

**IN VITRO SHOOT MULTIPLICATION AND TUBERIZATION OF
Dioscorea alata AND *Dioscorea bulbifera***

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ABSTRACT

Some Sri Lankan *Dioscorea spp.* Such as *Gonala*, *Hiritala*, *Kitala* and *Viala* are gradually disappearing from nature due to lack of concern and poor conservation. Therefore, this investigation was carried out as an initial step for sustainable long term conservation method through *in-vitro* multiplication and tuberization on *Dioscorea alata* (accession no: 11) and *Dioscorea bulbifera* (accession no: 117). For multiplication, full strength Murashige and Skoog (MS) salt medium supplemented with four different concentrations 6-benzylaminopurine (BAP) as 0 mg/L (DM1), 1 mg/L (DM2), 2 mg/L (DM3) and 4 mg/L (DM4) while concentration of α -naphthalene acetic acid (NAA) kept constant at 0.5 mg/L with 3% sucrose. After four months, the highest shoot length and highest aerial root count were observed in DM1 with mean values of 1.34 cm and 1.07 respectively. DM3 result the best shoot multiplication rate for accession no: 11 and 117 as 132% and 105%, respectively. For tuberization, half strength MS with 4% sucrose supplemented either with 1 mg/l BAP, 0.5 mg/l NAA and 0.1 mg/l Gibberellin (DT1) or with 3 mg/l BAP (DT2). After eight months, DT1 gave the highest micro-tuber count of 0.97 and proved better on tuberization. In conclusion, DM3 was ideal for shoot multiplication and DT1 proved better on micro-tuberization for both accessions.

Key words: *Dioscorea spp.*, Growth regulators, Multiplication, Microtubers, Yam.

INTRODUCTION

Dioscorea commonly known as yams is a monocotyledonous genus contains over 600 species. They are mainly cultivated in South America, Asia and West Africa (Ayensu and Coursey, 1972). Among *Dioscorea spp.*, some are important as nutrient and pharmaceutical resources. Tubers from specific yam species are used in pharmaceutical purposes as a source of diosgenin derivatives (Ishida, 1988; Deliu *et al.*, 1992), which used for the commercial

synthesis of sex hormones and corticosteroids, (Yongqin *et al.*, 2003) which are widely used for anti-inflammatory, androgenic and contraceptive drugs. The edible species are important food crops in many tropical countries especially in West Africa, Southeast Asia including China, Japan and Oceania and the Caribbean (Trousnot, 1985; Mitchell *et al.*, 1995).

In Sri Lanka, roots and tuber crops are one of the most important food crops after cereals. Among tuber crops, *Dioscorea spp.* finds an important place in the dietary habits of small and marginal farmers, especially in food insecure, rural marginal communities in Sri Lanka. As it is a seasonal crop, it can be grown in *Yala* season and harvest in the *Maha* season. Special character of *Dioscorea spp.* is it remains dormant until planting. Less inputs, easy management, minimum pest and disease attack, less attention, medicinal value and suitability in marginal lands give high demand or interest among farmers for cultivate *Dioscorea spp.* (Department of Agriculture, 2006).

Micro-tubers are small tubers originating from plant tissues *in-vitro* and offer several advantages over *in-vitro* grown plants. They are more tolerant to light and temperature variations than *in-vitro* plantlets, they do not have the requirement of frequent sub-culturing as is needed for maintaining plantlets and conservation and they are easier to handle and exchange than plantlets. Other than field conservation, crop germplasm can be conserved in seed banks, *in-vitro* gene banks under slow growth and reduced temperature. The *in-vitro* gene banks under slow growth conservation are however for short to medium term conservation, after which the plantlets are sub cultured when signs of deterioration are visible. Artificially encapsulated somatic embryos, shoots or other tissues are able to grow into plantlets after sowing under *in-vitro* or *ex-vitro* conditions (Standardi and Piccioni, 1998). To achieve long term storage, cryopreservation protocols have been developed for *D. Alata*, *D. Rotundata*, and *D. Cayenensis* shoot tips and artificial seeds using different techniques. Natural forming organs of the crop also can be conserved in *in-vitro* form with a long passage period more than one year (Mandal *et al.*, 2008).

