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Original Article

Propagation of edible *Dioscorea* species *in vitro*

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ABSTRACT

The three yam species *Dioscorea alata* (greater yam), *Dioscorea rotundata* (lesser yam) and *Dioscorea esculenta* (white yam) are most commonly cultivated edible yams and are propagated vegetatively. The tubers are rich in carbohydrate and are better source of protein than other tuber crops. Continuous vegetative propagation through tuber sets, prevalence of disease, and low viability severely hinder productivity of yams. To overcome the limitations, experiments have been carried out to optimize the culture conditions for *in vitro* propagation of different yam species. Nodal explants of fast growing vines and tuber sprouts of released varieties *viz*. Sree Shilpa of *D. alata*, Sree Subhra of *D. rotundata* and Sree Latha of *D. esculenta* were cultured in Murashige and Skoog (MS) media containing different combinations and concentrations of growth regulators. Response was better with tuber sprouts. Percent explant response towards axillary shoot proliferation was observed to be highest in *D. esculenta* (84%), followed by *D. alata* (68%) and *D. rotundata* (60%). Mean shoots per explant was recorded as high as 6.8 to 7.8 in *D. rotundata* and *D. esculenta,* respectively in media supplemented with relatively high concentration of Gibberellic acid ($GA_{3)}$ (2 mg/L) and 1-Naphthaleneacetic acid (NAA) (0.5 mg/L) and low concentration of 6-Benzyl Adenine (BA)(1 mg/L) while *D. alata* showed highest mean shoots per explant (7.4 ± 0.78) in media with BA (2.5 mg/L), GA_3 (1 mg/L), NAA (0.25 mg/L) and 2,4-D (0.5 mg/L). Regeneration was also achieved through callusing and organogenesis. Shoot organogenesis could be enhanced (20-24%/ 50 mg callus) by optimizing concentrations of BA (2-2.5 mg/L). Field establishment was achieved in high frequency (85-90%) using Hoagland nutrient prior to transplantation in sterilized soil: sand (1:1) mixture. Yield, tuber characters like starch content and dry matter content as well as RAPD analysis shows homology between regenerants and the source plants. Thus the study on axillary shoot proliferation could affect faster production of disease free quality planting materials. Moreover the results of the study on callusing and shoot organogenesis can have a greater impact in future bio-technological development of yams. These techniques may facilitate faster screening of large germplasm for superior transgenic lines.

Key words: *Dioscorea*, axillary shoot proliferation, callusing, regeneration, hardening.

INTRODUCTION

Dioscorea rotundata Poir, *D. esculenta* and *D. alata* L. are monocots and belong to the family *Dioscoreacea*. They produce underground edible tubers. Yams are important not only for their nutritional value but have been reported to have medicinal value also (Coursey, 1967; Hegde, 1981). Propagation in yams is generally vegetative through tuber sprouts. In general, a major hindrance in the vegetative propagation in yams is rapid spread of diseases, which causes substantial loss in harvest. In yams, yield loss due to fungal attack may be as high as 70 to 80% (Ghosh *et al.*, 1988), while yield loss due to yam mosaic virus is as much as 50% (Craig, 1964). Thus, production of healthy planting material is becoming difficult, which is otherwise hindered due to biotic and abiotic stresses, sexual degenerancy and absence of true seeds in successive cultivars. International germplasm exchange plays a unique role in crop improvement programmes, introducing genetic variability and has implications for nutritional security. Plant materials free from pests and disease-causing organisms can only be used for germplasm exchange, and thus serves as a medium for introducing variability and vigour. In wake of the problems in conventional breeding, i*n vitro* exploitation of yams will not only eradicate the problems arising in vegetative propagation, but, will also help in faster screening and field establishment of the crop. Meristems are usually free from diseases affecting the mother plant. Regeneration of plantlets from meristems, thus, offers the possibility of producing disease-free plantlets. Regeneration from the *in vitro* established cultures is successfully achieved with the help of phytohormones. Mantell *et al.* (1980) reported the regeneration of plantlets from meristems

of *D. alata* on modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Regeneration from nodal explants of *D.alata* were obtained in MS media supplemented with 1 μM NAA and 5 μM BAP (benzyl aminopurine). Micro-tubers were obtained from aging cultures in 1 μM NAA and BAP supplemented media (Nair *et al.,* 1985). These micro-tubers gives very high rate of regeneration, when transferred to fresh media (Mukherjee, 1999). MS media supplemented with 1 μM NAA and 10 μM BAP were found to be suitable for shoot initiation in axillary nodes of *D. esculenta*. Rooting was however established in media supplemented with 1 μM BAP, 0.2 μM NAA, 0.5 μM IBP and 1 μM 2, 4-D (Nair *et al.,* 1985). Ng and Hahn (1985) obtained plantlets from meristems of *D. rotundata* cultured on MS basal medium supplemented with 1 μM NAA, 0.6 μM BAP and 0.2 μM GA_3. Plantlets were obtained in 16 to 24 weeks. 1 μM NAA and BAP supplemented media was reported to be best for regeneration from nodal explants of *D. rotundata* (Nair *et al.,* 1985). The axillary bud thus developed gives whole plantlet with roots when transferred to fresh media. Microtubers were also reported in aging cultures in the same media.

