase of sodium hypochlorite concentration with long exposure time, showed a negative effect on plantlets, resulting in brownish colour with a lower survival rate (0%). The occurrence of high contamination rate of the culture at a relatively lower concentration and shorter exposure time treatment combinations was possibly due to

the insufficiency of sterilant concentration and exposure duration to remove or kill the contaminant agents mainly fungi and bacteria.

From the present study, 1% sodium hypochlorite in combination with about 2-3 drops of tween-20 for 15 minutes, is the best of all sterilisation regimes tested, with the lowest contamination and highest survival response of the variety. NaOCl is believed to be a very effective sterilising agent and is used frequently for surface sterilisation of plants for *in vitro* culture (Talei *et al.*, 2011). These results are confirmatory with earlier works (Oyebanji *et al.*, 2009), which reported sodium hypochlorite as a good sterilising agents for many others plants.

Other studies reported that ethanol and NaOCl were good sterilising agents for the plant-like peach, soft rush, oil palm, and other plants (Ogero *et al.*, 2012). Alam *et al.* (2016) further reported that the requirements for sterilisation are different and depend on the tissues and the type of the explants used for micropropagation. Many sterilisation procedures based on sodium hypochlorite solution and 70% ethanol rinse are usually considered good sterilising procedures for the

TABLE 1. Interaction effect of sodium hypochlorite concentrations and time of exposure on sterilisation of *Dioscorea* species (*Dioscorea alata*)

Sodium hypochlorite concentration (%)	Time of exposure (minute)	Contamination (%)	Clean culture (%)	Tissue survival rate (%)
0.5	5	100 <sup>a</sup>	0 <sup>h</sup>	0 <sup>h</sup>
0.5	10	93.33 <sup>b</sup>	6.67 <sup>g</sup>	13.33 <sup>g</sup>
0.5	15	86.66 <sup>c</sup>	13.34 <sup>f</sup>	26.66 <sup>f</sup>
1	5	80 <sup>d</sup>	20 <sup>e</sup>	53.33 <sup>c</sup>
1	10	60 <sup>e</sup>	40 <sup>d</sup>	86.66 <sup>b</sup>
1	15	0 <sup>h</sup>	100 <sup>a</sup>	100 <sup>a</sup>
1.5	5	13.33 <sup>f</sup>	86.67 <sup>c</sup>	46.66 <sup>d</sup>
1.5	10	6.66 <sup>g</sup>	93.34 <sup>b</sup>	33.33 <sup>e</sup>
1.5	15	0 <sup>h</sup>	100 <sup>a</sup>	0 <sup>h</sup>
CV		7.02	6.03	5.89

Means with the same letter in the same column are not significantly different, CV = Coefficient of Variation

*in vitro* culture of many plants (Aasim *et al.*, 2013).

**Shoot initiation.** There was a highly significant interaction effect ( $P < 0.0001$ ) of BAP and NAA (BAP\*NAA) on rate of shoot induction (Table 2). The basal medium (control) failed to stimulate bud break response in the cultured explants, even when the cultures were maintained further than the normal observation period. BAP at 0.5 mg l<sup>-1</sup> combined with 1mg l<sup>-1</sup> NAA resulted in the highest response in which an average of 100 % ex plant response with a mean shoot length of 4.42±0.02 cm was obtained (Table 2, Fig. 2).

The second best shoot induction of 86% was obtained in the MS medium supplemented with 0.25 mg l<sup>-1</sup> BAP, combined with 1 mg l<sup>-1</sup> NAA; with a mean shoot length of 3.55±0.04 cm (Table 2, Fig. 2). Among all the treatments, the minimum rate of shoot induction (20%)

was observed on MS containing 1 mg l<sup>-1</sup> BAP, combined with 1.5 mg l<sup>-1</sup> NAA. In this case, the percent of shoot induction was significantly lower than that of the first two combinations of plant growth regulators.

