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Micropropagation of *Dioscorea alata* L. through nodal segments

Supriya Das[1*], Manabendra Dutta Choudhury[1] and Pranab Behari Mazumdar[2]

[1]Department of Life science and Bioinformatics. Tissue Culture Laboratory, Assam University, Silchar.
[2]Department of Biotechnology. Assam University, Silchar.

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Yams (*Dioscorea*) are well known for their medicinal use as well as nutritional values. *Dioscorea* plants are rapidly vanishing from nature due to their over-exploitation by human beings. In order to conserve *Dioscorea* plants, the present investigation was carried out with a view to regenerate plantlet of *Dioscorea alata* L. through *in vitro* culture using full strength Murashige and Skoog (MS) medium and indole-3-acetic acid (IAA) with and without sucrose. Nodal vine segments of *D. alata* were used as explants and nodal segments were cultured on MS (Murashige and Skoog's) medium supplemented with different concentrations of auxin (IAA) for axillary bud proliferation. Best shoot proliferation was observed in MS medium containing 1.5 mg/L kinetin + 2 mg/L IAA with highest rate of shoot multiplication (average of 9.90 shoots/explants). Micro shootlets were inoculated in half strength MS basal medium supplemented with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) of IAA and best rooting was observed in medium supplemented with 2.5 mg/L IAA with highest root length (8.14 cm). Regenerated plants were transplanted in hardening medium containing Brick bats + Charcoal + Dried moss + Leaf molds + Soil in 1: 1: 1:1:1 proportion. Maximum survival percentage was observed as 85 to 87% after one month of transfer in hardening medium. This work proposes an economic technique for the conservation of *D. alata.*

Key words: Yams, *Dioscorea alata* L., *in vitro culture,* growth regulators.

INTRODUCTION

Monocotyledonous *Dioscorea* is known as yam. The genus *Dioscorea* includes 600 species and is of considerable economic importance (Ayensu, 1972). Many wild *Dioscorea* species are a very important source of secondary metabolites used in pharmaceutical industry and medicine. A number of *Dioscorea* wild species are sources of compounds used in the synthesis of sex hormones and corticosteroids (Coursey, 1967) and cultivated species are the sources of food in some countries (Coursey, 1976). *Dioscorea alata* L. is an important tuber crop and is a staple food for millions of peoples in tropical and subtropical countries (Edison et al., 2006). Root and tuber crops are the most important food crops after cereals. Tuber crops find an important place in the dietary habits of small and marginal farmers especially in the food security of tribal population. India holds a rich genetic diversity of tuber crop especially yam (Hann, 1995).

Yams are valuable source of carbohydrates, fibers and low level of fats which make them a good dietary nutrient; they are also processed into various staple intermediate and end product forms (Jaleel et al., 2007). *D. alata* L. is a slender creeping vine reaching a length of several meters; it is a glabrous twinner, with a peculiar 4 winged stem. Its leaves are simple, opposite, ovate, cordate and

*Corresponding author. E-mail: Supriya1august@gmail.com. Tel: +91 9401238157.

acuminate. The flowers are unisexual and fruits are capsules. The plant has many aerial tubers that also aid in reproduction. Underground tubers are large and have many branches; the colour is dark brown and they are fleshy. It is edible and used as a staple food. Powdered tubers are used as a remedy for piles, gonorrhea and are applied externally to sores.

The tubers of some species of *Dioscorea* are important sources of diosgenin, a chemical used for the commercial synthesis of sex hormones and corticosteroids, which are widely used for anti-inflammatory, androgenic and contraceptive drugs (Satour et al., 2007). Many species of *Dioscorea* genus are economically important crops worldwide. For example, *D. alata, D. Cayenensis,* and *D. rotundata* are main crops in Caribbean Central and South America and West Africa (Tor et al., 1998). Out of six hundred species of *Dioscorea,* 14 are used as edible tubers. Tubers have a dual agricultural function. They supply nourishment as a source of food and also act as planting materials (Craufurd et al., 2006). *Dioscorea* species are vegetatively propagated by using tuber pieces. The production of tuber is hampered by several significant virus and fungal diseases (Saleil et al., 1990).

