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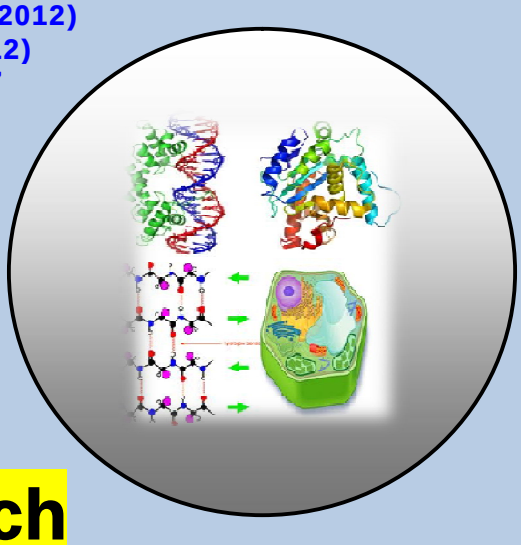

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Micropropagation of *Dioscorea alata* L. (Yam) from Shoot-Tip and Nodal Explants

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ABSTRACT

Dioscorea alata L. (Yam) is a member of genus Dioscorea and monocotyledonous plant belonging to the Dioscoreaceae family which comprises about 600 species. D. alata is an important crop plant that produces tuberous roots used as a source of food and medicine. Inefficiency of traditional methods of propagation and lack of planting materials are the main constraints for implementing large-scale cultivation. In this study, a micropropagation protocol for D. alata has been developed from shoot tip and nodal explants. Explants were sterilized using different concentrations of NaOCl for different time exposure. MS culture media supplemented with different types and concentration of auxins and cytokinins were used for culture initiation, shoot multiplication and root induction. Sodium hypochlorite (NaOCl) at a concentration of 3 and 2% at exposure time of 5 and 20 minutes gave the highest percentages (76.60±0.36%, 72.66±0.85%) of survived explants for nodal and for shoot tip, respectively. BAP at 1.5 mg/L + NAA 1.0 mg/l (93.2%) for nodal and BAP 1.0 mg/l + NAA 1.0 mg/l (87.1%) for shoot tip was found to be an optimum concentration for shoot induction. The combination of 2.0mg/L KIN, 1.0mg/l BAP and with 0.5mg/l NAA was found to be the optimum concentration yielding 8.4 and 9.5 shoots per explants for shoot tip and nodal respectively for shoot multiplication. Half strength MS medium with 2.0 mg/l IBA gave the highest rooting percentage and with 2.0mg/l NAA gave optimum root number and length. Up on acclimatization and transplanting, 90% survival efficiency was observed on soil mix ratio of 2:2:1 decomposed coffee husk, sand and red soil respectively. There were no observable variations with respect to morphology and growth characteristics to the greenhouse raised parent plant. This developed micropropagation protocol permits the development of mass propagation under field conditions for large scale commercial production, and also provide a possible system towards genetic improvement of the crop.

Key words: Dioscorea alata L., Shoot-Tip, In Vitro Propagation, MS Medium and Plant Growth Regulators.

INTRODUCTION

Dioscorea alata (water yam or greater yam) belongs to the Dioscoreaceae family, consists of about 600 species. The species are being widely distributed in Madagascar, Central America, Indo-Malaysia, Micronesia and Europe (Coursey, 1967; Ayensu, 1972). Greater yam is one of the most important and essential staple food crop for people in almost all tropical and subtropical countries (Han *et al*., 2000). Yam ranks at nine positions among staple vegetable crops with about 58,754,533 tons produced globally and 56,500,776 tones with an average yield of 11,766.8 kg /ha from African countries. According to recent statistics (FAO, 2012), large quantities of Yam are grown in West African countries. Nigeria is the leading country from Africa and Ethiopia is the center of yam cultivation in East Africa (Norman et al., 1995), and the crop plays a vital role in local livelihood particularly in the densely populated areas of southern, southwestern, and western parts of the country (Hildebrand et al., 2002; Tamiru, 2006). In Ethiopia 350,000 tones are produced annually from about 41,338 hectares with an average yield of 8,466.8 Kg/Ha (FAO, 2012). The conventional multiplication of *Dioscorea* species is by tuber seeds, a tuber fragment that grows and develops into a new tuber. The absence of viable seeds, the long period required for obtaining usable tubers and phytosanitary problems are some of the factors that limit the rapid conventional propagation and economic exploitation of *Dioscorea* species (Tschannen et al., 2005; Balogum et al., 2006). In traditional systems of producing yams (*Dioscorea* spp.), farmers often encounter shortages of yam planting material, especially following cyclones, droughts, and disease epidemics (Jill, 1989). Tuber yield is drastically reduced by viral and fungal infections through infected tubers transmitted to the next generation (Saleil, 1990; Ng, 1992) deteriorating the quality of the tuber (Mitchell and Ahmed, 1999). Therefore, there is need to develop plant tissue culture techniques for its application in further genetic improvement of the species and in the production of available and disease free plant materials under controlled conditions in small spaces with reduced labor requirement (Abraham, 2009). Moreover, each variety requires its own regeneration protocol (Gonzalez et al., 1999). *In vitro* propagation of *Dioscorea* species has been developed using different explants such as immature leaves (Kohmura et al., 1995), zygotic embryos (Viana and Mantell, 1989), nodal cuttings (Alizadeh et al., 1998; Yongqin et al., 2003), bulbs (Asokam et al., 1983), roots (Twyford and Mantell, 1996), cells and protoplasts (Tor et al., 1998). However, few studies have been reported on the micropropagation of *D. alata* using nodal segments and root explants with limited auxin and cytokinin types, concentrations and combinations (Twyford and Mantell, 1996; Wheatley et al., 2003; Borges et al., 2004; Jova et al.,2011; Das et al., 2013). Therefore, the present investigation was made on *in vitro* propagation *D. alata* using different concentrations, types and combinations of auxin and cytokinin in order to increase the rapid production of disease free and excess plant materials.