Shoot bud development capacity of nodal cultures increased with increase in concentrations of both NAA and BAP from zero to 1 and 0.5 mg l<sup>-1</sup>, respectively; and reduced with further addition of both hormones (Table 2). This result conforms to the report of Fotso *et al.* (2013) who considered 0.5 mg l<sup>-1</sup> of BAP together with 1.0 mg l<sup>-1</sup> of NAA as the best-supplemented medium for nodal cuttings derived shoot regeneration. A similar result has also been recently reported in the case of micropropagation of other *Dioscorea* species like *Dioscorea composita* (Alizadeh *et al.*, 1998), *Dioscorea floribunda* (Borthakur and Singh, 2002), *Dioscorea batatas* (Koda and Kikuta, 1991) and *Dioscorea abyssinica* (Mar-tine and

TABLE 2. Effects of BAP and NAA on *in vitro* shoot induction rate of *Dioscorea* species (*Dioscorea alata*), from nodal explants culture on MS

Plant growth regulators (mg l <sup>-1</sup> )		Shoot induction (%)	Shoot length(cm)
BAP (mg l <sup>-1</sup> )	NAA (mg l <sup>-1</sup> )		
0	0	0.00 <sup>l</sup>	0.00±0.34 <sup>j</sup>
0.25	0.5	26.66 <sup>j</sup>	0.97±0.01 <sup>hi</sup>
0.25	1	86 <sup>b</sup>	3.55±0.04 <sup>b</sup>
0.25	1.5	53 <sup>f</sup>	1.46±0.03 <sup>c</sup>
0.5	0.5	59.99 <sup>e</sup>	1.74±0.01 <sup>d</sup>
0.5	1	100 <sup>a</sup>	4.42±0.02 <sup>a</sup>
0.5	1.5	32.66 <sup>i</sup>	1.12±0.19 <sup>gh</sup>
0.75	0.5	46 <sup>g</sup>	1.36±0.40 <sup>ef</sup>
0.75	1	73 <sup>c</sup>	2.91±0.02 <sup>c</sup>
0.75	1.5	40 <sup>h</sup>	1.27±0.00 <sup>fg</sup>
1	0.5	33.33 <sup>i</sup>	1.15±0.02 <sup>g</sup>
1	1	66.66 <sup>d</sup>	1.84±0.03 <sup>d</sup>
1	1.5	20 <sup>k</sup>	0.9±0.06 <sup>i</sup>
CV		7.29	6.94

BAP = Benzyl Amino Purine. NAA = Naphthalene Acetic Acid, Means with the same letter in the same column are not significantly different, CV = Coefficient of variation