Malaurie *et al.* (1995) obtained 18% shoot regeneration at 12 weeks from cultured meristems of *D. cayenensis- D. rotundata* complex and *Dioscorea praehensilis* in modified MS medium supplemented with 2.69 μM NAA and 0.44 μM BAP. These studies indicate long period to shoot/plantlet regeneration or low shoot/plantlet regeneration, or both in yams. Results involving several monocots suggest that the level of shoot/plantlet regeneration reported for *Dioscorea* could be increased considerably and the time to regeneration drastically reduced. The study reported herein explored optimum combinations of growth regulators for high and rapid regeneration of three edible species of yam *viz. D. rotundata, D. esculenta* and *D. alata*, their field transfer, establishment and multiplication.

MATERIALS AND METHODS

Mini-tubers of *D. alata, D. rotundata* and *D. esculenta* were grown in field. At 4 to 5 months after planting, the apical and axillary buds of the yam vines were collected. Tuber sprouts were collected from the stored tubers. Explants were surface sterilized, first in 70% (v/v) ethanol for 5 min, transferred into 5% (v/v) and 10% (v/v) sodium hypochlorite for 10 and 20 min, respectively, and finally rinsed thrice in sterile distilled water. Meristems were excised from both the apical and axillary buds. Murashige and Skoog (MS) (1962) medium was modified by supplementing with combinations of plant growth regulators at different concentrations. In the first study, four experiments were conducted involving phytohormones in different combinations. BA had four levels (0.5, 1.0, 2.0 and 2.5 mg/L), NAA had two levels (0.5 and 0.25 mg/L), GA_3 had two levels (1 and 2 mg/L) and ascorbic acid and 2,4-D was added in singe treatment of 1000 mg/L and 0.5 mg/L, respectively. In the second study, callusing was studied with wide range of media with varying degrees of BA (0.5-2.5 mg/L), NAA (0.25-0.5 mg/L), GA_3 (0.5-4 mg/L) and 2,4-D (0.5-1 mg/L), while for shoot organogenesis two, media are formulated varying only in levels of BA (2 and 2.5 mg/L). Media were sterilized for 15 min in an autoclave at a temperature of 121 °C and 15 lb pressure. A volume of 10 ml medium was dispensed per test tube. Two meristems of about 0.6 mm size were cultured per test tube. Cultures were incubated at 25 °C with 12 h photoperiod, under 4,000 lux light intensity. Experimental design was randomized complete block with ten replications. Numbers of buds, shoots and plantlets were counted at 3 and 5 weeks after culture (WAC) and expressed as percentages of number of meristems plated. At 5 WAC, regenerated shoots were sub-cultured in a modified MS proliferation medium for rooting as described by Ng and Hahn (1985) to generate 25 plantlets from each genotype. Regenerated plantlets were observed for uniformity as well as compared to their respective mother plants. Field acclimatization of regenerated plantlets was done using the yam post-flask technique described by Ng *et al*. (1994). At maturity, the tuber characteristics were described using the yam descriptors of the International Plant Genetic Resources Institute (IPGRI) (IPGRI, 1997). Tubers were tested for starch, and dry matter content was established by incubating tubers for 3 days in 35-40 °C for drying, prior to weighing.

RESULTS AND DISCUSSIONS

Plantlet induction was tried for three of the edible yam species using nodal explants of vine tip, and tuber sprouts. *In vitro* cultures were established of the yam species *viz. D. esculenta*, *D.alata* and *D. rotundata*. The establishment of cultures was realized in two pathways. The first was being, direct axillary shoot proliferation and the other being through callusing and organogenesis. The regeneration rate obtained was found to be much higher during callusing and oraganogenesis than in direct axillary shoot proliferation as have been observed in other tuber crops like taro (Mukherjee *et al.,* 1998), sweet potato (Mukherjee, 2002; Mukherjee *et al.,* 2012) etc. Of the two types of explants, tuber sprouts were found to be far superior than the nodal explants of the vine in terms of per cent of explants response and regeneration rate.