Induction and growth of micro tubers in *Dioscorea* has been found to be under the control of many factors. Among the environmental factors, photoperiod (Ng, 1988) is documented as important factor. *In vitro* propagation of some economically important *Dioscorea* sp. has been achieved using nodal cuttings (Chaturvedi, 1975), bulbils (Asokan et al., 1983), zygotic embryos (Viana and Mantell, 1989), meristem tips (Malaurie et al., 1995), immature leaves (Kohmura, 1995) and roots (Twyford and Mantell, 1996). Micro-propagation has been done in *D. abyssinica* (Martine and Cappadocia, 1991), *D. alata* (Mantell and Hugo, 1989), *D. batatas* (Koda and Kikuta, 1991), *D. Weightii* (Mahesh et al., 2010), *D. floribunda* (Sengupta et al., 1984). *D. alata* was also cultured by using liquid medium (Jova et al., 2011) and the effect of temporary immersion system on the growth of *D. alata* plantlet was also investigated (Yan et al., 2011).

Yam propagation by seeds using conventional methods is slow and not adequate for rapid multiplication. Tuber yield is drastically reduced by viral and nematode infections; through infected tubers it is transmitted to the next generation (Ng, 1992) and it also deteriorates the quality of the tuber (Mitchell and Ahmed, 1999). *In vitro* propagation may help to overcome constraints related to the availability of high quality of planting material (Wheatley et al., 2005; Vaillant et al., 2005). In the present investigation an attempt was made to cultivate *D. alata* L. *in vitro* using minimum number of growth regulators, so that the plant can be regenerated in mass for general use as well as commercial exploitation in minimum cost. Effect of indole acetic acid (IAA) on shoot and root initiation was also studied. It was noticed from review of literature, many workers propagated *D. alata* using growth regulators kinetin, benzyl aminopurine (BAP), naphthalene acetic acid (NAA), indole butyric acid (IBA) but effect of IAA was not investigated; so this work was done to explore this ground.

MATERIALS AND METHODS

Explant source

Field grown plants of *D. alata* L. propagated from a wild tuber were used as source of explants for *in vitro* culture.

Sterilization

The nodal segments were washed in tap water for some time and disinfected with 0.1%(w/v) mercuric chloride ($HgCl_2$) for 5 min followed by thorough rinsing in autoclaved distilled water for at least 7 to 8 times. The surface sterilized explants had length of 0.5 to 1cm containing single node.

Culture medium and condition

MS medium was selected for *in vitro* culture of explants; the pH of the medium was adjusted with 1N HCl and 1N NaOH solution between 5.7 to 5.8. After adjusting the pH, agar powder was mixed with the medium and boiled for some time to obtain clear solution. Thereafter, 40 ml of medium was taken in each 100 ml screw capped bottle autoclaved at 121°C at 15lbs/sq inch for 20 min.

The screw capped bottles containing the medium were then allowed to cool for 24 h in the culture laboratory. The surface sterilized explants were placed vertically on the MS media with and without growth regulators. Various concentrations of indole acetic acid (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) and kinetin (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) were used to observe its effect on axillary bud proliferation, shoot initiation and root initiation.

The cultures were maintained at 25 ± 1°C under 16 h photoperiod provided by white fluorescent tubes. After every two weeks, the culture materials were transferred into new medium for better growth of plants.

Acclimatization

The plants were found to be ready for transplanting in hardening medium after five months as they developed sufficient shoots and roots. Rooted plants were removed from the culture tube and roots were washed thoroughly to remove the medium. Then the plantlets were transferred to sterile small plastic cups containing vermiculite (a sterile inert medium for planting transferred plants) and kept inside the tissue culture room for acclimatization before exposing to the natural environment. During this period, the plants were sprayed with liquid MS medium without agar and sugar, thrice in a week. After one month. The plants were transferred to acclimatization media and medium comprised pre sterilized Brick bats + charcoal + dried moss + leaf molds + soil (1:1:1:1:1).

Statistical analysis

Ten explants were used per treatment on each multiplication and rooting medium. Experiments were repeated thrice and collected data were analyzed by using ANOVA; variation among means was compared by the Post- Hoc Multiple Comparison test at $P < 0.05$ level of significance.

RESULTS

The effect of various concentrations of IAA (0.5, 1.0, 1.5,

Table 1. Effect of various concentrations of IAA on axillary bud proliferation (Data scored after 40 days, 10 replicates for each treatment, repeated thrice).

Treatment	Hormonal supplements IAA (mg/L)	Percentage of explants response	Days to bud break
T0	0	46	35-37
T1	0.5	40	28-30
T2	1.0	53	15-17
T3	1.5	62	12-14
T4	2.0	68	7-9
T5	2.5	59	11-13

Values represent mean ± SE.

Table 2. Shoot formation in nodal explants of *Dioscorea alata* L. cultured on MS medium supplemented with various concentrations of Kinetin and IAA (10 replicates per treatment, data scored after three month, repeated thrice).