In Sri Lanka, more than 100 accessions of yam germplasm including wild relatives, landraces have been conserved in the form of *in-vitro* and *ex-vitro* as a mandatory duty of Plant Genetic Resources Centre (PGRC) under the Department of Agriculture. These germplasm have been *in-vitro* conserved via shoot tips which are having short (4-6 months) sub-culture period resulting frequent transferring which is laborious and costly. Therefore, it is essential to increase the passage period of *in-vitro* conserved *Dioscorea* accessions. However, micro-tubers can be stored for a long period of time without losing their viability. And, micro-tubers may represent a very attractive alternative to plantlets as a means of conservation, commercialization and international exchange of virus-free germplasm.

Therefore, the current research was carried out to optimize *in-vitro* shoot multiplication and tuberization protocols for two selected yam species; *Dioscorea alata* and *Dioscorea bulbifera* with the long-term goal of applying for other species as well.

MATERIALS AND METHODS

This study was conducted at the Tissue Culture Laboratory of the Plant Genetic Resources Centre, Gannoruwa from January 2016 to February 2017.

Source of explants

Study was performed using *in-vitro* initiated shoot tip cultures of *Dioscorea alata* (*Kiriwel ala*) and *Dioscorea bulbifera* (*Bakamunu ala*) accessions. Mother plant cultures were initiated on a medium containing basic MS supplemented with 0.1 mg/l NAA, 0.5 mg/l BAP, 0.1 mg/l Gibberelic Acid (GA₃), 30 g/l sucrose and 8 g/l agar. Cultures were maintained at 24°C temperature, 60 to 65% relative humidity under 16 hour light/8 hour dark photoperiod and a light intensity range of 2000-2500 Lux.

Preparation of culture medium

Four different types of multiplication media were used to determine the best multiplication medium for *Dioscorea spp.* Culture medium was made with MS basal salts along with 30 g/l of sucrose. All media were

supplemented with 0.5 mg/l NAA and varying BAP concentrations [0 mg/l (DM1), 1 mg/l (DM2), 2 mg/l (DM3) and 4 mg/l (DM4)].

Effects of two different tuberization media were investigated to find out the most suitable medium for the induction of microtubers of *Dioscorea spp.* Both tuberization media consisted of half strength MS with 40 g/l of sucrose and supplemented one with 1 mg/l BAP, 0.5 mg/l NAA and 0.1 mg/l Gibberelic Acid (GA3) (DT1) while other supplemented only with 3 mg/l BAP (DT2). After adjusting the pH to 5.7-5.8, the medium was solidified with adding of 11 g/l of agar. Thereafter, 30 ml of medium was poured into each jar and sealed with polypropylene polythene and autoclaved at 121°C at 1.05 kg/cm² for 25 minutes.

Culture of explants

Healthy, disease free *in-vitro* initiated mother plant cultures were selected for the multiplication. Shoots height of *Dioscorea alata* and *Dioscorea bulbifera* were maintained around 2-3 cm. Selected shoots were inoculated aseptically under laminar flow cabinet, into prepared multiplication media. Each treatment was consisted of five replicates and each replicate consisted of three plantlets. Treated samples were kept for four months of incubation period to multiply. Cultures conditions were maintained as same for explants.

For the tuberization, mature shoots of *Dioscorea alata* and *Dioscorea bulbifera* were excised from healthy, disease free *in vitro* conserved mother plant cultures. Selected shoots were cultured aseptically under laminar flow cabinet, on prepared tuberization media. Each treatment consisted of five replicates and each replicate contained three plantlets. Treated samples were kept for eight months in incubation room for micro-tubers initiation. Cultures conditions were maintained as same as in explants.

Data analysis

The data was analyzed using ANOVA for Complete Randomized Design (CRD) as two factor factorial experiment using SAS. Means were separated using, Duncan's Multiple Range Test (DMRT).

The multiplication rate calculated using the formula,

$$\text{Multiplication rate} = \frac{\text{Total no of shoots produced*}}{\text{No of shoots at culture initiation}} \times 100$$

*4 months after treatment application

RESULTS AND DISCUSSION

Effect of growth regulators on shoot height and aerial root count

After four months in treatment cultures, height of shoot tips was highest on MS medium supplemented only with 0.5 mg/l NAA with mean shoot height of 1.34 cm when compared to MS supplanted with 0.5 mg/l NAA and 2 mg/l BAP and MS medium supplemented with 0.5 mg/l NAA and 4 mg/l BAP. MS medium supplemented with 0.5 mg/l NAA and 1 mg/l BAP also showed a significant difference over MS supplemented with 0.5 mg/l NAA and 4 mg/l BAP (Table 1).