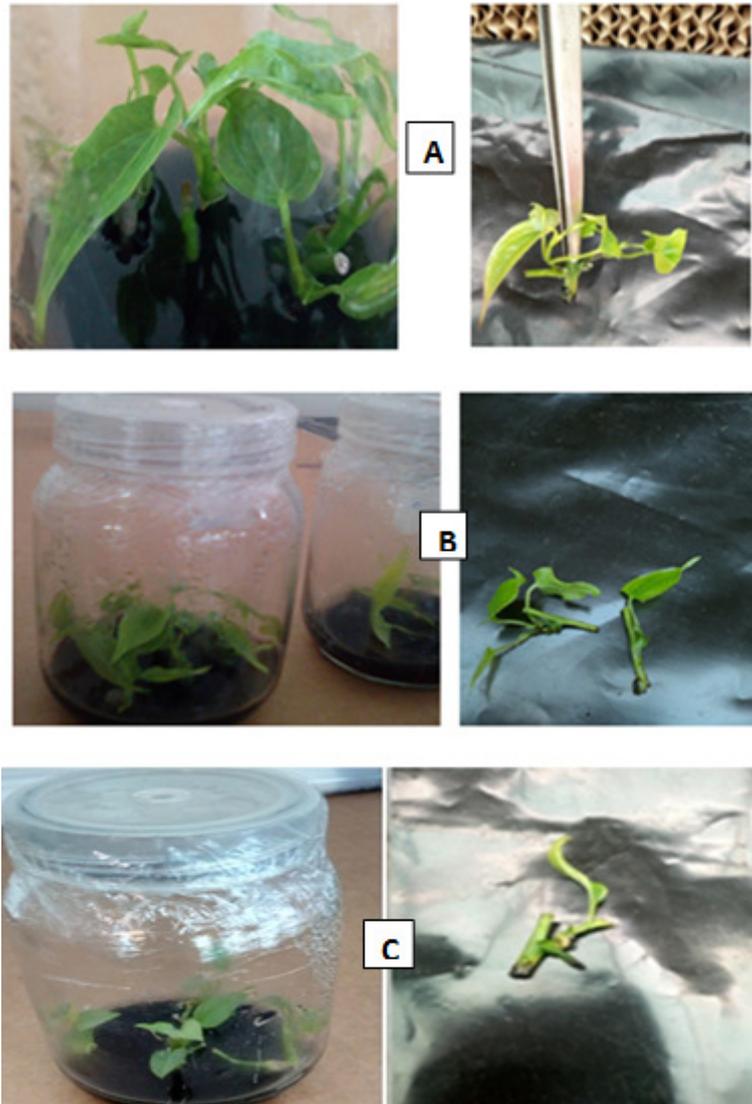


Figure 2. Response of Nodal explants of *Dioscorea alata* on initiation medium with different combinations of BAP and NAA. MS medium with 0.5 mg l<sup>-1</sup> BAP and 1 mg l<sup>-1</sup> NAA (A), MS medium with 0.25 mg l<sup>-1</sup> BAP and 1 mg l<sup>-1</sup> NAA (B), MS medium with 1 mg l<sup>-1</sup> BAP and 1.5 mg l<sup>-1</sup> NAA (C).

Cappadocia, 1991). Therefore, BAP (a cytokinin) with NAA (auxin) were good hormone combination for shoot initiation of Ethiopian yam, Bulcha variety.

In the present study, cultured nodal explants of *Dioscorea* spp. in solid MS medium started to develop micro-leaves after two weeks of culturing, with varying growth responses for the initiation medium, cultured nodal segments to all of the culture media compositions; except

the control (without plant growth regulators) (Table 2). The combination of 0.5 mg l<sup>-1</sup> BAP and 1 mg l<sup>-1</sup> NAA produced the highest shoot bud induction. George *et al.* (2008) similarly reported that, for shoot initiation cytokinins act best when combined with auxins.

**BAP and shoot multiplications.** The effect of different concentrations of BAP on shoot multiplication of Ethiopian yam is shown in

Table 3. Increasing BAP from 0.5 to 1.00 mg l<sup>-1</sup> presented a significant increase from 4.54±0.2 to 6.3±0.45 shoots per explant, from 4.06±0.26 to 6.12±0.52 cm shoot length and from 3.38±0.18 to 5.01±0.35 leaves per shoot (Table 3). MS media, supplemented with BAP (1.5 mg l<sup>-1</sup>), induced maximum number of shoots per explants (7.28±0.07) with highest shoot length of 8.72±0.43 cm and number of leaf (Table 3, Fig. 3). MS media with 1 mg l<sup>-1</sup> BAP gave the second highest shoot number (6.3±0.45) with shoot length 6.12±0.52 cm and highest leaf number 5.01±0.35.

The present results were in line with the finding of several other reports (Poornima and Ravishankar, 2007; Chu *et al.*, 2002, Mahesh *et al.* 2010) whereby the nature and amount of the cytokinin significantly determined growth of various genotypes of *Dioscorea* spp. Manoharan *et al.* (2016), on the other hand obtained MS media with 0.4 mg l<sup>-1</sup> BAP as the optimum concentration for shoot proliferation. According to Gopitha *et al.* (2010), low concentrations of cytokinin promote shoot multiplication and elongation. The difference might be due to the difference in type of media and yam variety.

The minimum shoot number (2.12±0.08) with minimum shoot length (1.97±0.12), and minimum leaf number (2.51±0.35) were

recorded on MS media with 2.5 mg l<sup>-1</sup> BAP (maximum concentration) (Table 3, Fig. 3). Increasing BAP above 1.5 mg l<sup>-1</sup>, resulted in shoots with stunted features, which were generally unusable. These effects were attributed to high dosage of hormone that disorderd the metabolism of the shoots and inhibited cell division and multiplication (Gopitha *et al.*, 2010). This is agreement with the work of Hussein *et al.* (2018) where increasing or decreasing of the BAP concentration from 1.50 mg l<sup>-1</sup> led to decline in the growth parameters like leaf number, shoot height and shoot number.