MATERIAL AND METHODS

Plant material

Healthy tubers of Yam were collected from Awassa Agricultural Research Center (located in South Nation and Nationalities People's Regional State, Ethiopia), planted and grown in a

flowering pot contained a sterilized soil mixture of coffee husk, sand and nursery red soil 2:2:1 respectively) under greenhouse condition at average temperature of 25^0c $\pm$ 2^0C.Very young, healthy and vigorous sprouts were used as a source of explants 2 months after cultivation. The tissue culture experiments were conducted under the laboratory of plant tissue culture, Department of Biology, College of Natural Science, Jimma University (JU)**.**

Figure 1. Mother Stockplant under greenhouse condition.

Culture medium preparation

MS powder (Murashige and Skoog medium, 1962) 4405.19 mg was weighed for one litter, sucrose and growth regulators were added to the double distilled water kept in a vessel. The final volume was adjusted in a graduated cylinder/beaker by adding double distilled water. The pH of the solution was adjusted to 5.7-5.8 using 0.1 N HCl or 1N KOH. For solidification of the medium, agar powder (Tissue culture grade; agar-agar type) 0.8% (w/v) was added to the luke warm solution and then, melted for proper dissolving. Then the medium was poured in glass vessels borosilicate test tubes. After plugging, the test tubes were covered with aluminum foil and autoclaved at 121^0C for 20 minutes in a vertical autoclave.

Growth regulators stock preparation

All the plant growth regulators were prepared by weighing 100 mg powder and dissolved in 3-5ml 1N NaOH and 1N HCl for auxins and cytokinins, respectively. Then, the final volume was adjusted to 100 ml stock by adding double distilled water. Each milliliter solution contains one milligram PGR. All the stock solutions were stored in refrigerator at $+4^o$C temperature to be used for a maximum of one month period.

Plant material collection and sterilization

Shoot tip and nodal segment explants (1.5-2.0cm) were collected from healthy and vigorously growing tuber sprout. The excised explants were washed three times with tap water and transferred to the laminar flow hood cabinet and surface sterilized in 70% (v/v) Ethanol for 30 seconds, then the treated explants were rinsed repeatedly four times with sterilized distilled water and surface sterilized further using NaOCl disinfectant solutions for the specified time, as per the treatment combinations. The sterilized shoot and nodal explants were further cut down to 10 to 15 mm size by removing all the dead and chlorine affected tissue prior to culture.

Culture initiation

Shoot tip and nodal segment explants was inoculated in magenta jar or large test tubes containing full MS (Murashige and Skoog, 1962) media supplemented with BAP (0.5, 1.0, 1.5 and 2.0, mg/L) and Kinetin (1, 1.5, 2.0, and 2.5 gm/L), in combination at each concentration, 3% sucrose (30 gm/L), and Agar-agar (8 gm/L) One explants per jar was cultured with 5 replicates per treatment.

The jars were incubated in the light condition and data on the percentage of initiated shoots per explants was scored after 3 months of culture. Initiations of the explants were stayed for four months and tissue browning regeneration was observed due to phenol compounds or secondary metabolites.

Shoot multiplication

All healthy initiated shoots was transferred to shoot multiplication MS fresh media supplemented with a treatment combination of three different concentrations of Kinetin (1, 2.0 and 3.0 mg/l) and three different concentrations of BAP (0.5, 1.0, and 1.5mg/l) alone and or in combination with 0.5 mg/l NAA at each concentration were used and four replicates with best proliferated shoots each was cultured in each medium. The cultures were maintained at 25 ± 2^0C with a 16 hour photoperiod at a light intensity of 2500-3000 lux from cool white florescent 60 watt bulbs. After 2 months of culture on shoot multiplication media, the number of shoots was recorded. Therefore, the experiment was 3x3x2 factorial combinations in CRD and treatment with free PGRs.

Root formation

The regenerated shoots was cultured on half-strength MS media supplemented with NAA (0, 1, 1.5, 2.0 and 2.5 mg/l) and IBA (1, 1.5, 2.0 and 2.5 mg/l) for root induction. Well developed microshoots with minimum length of 1.10 cm obtained from experiment three were transferred to experiment four for rooting. For rooting experiment, agar solidified (0.8% agar-agar) half strength MS basal medium added with 3% sucrose was supplemented with different concentrations of IBA and NAA.