Treatment	Hormonal supplements (mg/L)		Mean no. of shoot /explant	Mean no. of shoot/explant ± SE	Mean shoot length (cm)	Mean shoot length ± SE
	IAA	KN				
T0	0	0	1.5	1.5±0.22	1.50	1.50±0.02
T1	2.0	0.5	1.9	1.9±0.23	6.62	6.62±0.12
T2	2.0	1.0	5.6	5.6±0.30	7.09	7.09±0.12
T3	2.0	1.5	7.7	7.7±0.29	9.90	9.90±0.11
T4	2.0	2.0	3.8	3.8±0.19	5.86	5.86±0.05
T5	2.0	2.5	3.1	3.1±0.23	4.90	4.90±0.05

Values represent mean ± SE.

2.0 and 2.5 mg/L) on axillary bud breaking of *D. alata* L. is listed in Table 1. Explants cultured on MS medium without IAA showed proliferation of axillary bud but required maximum time (35 to 37 days). Addition of low concentration (0.5 mg/L) of IAA was less effective in bud breaking and it took 28 to 30 days for proliferation, which was the second highest time period for bud proliferation. Nodal segments cultured on media with IAA (concentration 2.0 mg/L) proliferated within 7-9 days and percentage of explants' response (68) was also very satisfactory. Nodal segments cultured on MS medium with 1.5 mg/L IAA took a second minimum time period (12 to 14) for bud proliferation and it showed impressive response (62%) of explants also.

Bud proliferation was enhanced by the addition of IAA. After bud proliferation, for further growth, cultured plants were transferred to the media supplemented with kinetin and auxin (IAA). Response of different concentrations of kinetin with 2.0 mg/L concentration of IAA was recorded in terms of number of shoot and shoot length. 2.0 mg/L IAA when supplemented with MS media showed effective result in bud breaking. This concentration of IAA combined with different concentrations of kinetin was selected to study its effect on shoot proliferation in terms of shoot length and number of shoot per explants. Data obtained from the study are shown in Table 2.

MS medium with growth regulators produced better result in terms of percentage of explants' response, number of shoots/explants and average shoot length. Of the combination tested, MS+ kinetin (1.5 mg/L)+ IAA (2.0 mg/L) elicited optimal response, in which an average of 7.7± 0.29 shootlets with a mean shoot length of 9.90 ± 0.11cm per explant was recorded. The second highest shoot proliferation in terms of shoot number and shoot length was observed in the MS medium +Kinetin (1.0 mg/L)+ IAA (2.0 mg/L), in which 5.6±0.30 shootlets per explants with shoot length of 7.09 ± 0.12 cm was recorded. The well grown shoots were transferred to half strength MS medium containing IAA. The rooting responses of shoots in different concentrations of IAA were measured in terms of days required for root initiation, mean no. of roots/shoots and mean root length. Data are represented in Table 3. In different concentrations of IAA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L), the response of shoots was recorded.

IAA enhanced rooting and data were recorded. Shoots cultured on half strength MS media without any hormonal supplementation were unable to produce root; in some shoots, rooting was observed but in very negligible amount. Media supplemented with lower concentration (0.5 mg/L) of IAA produce few (mean 2.6±0.16) number of root with minimum root length (mean1.39 cm). Media treated with higher concentration of IAA (2.0, 2.5 and 3 mg/L) respond well .Half strength MS + 2.5 mg/L showed more impressive result where about 62% explants responded with average root length of 8.14 cm. In terms of response of explants, two concentrations of 2.5 and 3.0 mg/L showed

Table 3. Influence of different concentrations of IAA on rooting of *in vitro* generated shootlets of *Dioscorea alata* L. (Data scored after 3 month of inoculation, 10 replicates per treatment, repeated thrice).

Treatments	Concentration of hormone mg/L	Days to root initiation	% of explants response	Mean root number	Mean root number ± SE	Mean root length (cm)	Mean root length ± SE
T0	0	-	-	-	-	-	-
T1	0.5	60-62	46	2.6	2.6±0.16	1.39	1.39±0.02
T2	1.0	51-53	49	3.8	3.8±0.13	2.05	2.05±0.03
T3	1.5	43-45	58	4.9	4.9±0.17	3.73	3.73±0.07
T4	2.0	36-38	63	5.7	5.7±0.15	7.77	7.77±0.09
T5	2.5	22-24	62	6.6	6.6±0.16	8.14	8.14±0.06
T6	3.0	32-34	59	4.1	4.1±0.23	5.36	5.36±0.07

Values represent mean ± SE.