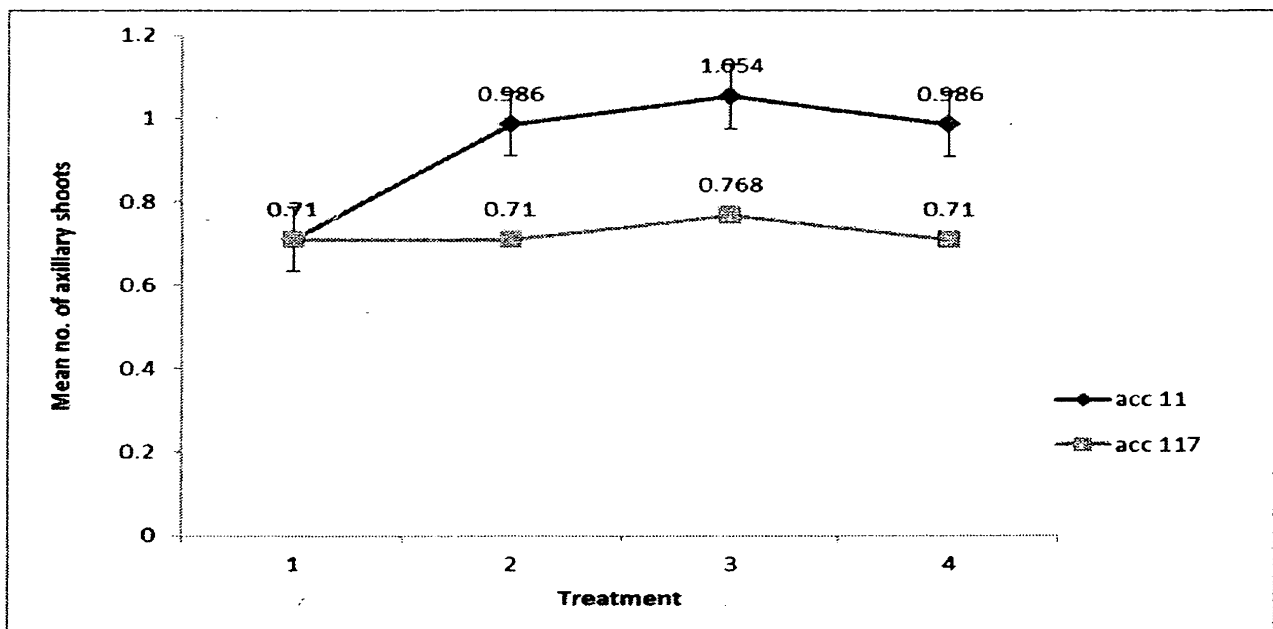
NAA promotes cell elongation and growth in plants. Higher explants height on BAP absent medium could be due to plants get elongated in the presence of auxin without cell division (Campbell *et al.*, 2008). However, high levels of auxin suppress organized growth promoting meristem-like cells. Higher concentrations of BAP (4 mg/l) with 0.5 mg/l NAA cause stunted shoots with fewer numbers of shoots and could be the reason for poor growth of explants in MS medium supplemented with 0.5 mg/l NAA and 4 mg/l BAP.

Table 1. Effect of BAP levels in MS medium on shoot height and aerial root count.

Treatments	BAP (mg/l)	Mean shoot height	Mean no. of aerial roots
DM1	0.00	1.34 ^a	1.07 ^a
DM2	1.00	1.22 ^{ab}	0.73 ^b
DM3	2.00	1.02 ^{bc}	0.74 ^b
DM4	4.00	0.94 ^c	0.73 ^b

Note: *Means of the same letters are not significantly different at 0.05 probability level. DM1-0.5 mg/l NAA+0 mg/l BAP, DM2-0.5 mg/l NAA+1 mg/l BAP, DM3-0.5 mg/l NAA+2 mg/l BAP, DM4-0.5 mg/l NAA+4 mg/l BAP.

The data in Table 1 also revealed that among all treatments, MS medium supplemented only with 0.5 mg/l NAA showed the effect on aerial root number with a mean value of 1.07. The ratio of auxin to cytokinin plays an important role in plant growth; when higher the auxin to cytokinin ratio, higher the root initiation and its growth. According to the experiment, highest auxin:cytokinin ratio was recorded in MS supplemented only with 0.5 mg/l NAA, reporting highest aerial root count (Campbell *et al.*, 2008).

**Figure 1. Treatment-Accession interaction plot for number of axillary shoots.**

Note: Treatments: 1-0.5 mg/l NAA+0 mg/l BAP, 2-0.5mg/l NAA+1 mg/l BAP, 3-0.5 mg/l NAA+2 mg/ BAP, 4-0.5 mg/l NAA+4 mg/l BAP.