Acclimatization

Rooted plantlets were removed from the medium. The roots were carefully washed with running tap water and warm water. The plantlets were then planted into pots (4.5 cm x 3.5 cm) filled with a mixture of coffee husk, sandy and red soil in different proportions and acclimatized for 3 months under natural diffuse sunlight and 70% humidity in a glasshouse. They were then treated in light polyethylene tunnel covered by 70 % shade net above it and without shade net. The system was designed to give high humidity (80 - 90 %) to prevent desiccation for ten days, prior to their transfer to a shade house. In the tunnel, the water was sprayed daily without creating water logging and meant to maintain relative humidity (RH) as high as possible.

Experimental design and Treatments

All experiments were laid in a Completely Randomized Design (CRD) with factorial treatment combinations, having three replications per treatment and five explants per jar under each replicate. All the experiments were repeated two times to ensure reproducibility of the results and the average of these two were considered for analysis. Prior to laying the multiplication and rooting experiments, sufficient explants were made to multiply till the desired numbers of explants are gained.

At all times, explants were cultured on a PGR-free medium prior to their use for an experiment; so as to avoid any sort of carryover effects from the previous culture medium they were retained. Controls were set for each experiment with zero concentration of the analyte considered.

Data recording

After sterilization experiment, the number and percentage of explants affected by contamination and tissue death was recorded during the first two weeks of culture for shoot tip and nodal explants independently. For the second experiment, the number and/or percentage of explants forming shoot buds was recorded after four weeks for shoot tip and nodal explants independently. Number of shoots proliferated from each shoot bud on multiplication media was counted at three weeks interval during sub culturing. The number of roots (including the main roots and their branches), shoot length and the length of the roots was recorded after three weeks of culture.

Data Analysis

Average of the data collected from the two repetitions for each experiment were independently subjected to statistical analysis using the SAS statistical software (version 9.2) and ANOVA was constructed, followed by mean separation using appropriate procedures (REGWQ). When ANOVA indicated significant treatment effects (5%, 1% or 0.1%) based on the F-test, probability level of 0.05 ($p \leq 0.05$) was considered to determine which treatments were statistically different from the other treatments..

RESULTS AND DISCUSSION

Effects of NaOCl concentrations and exposure time on sterilization of *D. alata* explants

The effect of different concentrations of sodium hypochlorite solutions and different time durations of explants exposure to the sterilants were evaluated for determining the most effective treatment combination on sterilization of shoot tip and nodal explants.

The analysis of variance showed that the concentration of sodium hypochlorite solution, time duration of explants exposure to the sterilants and interaction of concentrations to time duration had highly significant effect ($p < 0.0001$) on both of contamination and tissue death of shoot tips and nodal explants. The highly significant difference was also revealed between the two types of explants (treatment:-* explants = $p < 0.0001$) indicating that the level of contamination and tissue death was influenced by explants type and the mean average value for contamination and tissue death of shoot tips exceeded that of node explants. The highest rate of contaminant free culture (76.60±0.36% and 72.66±0.85%) was obtained from treatment combinations of three and two percent concentration of NaOCl and five and 20 min exposure duration, respectively for shoot tip explants. For nodal explants 3% concentration and five minute exposure duration were found to be the most effective treatment combination with mean average result of 72.00±0.20% contaminant free lively cultures (Table 1). Increasing the sterilant concentration from 1.0 to 3.0% active chlorine maintaining five minute exposure duration constant had reduced the rate of contamination from 88.00 ± 1.00% to zero and from 90.00 ± 0.50% to zero for shoot tips and nodal explants, respectively.

This effect was mainly attributed to the high level of tissue death i.e., 77.40 ± 0.45% for shoot tips and 70.00 ± 1.00% for nodal explants caused by the maximum concentration of the sterilant solution. Exposure duration of explants to the sterilant chemical also had significantly affected the effectiveness of the chemical in that it increased in time from five to 10 minute at two percent constant concentration had decreased the percentage of clean lively culture from 36.50 ± 0.50 to 22.60 ± 0.60 and from 50.00 ± 0.30 to 30.00 ± 0.50 for shoot tip and nodal culture, respectively increasing the rate of tissue death (Table 1). The rate of tissue death severely affected shoot tip explants than nodal explants as shoot tip tissues are relatively young and more susceptible to the chemical action.

Table1. Interaction effect of Sodium hypochlorite concentrations and its time of exposure on sterilization of Shoot tip and Nodal explants of *D. alata.*