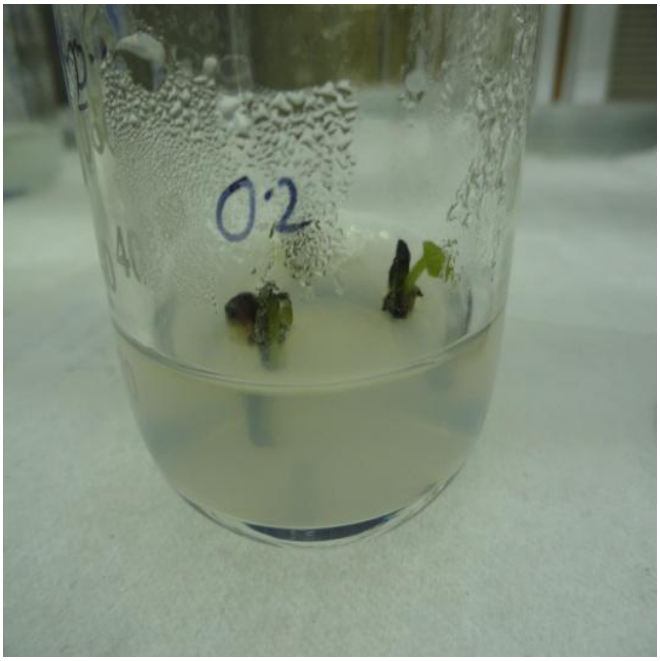

Figure 1. *Dioscorea alata* L. showing bud proliferation.

more or less similar result (62 and 59%, respectively). Shoots treated with 2.0 mg/L IAA showed the second highest response with root number of 5.7 and mean root length of 7.7 cm.

Plants were found to be ready for transplanting in hardening medium after five months. Rooted plants were removed from culture tube and washed thoroughly to remove adhering gel. They were then transplanted to sterile plastic cups containing vermiculite and kept inside the growth chamber. During this period, the plants were sprayed with liquid MS medium without agar and sugar. The plants were dipped in 2 mg/L Diethane for two minutes as precaution to resist fungal infection. After 1 month they were transplanted to earthen pots containing mixture of Brick bats + soil + Charcoal + Dried moss + Leaf mold (1:1:1:1:1).

The acclimatized plants grew normally without any morphological variation. 85 to 87% acclimatized plants survived.

DISCUSSION

Micro-propagation of various plant species including many medicinal plants have been described by many authors during the last two decades (Skirvin et al., 1990). In the present investigation micro-propagation of *D. alata* was done and for that nodal segment was used as explants. MS media supplemented with IAA and kinetin was used for root and shoot growth. In this work, we cultured *D. alata* in solid MS medium; similarly *in vitro* propagation of other yam species is tried by Martine and Cappadocia (1992) using solid medium.

Nodal segments of *D. alata* were cultured on MS media supplemented with auxin (IAA); within 7 to 9 days (Table 1) axillary bud proliferated (Figures 1 to 4) in culture media supplemented with IAA (2 mg/L). Results of many literatures indicate that addition of either IAA or NAA in culture medium improved shoot growth in a number of species and completely supported the effect of IAA on bud breaking in the present investigation. MS media was selected as culture media. Chen et al. (1995), while working with *Eucommia colomoides,* reported that MS medium is effective than WPM (Woody Plant Medium) and their report supports the findings of the present work where also MS medium was used as culture medium and satisfactory growth was observed using nodal segments as explants. For axillary bud proliferation nodal segments culture is recommended (Narula et al., 2007) for rapid clonal propagation when working with *D. bulbifera.*

In the present work, two growth regulators were added with full strength MS medium and results indicated that MS media supplemented with Kinetin (1.5 mg/L) and IAA (2.0 mg/L) induced maximum number of shoot per explant (mean no. 7.7) with highest shoot length of 9.90 cm (Table 2, Figures 5 and 6). Effects of different concen-concentration of Kinetin and indole acetic acid are shown in Table 2. It is observed that for shoot proliferation cyto-

Figure 2. Multiple shoot regeneration.

Figure 3. Showing Growth after 45 days.

Figure 4. Showing Growth after 75 days.

Figure 5. Showing Shoot number in regenerated plants.

cytokinin required optimal quantity in many genotypes but addition of low concentration of auxin with cytokinin triggered shoot proliferation (Sengupta et al., 1984). In this study, low concentration (1.5 mg/L) of kinetin with high concentration (2.0 mg/L) of IAA induced shoot proliferation; further increase of kinetin concentration after optimal concentration (1.5 mg/L) decreased shoot length. The result was supported by this work which shows that suppression of kinetin increases shoot length, node number and root length (Jha and Jha, 1998). In *Dioscorea composita* only auxin NAA, IAA and IBA at 1.25 and 2.5 mg/L account for promotive effects of *in vitro* shoot growth (Ondo et al., 2007).