Emergence of axillary shoots was enhanced by the addition of NAA and BAP in combination. Cytokinin alone has no effect on plant growth. Auxin stimulates cell division if the medium contains cytokinin. Higher cytokinin to auxin ratio enhances the growth of shoot buds. There is a significant difference in treatment-accession interaction on number of axillary shoot development. There was no significant difference in accession 11 at MS supplemented with 0.5 mg/l NAA and 1 mg/l BAP, MS supplemented with 0.5 mg/l NAA and 2 mg/l BAP, MS supplemented with 0.5 mg/l NAA and 4 mg/l BAP. But all above combinations were significantly differing from accession 117 at any treatment level saying that accession 11 is highly responded to plant growth regulators on axillary shoot emergence (Figure 1).

Shoot multiplication rate

Of all the tested treatments, each and every treatment level showed 100% or above; which concludes, even with low levels of growth regulators, better multiplication rate can be obtained. Among them, MS medium with 0.5 mg/l NAA and 2 mg/l BAP elicited the optimal response in multiplication rates for accession 11 and 117 as 132% and 105% respectively (Table 2).

Table 2. Multiplication rate of accessions according to respective treatment.

Accession	Treatment	Multiplication Rate (%)
11	DM1	100
	DM2	128
	DM3	132
	DM4	125
117	DM1	100
	DM2	100
	DM3	105
	DM4	100

Note: Probability level is 0.05. *DM1-0.5 mg/l NAA+0 mg/l BAP, DM2-0.5 mg/l NAA+1 mg/l BAP, DM3-0.5 mg/l NAA+2 mg/l BAP, DM4-0.5 mg/l NAA+4 mg/l BAP.

For the micropropagation of *Dioscorea* spp., MS medium has been commonly used and BAP is more responsive than kinetin for shoot multiplication in *Dioscorea alata*. *Dioscorea* spp. Is rich in phenolic compounds however, in this study; phenolic exudation in the multiplication medium was effectively controlled by frequent transferring into fresh medium. Yam shoot development found to be effective in liquid or agar-solidified medium and this might be due to the stimulation of photosynthesis *in-vitro* by aeration.

Effect of growth regulators on number of leaves

Number of leaves were not affected either by any applied treatment or accession ($P>0.05$) (Table 3).

Table 3. Effect of BAP in MS medium on leaf count.

Treatments	BAP (mg/l)	Mean no. of leaves
DM1	0.00	2.30 ^{ab}
DM2	1.00	2.84 ^{ab}
DM3	2.00	2.70 ^{ab}
DM4	4.00	2.01 ^b

Note: *Means of the same letters are not significantly different at 0.05 probability level. DM1-0.5 mg/l NAA+0 mg/l BAP, DM2-0.5 mg/l NAA+1 mg/l BAP, DM3-0.5 mg/l NAA+2 mg/l BAP, DM4-0.5 mg/l NAA+4 mg/l BAP.

Effect of growth regulators on microtuber initiation

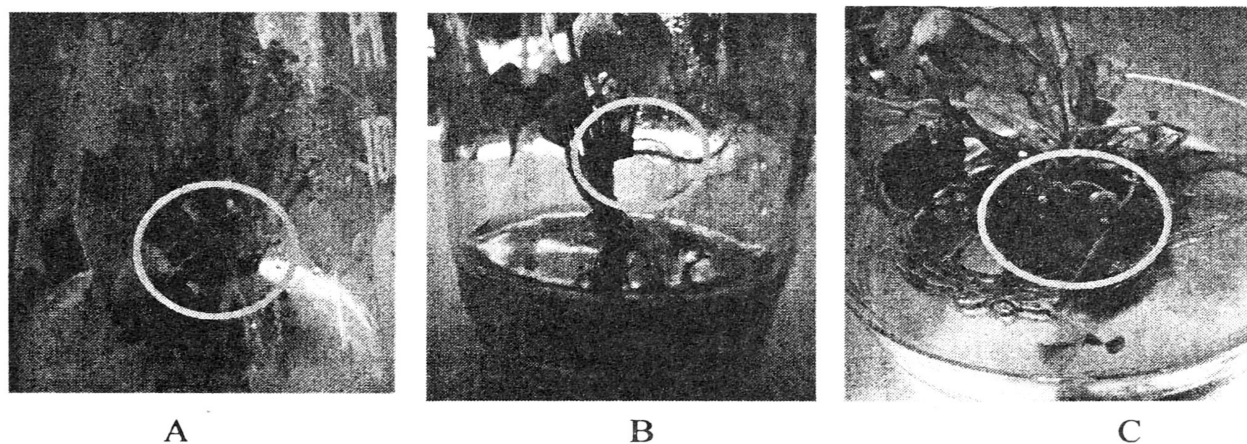
Microtubers develop from primary nodal complexes. In the leaf axils of old nodes, there are two axillary buds and one shoot primordial, which also later develop into an axillary bud. This primary nodal complex-initial has capacity for multiple bud production, roots and a tuberous storage organ (Mantell, 1989). Out of tested two media, medium which contained half strength MS supplemented with 1 mg/l BAP, 0.5 mg/l NAA and 0.1 mg/l GA₃ showed a significant difference over medium which contained half strength MS only with 3 mg/l BAP on microtuber induction and microtuber count. The mean value for number of microtuber per plantlet was recorded as 0.969 in MS supplemented with 1 mg/l BAP, 0.5 mg/l NAA and 0.1 mg/l GA₃ for both accessions (Table 4).