Con. (%)	Time (min)	% of Contamination.		% of Clean culture		% of Tissue Death	
		Shoot tip (Mean±Std)	Nodal (Mean±Std)	Shoot tip (Mean±Std)	Nodal (Mean±Std)	Shoot tip (Mean±Std)	Nodal (Mean±Std)
0	0	100.00±0.00^{a}	100.00±0.00^{a}	0.00±0.00^{k}	0.00±0.00^{o}	0.00±0.00^{k}	0.00±0.00^{k}
1	5	88.00±1.00^{b}	90.00±0.50^{b}	12.00±0.50^{j}	10.00±0.20^{n}	0.00±0.00^{k}	0.00±0.00^{k}
1	10	84.60±0.28^{c}	87.00±0.50^{c}	13.13±0.80^{j}	11.50±0.50^{m}	0.66±0.04^{j}	1.50±0.10^{j}
1	15	81.66±0.28^{d}	84.00±0.50^{d}	15.90±0.10^{i}	13.00±0.34^{m}	0.83±0.07^{j}	3.00±0.20^{i}
1	20	78.40±0.52^{e}	81.00±0.50^{e}	18.00±0.50^{h}	15.00±0.20^{l}	1.20±0.15^{i}	4.00±0.20^{i}
1.5	5	73.33±0.06^{f}	78.00±0.50^{f}	21.70±0.88^{g}	16.50±0.50^{k}	1.66±0.15^{i}	5.50±0.50^{h}
1.5	10	71.00±1.32^{g}	74.60±0.40^{g}	22.50±1.00^{f}	18.00±0.20^{j}	2.16±0.15^{h}	7.40±0.40^{g}
1.5	15	68.00±0.50^{h}	72.00±0.50^{h}	24.00±0.50^{f}	19.50±0.50^{i}	2.66±0.15^{h}	8.50±0.50^{g}
1.5	20	65.00±0.50^{i}	69.00±0.20^{i}	25.50±0.70^{e}	21.00±0.50^{i}	3.16±0.15^{g}	10.00±1.00^{f}
2	5	60.50±1.00^{j}	65.83±0.72^{j}	27.50±0.43^{d}	23.00±1.00^{h}	4.00±0.20^{f}	11.00±0.50^{f}
2	10	58.00±0.50^{k}	62.96±0.30^{k}	28.50±0.50^{d}	27.50±0.50^{g}	4.50±0.10^{e}	12.50±0.50^{e}
2	15	48.00±0.50^{l}	59.66±1.25^{l}	35.33±1.60^{c}	46.00±1.00^{d}	5.33±0.15^{d}	14.00±0.50^{d}
2	20	12.00±0.50^{m}	57.00±0.50^{m}	72.66±0.85^{b}	70.00±1.00^{b}	5.00±0.26^{d}	12.00±0.50^{e}
3	5	9.50±0.50^{n}	17.00±0.20^{n}	76.60±0.36^{a}	72.00±0.20^{a}	4.63±0.15^{e}	11.00±0.50^{f}
3	10	8.50±0.50^{n}	11.00±0.50^{o}	36.50±0.50^{c}	50.00±0.30^{c}	55.00±0.50^{c}	43.00±0.50^{c}
3	15	6.50±0.50^{o}	7.66±0.20^{p}	25.20±0.40^{e}	35.00±1.00^{e}	68.30±1.12^{b}	57.00±0.50^{b}
3	20	0.00±0.00^{p}	0.00±0.00^{q}	22.60±0.60^{f}	30.00±0.50^{f}	77.40±0.45^{a}	70.00±1.00^{a}
CV		1.15	0.85	2.49	2.08	2.47	3.23

Means with the same letters in a column are not significantly different from each other by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ) at α= 5% .

Generally, occurrence of high contamination rate of 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. High concentration of NaOCl and long exposure duration of explants in the sterilant solution resulted better removal of microbes

due to the powerful oxidant property of active chlorine that disintegrates the lipids in the cell wall of bacteria and fungi. The effect of the sterilant chemical could also alter or denature the shape and function of microbial enzymes (George et al., 2008). However, the increase in sterilant concentration and exposure time above certain optimum limit cause loss of explants because of the oxidant chemical ingredient killing the plant tissue as well. Hence, the optimum treatment combination (concentration and time) for effective sterilization of explants should be determined based on the two aspects of the observations, i.e. a relatively minimum level of contamination as well as tissue death that gives the maximum percentage of clean lively culture as indicated in Table 1.

Table 2. **Effects of BAP and NAA on *in vitro* shoot induction rate of *D. alata* from shoot tip and nodal explants culture on MS.**

CONC. OF PGRS (mg/l)		Explants	
BAP	NAA	Shoot tip	Nodal
0	0	6.55±0.99^{q}	43.67±0.63^{h}
0.5	1	25.95±0.85^{n}	41.25±0.90^{i}
0.5	1.5	33.65±0.47^{k}	54.75±1.07^{e}
0.5	2	43.67±0.63^{h}	62.75±0.82^{d}
0.5	2.5	8.12±0.39^{p}	7.90±0.14^{p}
1	1	87.12±0.62^{a}	64.00±0.81^{c}
1	1.5	82.25±0.53^{b}	72.57±0.84^{b}
1	2	27.40±0.43^{m}	50.70±0.52^{f}
1	2.5	20.60±0.48^{o}	36.82±0.23^{j}
1.5	1	77.25±0.53^{c}	93.20±1.07^{a}
1.5	1.5	30.60±0.48^{l}	30.62±0.68^{l}
1.5	2	55.02±0.68^{g}	33.65±0.47^{k}
1.5	2.5	37.12±0.62^{j}	27.15±0.75^{m}
2	1	67.32±0.53^{e}	43.07±0.83^{i}
2	1.5	64.25±0.52^{f}	20.57±0.46^{o}
2	2	73.47±0.49^{d}	25.95±0.85^{n}
2	2.5	41.10± 0.84^{i}	45.92±0.09^{g}
CV		1.98	1.78

Means within a column followed by the same letters are not statically significant at p< 0.01 by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ).

According to Tariqul Islaml et al. (2008), effective chlorine concentration with three percent and Tween 20 (2 - 3 drops) for 15 min minimizes the level of contamination from nodal explants. This is partially in agreement with present result of 3% active chlorine in NaOCl solution for five minute. Similarly, the nodal segments disinfected with 0.1% mercuric chloride (HgCl2) for five minute as effective treatment to remove microbes from shoot tip and nodal explants on initiation of aseptic culture *in vitro* (Supriya, 2013).