**NAA and IBA combinations on *in vitro* rooting.** The rooting responses of shoots on different media showed high significant differences (P<0.0001) due to the interaction of the phytohormones applied and for number of roots and root length over a period of four weeks (Fig. 4, Table 4). Rooting was better in the culture which had a combination of 2 mg l<sup>-1</sup> NAA with 1.5 mg l<sup>-1</sup> IBA; where 10.6±0.44 root number with an average number of 13.80±0.44 cm root length per plantlets were recorded (Fig. 4). The second highest performer in rooting was the combination of 1 mg l<sup>-1</sup> of IBA and 2 mg l<sup>-1</sup> of NAA; where 8.67±1.13 number of roots and shoots

TABLE 3. Shoot multiplication in nodal explants of *Dioscorea species* (*Dioscorea alata*) cultured on semisolid MS medium supplemented with various concentration of BAP

BAP (mg l <sup>-1</sup> )	Number of leaves	Number of shoot	Shoot height (cm)
0	2.09±0.09 <sup>f</sup>	3.05±0.37 <sup>e</sup>	2.53±0.14 <sup>e</sup>
0.5	3.38±0.18 <sup>d</sup>	4.54±0.28 <sup>c</sup>	4.06±0.26 <sup>c</sup>
1	5.01±0.35 <sup>b</sup>	6.3±0.45 <sup>b</sup>	6.12±0.52 <sup>b</sup>
1.5	7.12±0.22 <sup>a</sup>	7.28±0.07 <sup>a</sup>	8.72±0.43 <sup>a</sup>
2	4.40±0.19 <sup>c</sup>	3.64±0.25 <sup>d</sup>	3.44±0.44 <sup>d</sup>
2.5	2.51±0.35 <sup>e</sup>	2.12±0.08 <sup>f</sup>	1.97±0.12 <sup>f</sup>
CV	5.90	6.65	7.80

BAP = Benzyl Amino Purine. Means with the same letter in the same column are not significantly different, CV = Coefficient of variation