Table 4. Treatment response on number of microtuber per plantlet.

Treatments	BAP (mg/l)	NAA (mg/l)	GA ₃ (mg/l)	Mean no. of tubers/plantlet
DT1	1.00	0.50	0.10	0.96 ^a
DT2	3.00	0.00	0.00	0.71 ^b

Note: *Means of the same letters are not significantly different at 0.05 probability level. DT1- 1 mg/l BAP+0.5 mg/l NAA+0.1 mg/l GA₃, DT2- 3 mg/l BAP.

Our work on microtuberization identified several factors effect on this phenomenon. The MS basal medium formulation inhibited microtuberization in *D. alata* and *D. bulbifera*. Microtubers were however produced on glycine-free MS medium in *D. alata* while half-strength MS medium enhanced microtuberization in *D. alata*. Microtuber formation was also stimulated in *D. alata* in the absence of ammonium. Optimum basal medium formulation for microtuberization varied with other factors such as medium, sucrose concentration, light and temperature regimes. Higher concentration (4%) of carbon sources gave higher tuberization than lower (3%) concentration.

**Figure 2. Stages of microtuberization in Accession 117.**

Note: Aerial root stage, B- Green tuber stage, C- Brown tuber stage

Microtuberization was affected by plant growth regulators, the effect being greater when applied at culture initiation than at later stages (Balogun, 2009). NAA may enhance rooting through promoting cell division and growth

of the plant, which accelerates the maturity of plantlets and enters to the reproductive stage which is very much important in microtuber induction of *Dioscorea* spp. NAA, was also superior to IAA in microtuber induction in *D. alata* (Chang *et al.*, 1995).

Our work on microtuberization in yam can be improved by application of Jasmonic acid (JA). Previous studies found that, microtuberization are to be controlled by JA, which is a 12-carbon acid that has fragrant and plant growth-regulating properties, inhibiting growth and promotes senescence. In *D. alata*, JA synthesis is activated by short day length, while the peak of JA content coincided with initiation of microtuber enlargement and decreased significantly afterwards, although vigorous growth and enlargement of microtuber continued (Kikuno *et al.*, 2002).

Short days stimulate yam microtuberization depending on the growth stage while long days inhibit it. In *D. alata*, however, shorter day length (8 h) was necessary to consistently produce more and larger microtubers but only from basal nodes. Other nodes did at longer (16 and 24 h) photoperiods (Ng and Mantell, 1996). Application of above finding; decreasing the photoperiod from 16 hour to 8 hour will improve our work further through microtubers induction from basal nodal.

CONCLUSION

MS medium supplemented with 1 mg/l BAP, 0.5 mg/l NAA and 0.1 mg/l GA₃ (DT1) resulted best micro-tuber induction for both accessions. MS media supplemented with 0.5mg/l NAA and 2mg/l BAP (DM3), was better for shoot multiplication in both accession. Culture medium with MS and 0.5 mg/L NAA (DM1) significantly enhanced the shoot elongation and induction of aerial roots and hence, these plants are very much suitable for tuberization trials.

ACKNOWLEDGEMENTS

Authors wish to extend their sincere gratitude to Dr. K. Hettiarachchi, Director, Seed Certification & Plant Protection Centre, Gannoruwa, and Sri Lanka Council for Agricultural Research Policy for the financial support and the staff of *in-vitro* conservation laboratory.

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