The use of mercuric chloride, however, is highly discouraged as such chemicals have been known to have a serious harmful residual effect to both human and the environment. Thus, the trend is to try for the substitute with a relatively safe or less harmful chemical such as NaOCl solution as employed in this study.

Effect of different concentrations and combinations of BAP and NAA on shoot initiation from nodal and shoot tip explants of *D. alata*

Aseptic shoot tips and nodal cultures were transferred on MS media fortified with different concentrations of BAP in combination with NAA for three weeks to determine optimum medium for shoot induction of *D alata.* The analysis of variance obtained from the data (Table 2) showed that the interaction with BAP and type of explants had very highly significant effect ($P < 0.0001$) on the shoot induction rate. Interaction effect of explants type with BAP and NAA on rate of shoot induction was found to be highly significant (BAP*NAA *Explant).

The response of shoot tip and nodal explants to a given concentration of BAP was not the same; the nodal explants gave greater response than shoot tip explants (Table 2). The nodal explants gave greater response than shoot tip explants. Although it requires further investigation to be carried out, this might be related to the variation in endogenous level of auxin in shoot tip- and nodal sections of the given genotype (Hopkins and Huner, 2004). The highest rate of shoot induction (93.20±1.07%) was obtained on MS medium supplemented with 1.5mg/l of BAP and 1.0 mg/l of NAA from nodal explants while shoot tip (87.25±0.53%) on MS containing 1.0 mg/l of BAP and 1.0 mg/l of NAA (Table 2). MS supplemented with 1.0 mg/l of BAP and 1.0 mg/l of NAA for shoot tip and 1.5 mg/l of BAP with 1.0 mg/l NAA for nodal initiation were found to be the optimum media for *in vitro* shoot initiation of *D. alata* (Table 2). From all treatments the minimum rate of shoot induction was observed on MS containing 0.5 mg/l of BAP and 2.5 mg/l of NAA (8.12±0.39% and 7.90±0.14%) for shoot tip- and nodal explants. Shoot bud development capacity of both shoot tip- and nodal cultures increased with the increase in concentration of NAA from zero to 2 mg/l and reduced with further addition of NAA (Table 2). The nodal explants gave greater response than shoot tip explants (Table 2). Although it requires further investigation to be carried out, this might be related to the variation in endogenous level of auxin in shoot tip- and nodal sections of the given genotype (Hopkins and Huner, 2004). The highest rate of shoot induction (93.20±1.07%**)** was obtained on MS medium supplemented with 1.5 mg/l of BAP and 1.0 mg/l of NAA from nodal explants while shoot tip (87.25±0.53%) on MS containing 1.0 mg/l of BAP and 1.0 of NAA (Table 2). MS supplemented with 1.0 mg/l of BAP and 1.0 mg/l of NAA for shoot tip and 1.5 mg/l of BAP with 1.0 mg/l of NAA for nodal initiation were found to be the optimum media for *in vitro* shoot initiation of *D. alata* (Table 2).

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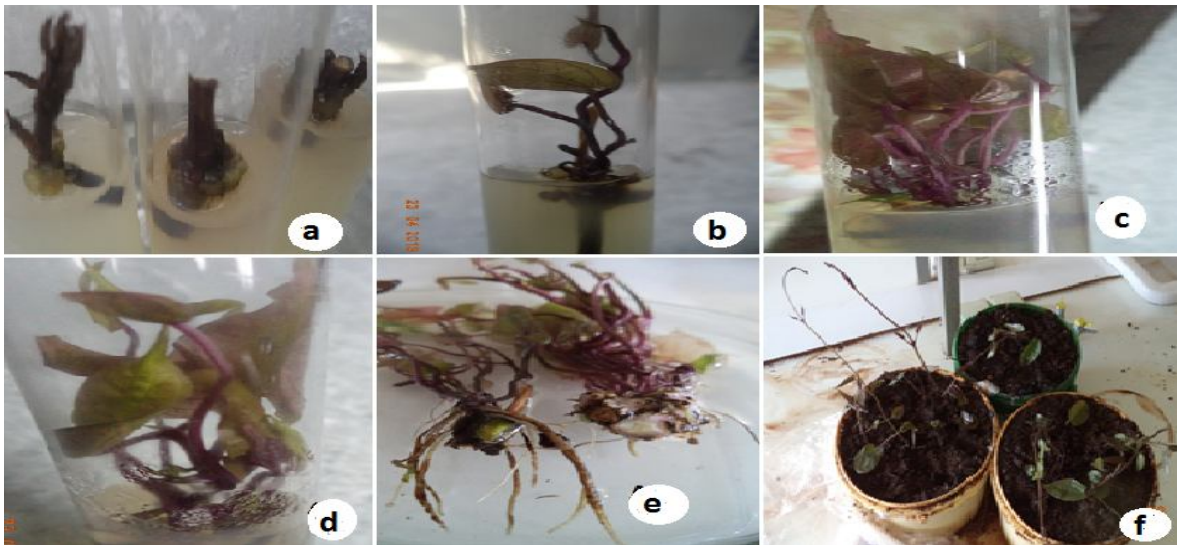