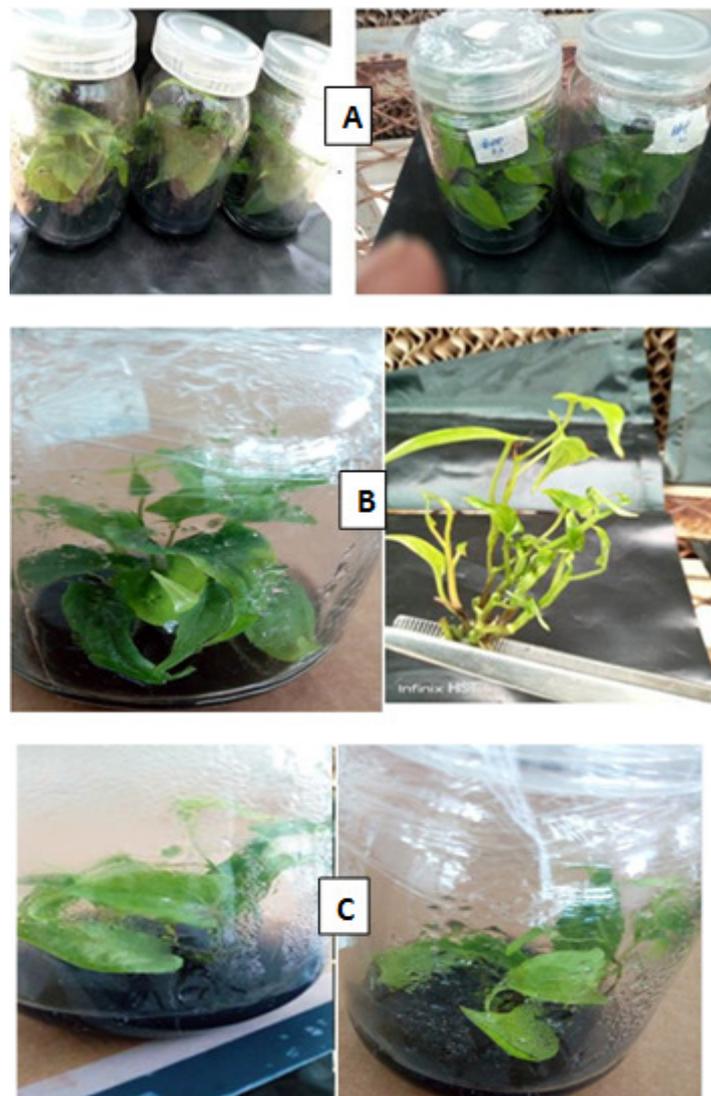


Figure 3. Effect of BAP on shoot multiplication, MS medium with 1.5 mg l<sup>-1</sup>BAP (A), MS medium with 1 mg<sup>-1</sup> BAP (B), MS medium with 2.5 mg<sup>-1</sup> BAP (C).

responded with  $11.50 \pm 0.21$  cm average root length per plantlet. However, increasing the concentrations of IBA and NAA from (1.5 and 2 mg l<sup>-1</sup>, respectively) decreased the percentage of rooted shoots consistently; but required only short days for root initiation.

Rooting at lower levels of NAA and IBA (0.5 mg l<sup>-1</sup>) showed semi-elongated and minimum root number, with minimum root length with poor root growth and with dwarf growth of shoots in the cultured shoots during

the four weeks of observation period. Shoot planted on auxin free medium developed very small amounts of roots ( $1.00 \pm 0.01$ ) with minimum average root length ( $1.00 \pm 0.029$  cm (Fig. 4). The well rooted plantlets were successfully acclimatised and cultivated in green house. About 90% of plantlets were survived and established as health plant (Fig. 5).

The production of plantlets with abundant rooting *in vitro* is very important for the