Our experimental result is completely different from the cited work. The effects of auxins and cytokinins on shoot multiplication of various medicinal plants are reported by many workers (Alizadeh et al., 1998; Ahuja et al., 1982). Explants culture in 2.0 mg/L KN + 1.0 mg/L BAP + 0.5 mg/L NAA showed that highest rate of shoot multiplication is reported when working with *D. alata* (Borges et al., 2009) and rooting is more profuse in half strength MS basal media with 2.0 mg/l NAA. But in the present investigation, it was clearly observed that MS media supplemented with only auxin (IAA 2.0 mg/L) was effective in axillary bud proliferation and it initiated shoot formation within 7- 9 days. Kinetin was used to increase the shoot

Figure 6. Regenerated plants established in pots.

length. Explants cultured on media (MS + 2.0mg/L IAA + 1.5 mg/L Kn) showed highest shoot length within three month. Auxin (IAA) was very effective in bud breaking and shoots formation. Number of shoot induced per explant by the effect of IAA (2.0 mg/L) was also very satisfactory (Figure 5). Kinetin (1.5 mg/L) with IAA (2.0 mg/L) enhanced shoot multiplication and also increased number of node.

2 mg/L kinetin + 1.0 mg/L BAP+ 0.5 mg/L NAA + 100 mg/L ascorbic acid is supplemented with MS media for shoot proliferation of *D. oppositifolia* and results show 90% explants proliferate with highest rate of shoot multiplication (10.5 shoot/explant) (Behera et al., 2009). In our study, only one cytokinin (kinetin) was added with auxin (IAA) for shoot multiplication and IAA (2.0 mg/L) + Kinetin (1.5 mg/L) induced highest shoot length of 9.90 cm (Table 2) with 7.7 mean number of shoot per explant. Combination and interaction of BA and NAA plays an important role in *in vitro* propagation of nodal explants for multiple shoot induction (Shin et al., 2004). MS medium with 1.0 mg/L NAA and 0.5 to 1.0 mg/L BA is best concentration for induction of multiple shoot in *D. opposita*. The present study revealed that combination and interaction of Kinetin (1.5 mg/L) and IAA (2.0 mg/L) induced multiple shoot in MS media.

Half strength MS media supplemented with 2.5 mg/L IAA is most efficient for root initiation and it produces roots (average 6.67) with average length of 8.33 cm (Table 3). Addition of IAA, IBA or NAA to MS medium produces root (Barna and Wakhlu, 1988). Result of this work supports the observation of the present work. Shoot culture on full strength MS medium with auxin produces callus at the base of the shoots (Patra et al., 1998). The microshoots of various medicinal plants are rooted on only MS medium without growth regulators by many workers (Thomas and Maseena, 2006; Saxena et al., 1998). Observation of this work showed difference with the result of recent study because no rooting was observed in auxin free MS medium (Table 3). Rooting occurs in the presence and absence of NAA, IBA or IAA; it is also recorded that higher concentration of NAA, IBA or IAA (5.0 to 10 µM) induces root sooner than the lower concentration (0.1 to 1.0 µM/L) of IAA, NAA or IBA (Mao et al., 1995). The findings of this experiment are similar with the result of the present work. Nodal segments cultured on MS medium with IAA at concentration of 0.5 mg/L required 60 to 62 days to initiate rooting but IAA at concentration of 2.5 mg/L required only 22 to 24 days for root initiation. No rooting is observed in shoots planted on auxin free basal medium; lower concentration of auxin (NAA 0.5 mg/L) produced very few or no root (Behera et al., 2009). Result of this work show is similar with the present investigation because low concentration of IAA (0.5 mg/L) produced minimum number of root (average 2.56), with root length of 1.33 cm (Table 3). Work with *Saussurea lappa* indicated media containing 0.5 to 1.0µM NAA initiated rooting in 90% of culture shoots (Arora and Bhojwani, 1989).

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 Brick bats + soil + Charcoal + Dried moss + leaf mold (1: 1: 1: 1: 1) and survival rate was 85 and 87% after one month of hardening.

Here, an attempt was made to propagate *D. alata* using minimum number of growth regulators. In this Investigation, an efficient micropropagation technique (1.5 mg/L IAA + 2.0 mg/L KN for shooting and 2.5 mg /L IAA for rooting) was derived which may be useful for raising quality plants of *D. alata* for commercial purpose at lowest cost. This technique paves the way not only for *ex situ* conservation but also for the restoration of genetic stock of the species.

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