Figure 2. (a) 3% sodium hypochlorite concentration and 5 minute exposure time results for contamination free culture after two weeks, (b) Shoot induction on 1.5 mg/l of BAP and 1.0 mg/l NAA after 6 weeks of culture, (c) *In vitro* shoot multiplication on MS +2.0 mg of KIN + 1.0 mg/l of BAP + 0.5 mg/l NAA from shoot tip explants after eight weeks, (d) *In vitro* shoot multiplication on MS +2.0 mg of KIN + 1.0 mg/l of BAP + 0.5 mg/l NAA from nodal explants after eight weeks, (e) *In vitro* rooted shoot in half MS + 2 mg/l IBA, (f) transplanted and established plantlets in pot/ polytene tube after twelve weeks. The addition of optimum amount of auxin, however, together with relatively less amount of cytokinin might reduce shoot induction rate by inhibiting the availability of the required endogenous amount of auxin for shoot initiation as cytokinins do not act alone unless combined with auxins (George et al., 2008). Such an effect was observed in the present study, the combination of BAP and NAA produced very low response of shoot bud induction when the concentration of Auxins increases. The combination of 0.5mg/l of BAP and 2.5mg/l of NAA gave no or very low response of shoot bud induction rather than callus formation while 1.0mg/l of BAP with 1.0mg/l of NAA produced the highest shoot induction (Table 2). This recalls the knowledge that, it is the ratio of auxin to cytokinin, not the absolute level of cytokinin that initiates shoot bud growth (Hartmann et al., 2009). The present result of shoot induction rate is partially in agreement with the report of Fotso et al., (2013) who considered 0.5 mg/l of BAP with 1.0 mg/l of NAA as the best supplemented medium for nodal cuttings derived shoot regeneration.

Effect of different concentrations and combinations of KIN, BAP and NAA on shoot multiplication of *D. alata*

Those shoot buds that performed well on the prevailing shoot induction medium were transferred onto MS supplemented with Kinetin (1, 2, and 3mg/l) and BAP (0.5, 1 and 1.5mg/l) alone and in combination with NAA (0.5 mg/l). Cultures were subcultured twice and the effect of hormones on *in vitro* shoot multiplication of *D. alata*, cultivar was evaluated. In this study, the significance of KIN and BAP also the interaction of both with NAA were considered.

The ANOVA revealed that the concentration of KIN and BAP both alone and in combination with NAA had a highly significant effect ($p < 0.0001$) on shoot multiplication rate. Shoot buds raised from both shoot tip- and nodal explants responded almost similarly on shoot multiplication indicating the non-significant effect of explants at this stage. The proliferation rate had shown a progressive increase from the first sub-culture to the second by an average of 16.6%.

In this study, the best result (number) of shoot proliferation from nodal (9.50±0.29) was obtained on MS containing 2.0 mg/l of Kinetin, 0.5 mg/l of BAP and 0.5 mg/l of NAA (Table 3). Nevertheless, the micro shoots from both shoot tip and nodal in this medium were less vigorous and bushy as compared to MS prepared with 2.0 mg/l of KIN +1.5mg/l of BAP + 0.5mg/l of NAA (Figure 2) resulting in the production of 7.00±0.29 shoots (Table 3).

Table 3. Effect of different concentrations and combinations of KIN, BAP and NAA treatments on shoot multiplication of *D. alata*

Level of PGRs (mg/l)			Shoot tip		Nodal	
			Shoot number	Shoot length	Shoot number	Shoot length
KIN	BAP	NAA	Mean Std Dev	Mean ± Std Dev	Mean ± Std Dev	Mean ± Std Dev
1	0.5	0	2.00±0.18^{l}	1.60±0.18^{fg}	2.0±0.18^{ij}	1.30±0.14^{ij}
1	1	0	2.50±0.08^{k}	2.00±0.18^{f}	1.50±0.08^{kl}	2.05±0.12^{h}
1	1.5	0	2.70±0.14^{kjı}	2.70±0.14^{e}	2.70±0.14^{gh}	3.40±0.18^{fe}
2	0.5	0	3.00±0.08^{hji}	3.00±0.08^{de}	7.00±0.29^{b}	5.30±0.18^{b}
2	1	0	3.20±0.08^{gh}	5.00±0.14^{a}	3.20±0.08^{f}	3.80±0.18^{e}
2	1.5	0	3.50±0.35^{g}	3.30±0.08^{cd}	3.50±0.35^{f}	2.50±0.18^{g}
3	0.5	0	4.30±0.25^{ef}	1.60±0.14^{fg}	1.30±0.18^{l}	1.60±0.14^{ij}
3	1	0	3.00±0.18^{hji}	3.00±0.18^{de}	3.00±0.18^{gf}	3.00±0.18^{f}
3	1.5	0	3.15±0.26^{hgi}	3.15±0.26^{cd}	3.15±0.26^{gf}	1.20±0.18^{j}
1	0.5	0.5	3.50±0.11^{g}	3.50±0.11^{c}	3.50±0.11^{f}	3.00±0.11^{f}
1	1	0.5	4.50±0.21^{e}	4.50±0.21^{b}	4.50±0.21^{d}	4.30±0.21^{d}
1	1.5	0.5	5.00±0.29^{d}	5.10±0.43^{a}	5.00±0.29^{c}	4.75±0.42^{c}
2	0.5	0.5	6.40±0.18^{c}	4.57±0.09^{ab}	9.50±0.29^{a}	6.20±0.18^{a}
2	1	0.5	8.40±0.21^{a}	5.30±0.29^{a}	6.80±0.18^{b}	4.50±0.21^{dc}
2	1.5	0.5	7.00±0.29^{b}	3.50±0.18^{c}	2.40±0.18^{ih}	3.00±0.25^{f}
3	0.5	0.5	4.00±0.29^{f}	1.30±0.18^{g}	4.00±0.29^{e}	1.30±0.08^{ij}
3	1	0.5	2.60±0.08^{kj}	2.60±0.08^{e}	1.80±0.18^{kj}	2.50±0.18^{g}
3	1.5	0.5	2.05±0.20^{l}	1.80±0.18^{f}	1.50±0.08^{kl}	1.70±0.29^{ih}
CV			5.48	6.33	6.12	6.83