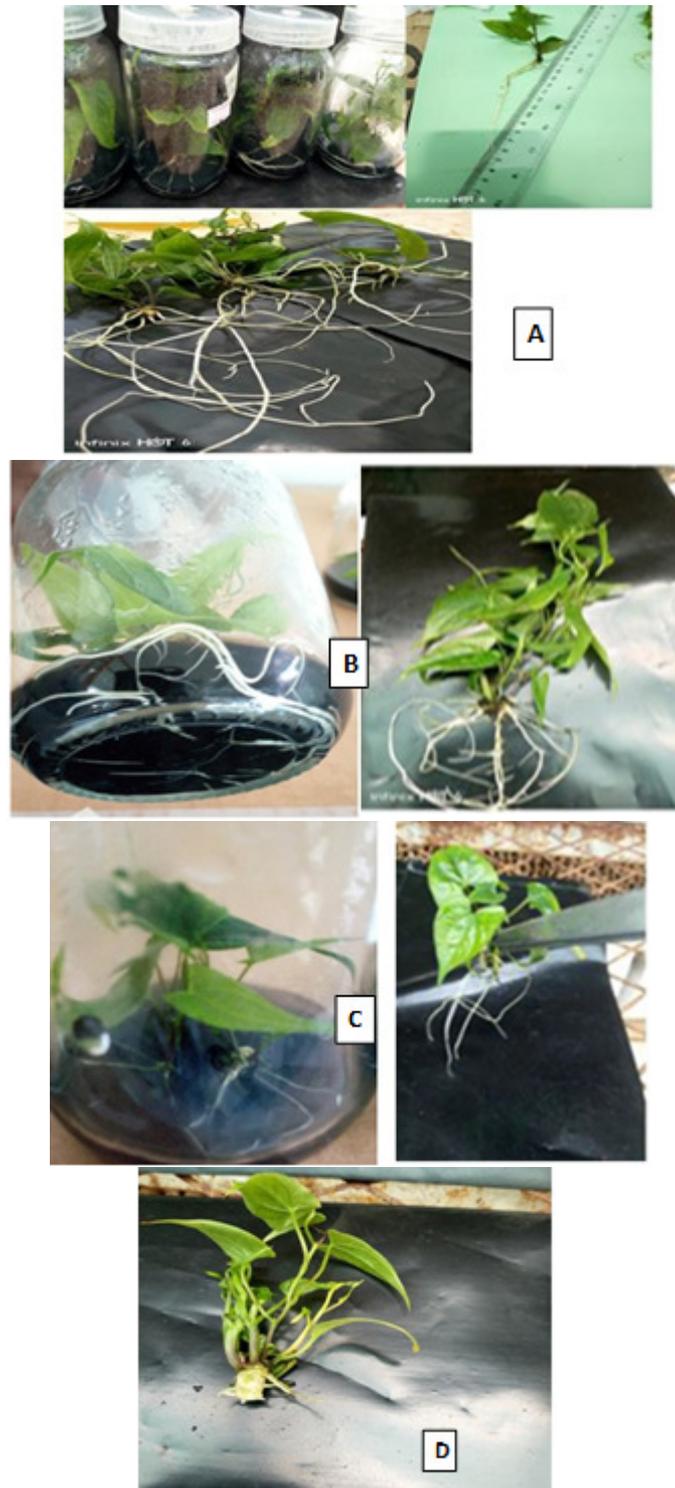


Figure 4. *In vitro* rooting of *Dioscorea alata* shoots on full strength MS medium containing combination of NAA and IBA. Root induction on 2 mg l<sup>-1</sup> NAA with 1.5 mg l<sup>-1</sup> IBA (A), Root induction on 2 mg l<sup>-1</sup> NAA with 1 mg l<sup>-1</sup> IBA (B), Root induction on 0.5 mg l<sup>-1</sup> NAA w.

TABLE 4. Influence of different combination of NAA and IBA on rooting of *in vitro* generated shoot lets of *Dioscorea* species (*Dioscorea alata*)

NAA (mg l <sup>-1</sup> )	IBA (mg l <sup>-1</sup> )	Root height (cm)	Root number
0	0	1.00±0.01 <sup>o</sup>	1.00±0.29 <sup>p</sup>
0.5	0.5	1.04±0.21 <sup>o</sup>	1.4±0.31 <sup>op</sup>
0.5	1	2.70±0.68 <sup>km</sup>	3.24±0.43 <sup>m</sup>
0.5	1.5	3.56±0.38 <sup>l</sup>	4±0.22 <sup>kl</sup>
0.5	2	3.22±0.40 <sup>lm</sup>	3.64±0.03 <sup>lm</sup>
1	0.5	4.90±0.54 <sup>ij</sup>	4.6±0.06 <sup>j</sup>
1	1	5.38±0.05 <sup>hi</sup>	4.76±0.06 <sup>ij</sup>
1	1.5	7.49±0.34 <sup>e</sup>	6.94±0.22 <sup>d</sup>
1	2	6.50±0.18 <sup>f</sup>	5.52±1.7 <sup>gh</sup>
1.5	0.5	6.95±0.03 <sup>f</sup>	6.7±0.22 <sup>de</sup>
1.5	1	8.07±0.74 <sup>d</sup>	7.68±0.23 <sup>c</sup>
1.5	1.5	9.11±0.11 <sup>c</sup>	8.19±0.54 <sup>cb</sup>
1.5	2	6.95±0.02 <sup>f</sup>	6.2±0.23 <sup>ef</sup>
2	0.5	6.50±0.15 <sup>f</sup>	5.94±0.04 <sup>fg</sup>
2	1	11.50±0.21 <sup>b</sup>	8.67±1.13 <sup>b</sup>
2	1.5	13.80±0.44 <sup>a</sup>	10.6±0.44 <sup>a</sup>
2	2	5.94±0.04 <sup>g</sup>	5.26±0.32 <sup>hi</sup>
2.5	0.5	4.50±0.41 <sup>j</sup>	4.26±0.18 <sup>jk</sup>
2.5	1	3.04±0.69 <sup>lm</sup>	3.39±0.23 <sup>m</sup>
2.5	1.5	1.94±0.30 <sup>n</sup>	2.6±0.35 <sup>n</sup>
2.5	2	1.46±0.10 <sup>n</sup>	1.6±0.54 <sup>o</sup>
CV		9.19	7.60

IBA = Indol-3-Butyric Acid, NAA= Naphthalene Acetic Acid. Means with the same letter in the same column are not significantly different, CV = Coefficient of Variation

successful establishment of regenerated plantlets in soil (Kumar and Nair, 1979). Behera *et al.* (2008) used 2 mg l<sup>-1</sup> NAA and 2 mg l<sup>-1</sup> IBA in half-strength MS medium for different species of *Dioscorea* and induced the highest rooting. Earlier authors also obtained similar findings that combinations of auxin NAA and IBA were needed for better production of roots in *Dioscorea prazeri* (Thankappan and Patell, 2011), *Dioscorea nipponica* (Chen *et al.*, 2007), *Dioscorea esculenta* (Belarmino and Rosario, 1991), (Obssi *et al.*, 2015), *Dioscorea rotundata* (Taha and Abdelaziz, 2017) and yam landreses (Hussein *et al.*, 2018). But Bahar *et al.* (2010) suggested that NAA or IBA alone

used to profuse rooting on *Dioscorea oppositifolia* and reported that NAA was more effective than IBA. Additionally, these results could be explained on the basis that auxin-induced responses which involved cell division, cell enlargement, protein and nucleic acids synthesis that increase the apical dominance, growth and elongation (Wilkins, 1989). On the other hand, Forsyth and Stadan (1981) reported that rooting relies on MS media with 5 mg l<sup>-1</sup> IBA and a results of Mao *et al.* (1995) showed that higher concentrations of NAA, IBA or IAA induced root growth rather than the lower concentrations.

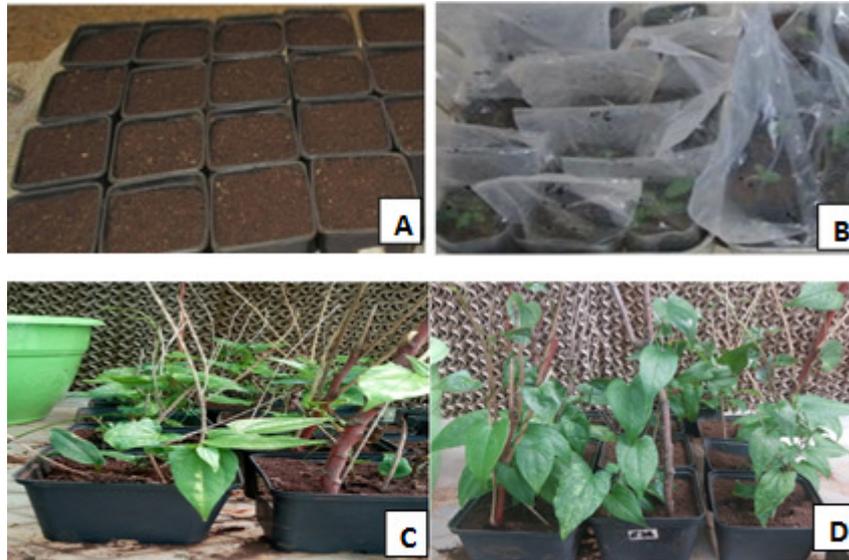


Figure 5. Acclimatisation in greenhouse: (A) plastic pots filled with sterilised soil, (B) first week of acclimatisation (as covered), (C) 15 days after covering plastic bag has been removed, (D) 30 days after covering plastic bag has been removed.

## CONCLUSION

The maximum of shoot induction (100%) was observed on MS medium supplemented with 0.5 mg l<sup>-1</sup> of BAP and 1 mg l<sup>-1</sup> of NAA. MS medium containing 1.5 mg l<sup>-1</sup> of BAP also produced the highest shoot number. Best rooting percentage was achieved on full strength MS basal media containing 1.5 mg l<sup>-1</sup> of IBA in combination with 2.0 mg l<sup>-1</sup> of NAA followed by MS basal media containing 2.0 mg l<sup>-1</sup> of NAA and 1 mg l<sup>-1</sup> IBA which resulted 8.67±0.13 root number, and 11.50±0.21 cm root length.

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