Percentage increases/decreases in shoot number, shoot height, root number and root height per explants is given by plus or minus (±). *Means within a column followed by the same letters are not statically significant at* $p < 0.01$ *by Ryan –Einot - Gabriel - Welsch Multiple Range Test (REGWQ).*

In MS containing 2.0 mg/l of KIN, 1.0 mg/l of BAP and 0.5mg/l of NAA 8.40±0.21 shoots from shoot tip with a good morphological appearance were observed. Shoot buds on MS prepared with 1.0 mg/l Kinetin, 0.5 mg/l of BAP and 0.5mg/l of NAA have changed into callus wh ile as the concentration of Kinetin or BAP increases at the same concentration of NAA had given better and good shoot proliferation of adventitious origin. An increase in number of shoots per shoot bud culture with increased concentration of Kinetin and BAP from 0.5 up to 3 mg/l Kinetin and 0.5 to 1.5mg/l BAP respectively might be due to the effect of both Kinetin and BAP in releasing primordia of lateral buds from dormancy or breaking apical dominance by inhibiting the level of endogenous auxins. Cultures on a higher level of above 2.0 mg/l KIN and 1.0 mg/l BAP had developed into bushy and ill defined shoot buds and they did not respond when subcultured on the same medium. The present results are in agreement with those of Behera *et al*. (2010) and Borges *et al*. (2009) who reported best proliferation rate of *D. alata* on MS + 2 mg/l KIN + 1.0 mg/l BAP + 0.5 mg/l NAA. On the other hand, Behera et al. (2008) reported that of the combinations tested BAP (2.0mg/l) + NAA (0.5 mg/l) with ascorbic acid 100 mg/l, elicited optimal response in which an average of 6.0± 0.18 shoot lets with a mean shoot length of 5.0± 029 cm per explants were recorded on *Dioscorea hispida Dennst* species. In the present study among all the combinations and concentrations, the longest shoots 6.20±0.18 and 5.30±0.29 were observed on the medium containing 2.0 mg/l KIN+ 1.0 mg/l BAP + 0.5 mg/l NAA and 2.0mg/l KIN + 0.5mg/l BAP + 0.5 mg/l NAA for both nodal and shoot tip explants respectively.

Effect of different concentrations of IBA and NAA for *in vitro* root initiation of *D. alata*

The highest rooting percentage 90.70±0.99% was obtained on half - strength MS medium at 2.0 mg/l of IBA followed by 86.75±0.64% at 2.0mg/l of NAA with minimum callusing. Among the given concentrations the above root inducing auxin hormones higher concentration resulted in less rooting percentages with greater diameter of callusing. Naphthalene acetic acid (NAA) at a concentration of 0.5 mg/l resulted in less percentage of rooting 48.62±0.85% that was less than the root induced from the same amount of IBA (Table 4). The longest shoots 5.00±0.18cm were obtained from a medium that contained 2.0 mg/l NAA followed by and 4.30±0.08cm from 1.0 mg/l of IBA. Smallest shoot height 1.40±0.08cm were obtained from 1.0 mg/l of NAA. The highest mean number 8.30±0.21 of roots were obtained from 2.0 mg/l of NAA followed by 10.61±0.21 on 2.0 mg/l IBA. Highest concentrations of auxins resulted in less number of root. Relatively, less number of roots 1.20±0.18 was obtained from IBA at 2.5 mg/l (Table 4) In this study, half - strength MS medium supplemented with NAA (0.5 1.0, 1.5, 2.0 and 2.5) and IBA (0.5 1.0, 1.5, 2.0 and 2.5) were evaluated and relatively 2.0 mg/L for both IBA and NAA were given good rooting percentage and the second highest percentage (76.2%) was at 1.5mg/l of IBA. These results are in agreement with those reported by Behera et al*.* (2010) concerning the better performance of half strength MS for *in vitro* rooting of *D. alata* in terms of rooting with the addition of 2.0 mg/L of either IBA or NAA . Also said, at lower level of NAA (0.5 mg/l) treatments, there was hardly any rooting in the cultured shoots during the 4 weeks of observation. However NAA at higher concentration (1.5 and 2.0 mg/l) and IBA (2.0 mg/l) was the best for inducing root. Behera *et al.,* (2008) also noted the same result on different species of Dioscorea (shootlets

of *D.hispida*) on 2mg/l NAA and 2mg/l IBA in half strength MS basal medium induce (80%) second highest rooting. For rooting Behera *et al*., (2009) *in vitro* micro shootlets of *D.oppositifolia* inoculated on half MS medium supplemented with 2mg/L NAA and profuse rooting was observed on this medium.

Table 4. Effect of various concentrations of IBA and NAA on rooting of proliferated shoots of *D. alata* cultured on half - strength MS medium.

Conc. of PGRs (mg/l)		Rooting Percent (Mean ± SD)	Shoot height (Mean (cm) ± SD)	Root number (Mean ± SD)	Root length(cm) (Mean ± SD)
NAA	IBA				
0	0	0.00±0.00^{l}	1.20±0.08^{h}	0.00±0.00^{i}	0.00±0.00^{g}
0	0.5	52.80±1.04^{g}	1.50±0.08^{g}	2.20±0.18^{g}	2.00±0.18^{e}
0	1	67.00±0.91^{e}	4.30±0.08^{b}	4.30±0.29^{e}	1.20±0.16^{f}
0	1.5	76.20±0.83^{c}	2.00±0.21^{f}	6.20±0.24^{c}	2.40±0.18^{d}
0	2	90.70±0.99^{a}	3.40±0.11^{c}	10.61±0.21^{b}	4.00±0.24^{b}
0	2.5	73.40±0.87^{d}	2.50±0.08^{e}	1.20±0.18^{h}	1.10±0.21^{f}
0.5	0	48.62±0.85^{j}	3.00±0.18^{d}	1.50±0.18^{h}	1.25±0.05^{f}
1	0	52.40±0.71^{h}	1.40±0.08^{hg}	3.50±0.18^{f}	3.00±0.18^{c}
1.5	0	63.10±0.73^{f}	2.50±0.18^{e}	4.05±0.23^{e}	5.00±0.18^{a}
2	0	86.75±0.64^{b}	5.00±0.18^{a}	8.30±0.21^{a}	2.00±0.18^{e}
2.5	0	47.70±0.72^{k}	3.50±0.18^{c}	5.00±0.21^{d}	4.00±0.18^{b}
CV		1.34	5.13	5.25	7.41

Percentage increases/decrease in shoot height, root number and root height per explants is given by plus or minus (±). Means within a column followed by the same letters are not statically significant at **α=** 5 % by Ryan - Einot – Gabrie Welsch Multiple Range Test (REGWQ).

Acclimatization and field establishment of *in vitro* derived plantlet

The plants were found to be ready for transplanting in hardening medium as they developed sufficient shoots and roots. Rooted plants were removed from the culture tube and roots were washed thoroughly to remove the medium. The well rooted plants were transferred to sterilized plastic cups containing vermiculite for hardening and kept under controlled condition. Production of plantlets with profuse rooting in *in vitro* is essential for successful establishment of regenerated plants in soil (Ohyma, 1970). Later, plants were transferred to earthen pots containing mixture of different types of soil and organic compounds for explants development.

The establishment of *in vitro* plantlets under different environmental conditions was greatly affected in terms of survival percentage of plantlets. In the present study, employing the acclimatization procedure indicated in the methodology, the plantlets were transferred to pots containing 1:1:1: coffee husk, sand and red soil respectively showed 75% survival efficiency.

Micropropagated plants were transferred to the pots containing mixture of coffee husk + sand + red soil in 2:1:1 ratio and 82.5% plants survived and the one transferred to the pots containing mixture of coffee husk + sand + red soil in 2:2:1 ratio about 90% plants survived. The plantlets transferred under net house conditions resulted in the best establishment, whereas no plantlets could be established under direct field conditions.

CONCLUSION

Sterilization of selected explants is a key step for removing any sorts of microbial contaminants in order to attain successful *in vitro* establishment of plant tissue culture. From different concentration of sterilant used for sterilizing yam nodal and shoot tip explants two and three percent sodium hypochlorite with five and 20 minute exposures of time was optimum for nodal and shoot tip explants of *D. alata*. So that optimum sterilant concentration and exposure of time is crucial for micropropagation of *D. alata* (Yam). The maximum percentage of shoot induction (93.20±1.07%) and (87.312±0.62%) was observed on an MS medium supplemented with 1.5.mg/l of BAP and 1.0mg/l of NAA for nodal and 1.0 mg/l of BAP and 1.0 mg/l of NAA for shoot tip explants respectively. MS containing 2.0 mg/l of BAP and 1.0 mg/l with 0.5 mg/l of NAA also produced 8.40±0.21% shoot number with good morphological appearance from shoot tip. Best rooting percentage was achieved on half strength MS basal media containing 2.0 mg/l of IBA which resulted mean values of 90.70 ± 0.99% with 10.61±0.61 root number, followed by half strength MS basal media containing 2.0 mg/l of NAA which resulted 86.75±0.64 with 8.30 ±0.21 root number, 5.00±0.18cm shoot height and 2.00 ± 0.18cm root length. Those plantlets well performed *in vitro* showed 90% survival efficiency after hardening and acclimatization on soil mixture by ratio of 2:2:1 decomposed coffee husk, sand and red soil respectively. This protocol provide large-scale production of *D. alata* cultivar, genetic improvement and high disease free yield of the crop using nodal and shoot tip explants sources.

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