1. Axillary Shoot Proliferation

Different growth regulators were supplemented with the basal MS media in varied concentrations, in an attempt to optimize conditions for *in vitro* regeneration. While attempting axillary shoot proliferation (**Fig. 1**), different growth regulators employed in their respective concentration range are, BA (0.5-2.5 mg/L), NAA (0.25-0.5 mg/L), GA_3 (1-2 mg/L), ascorbic acid (100mg/L) and 2,4-D (0.5 mg/L). Percent of explant response was found to be highest in *D. esculenta* (84.2%) followed by *D. alata* (68.5%) and *D. rotundata* (60.5%) (**Table 1**). Regeneration rate measured in terms of mean number of shoots produced per explant was found to be in the range of 2.8-5.4. *D. esculenta* and *D. rotundata* showed maximum regeneration in the media supplemented with BA (1 mg/L), NAA (0.5 mg/L), GA_3 (2 mg/L) and ascorbic acid (100 mg/L) and produced 7.8 and 6.8 shoots/per explant respectively. However, *D. alata* gave highest shoots per plant in the media supplemented with BA (2.5 mg/L), NAA (0.25 mg/L), GA_3 (1 mg/L) and 2,4-D (0.5 mg/L) (**Table 1**). Similarly, explants of *D. esculenta* and *D. rotundata* showed poor performance, and gave 2.9 and 3 mean shoots per explant, respectively in BA (2.0 mg/L), NAA (0.25 mg/L), GA_3 (1 mg/L) and 2,4-D (0.5 mg/L) supplemented media, while *D. alata* gave lowest mean shoots (3.2 ± 0.44) per explant in the media with BA (0.5 mg/L), NAA (0.5 mg/L), GA_3 (2 mg/L) and ascorbic acid (100 mg/L). *D. esculenta* and *D. rotundata* gave similar results with growth regulators. Both the cultivars, showed substantial decrease in mean shoots per explant (40-60 %), when subjected in media with 2,4-D. while *D. alata* showed significant increase(60-100%) in mean shoots per explant in 2,4-D supplemented media (Table 1). Ascorbic acid is an effective additive to the culture media, which can overcome browning effect, which occurs due to phenolic exudates during culture. Thus optimum culture conditions for regeneration in *D. esculenta* and *D. rotundata* are low BA and NAA content and high GA_3 content, while the same is true in *D. alata* in media with 2,4-D.

2. Callusing and Regeneration

Callus induction is an excellent method in introducing variation. The *in vitro* raised explants of three *Dioscorea* species were studied for callus induction in different media taking MS basal as control. The highest rate (**Fig. 2**) of callusing was observed uniformly in *D. esculenta* followed by *D. alata* and *D. rotundata*, for respective treatments. Maximum callusing was observed in the range of 48-70% for all the three cultivars. The dose of 1 mg/L 2,4-D,0.5 mg/L BA. 0.25 mg/L NAA and 0.5 mg/L GA_3 supplemented media was found to be optimum for callus induction (**Table 2**). *D. esculenta* showed highest response of 65-70% followed by *D. alata* (58-66%) and *D. rotundata* (48-54%). It was observed from the callusing response in T_1-T_3 that the callusing response decreased with enhanced doses of BA and GA_3. The rate of callusing continued to decrease with low doses of BA and GA_3, in the absence of 2, 4-D in treated media (T_4-T_6). Thus 2, 4-D was vital for callus induction in this study. Although, only *D. rotundata* and *D. alata* has been involved in studies by Ng and Hahn (1985) and Malaurie *et al*. (1995), the lower concentrations of phytohormone combinations for high rate of callusing obtained in the present study is comparable to the earlier results.

Rate of shoot organogenesis was enhanced 40-60% by optimizing concentrations of BA. All the three species gave 14.6-15.5 mean shoots per 50 mg of callus in media with 2 mg/L BA, while the same was enhanced to 20.5-24.4 in media with 2.5 mg/L BA (**Table 3**). Thus, 2-2.5 mg/L BA, 0.5 mg/L 2,4-D, 1 mg/L GA_3 media supplementation was found to be optimum for shoot organogenesis. All the induced shoots were successfully rooted in modified MS proliferation medium within 3-4 weeks.

3. Hardening and Acclimatization

Hardening was initially done in liquid Hoagland's nutrient. The plantlets were suspended in Hoagland solution for 1-2 weeks, and then transplanted to sterilized soil and sand (1:1) mixture. Field establishment was achieved in high frequency (85-90%) in all the three species of yam. The high percentage of acclimatization (**Fig. 3 and 4**) obtained by treating plantlets with Hoagland's solution prior to transplantation in soil mixture is comparable to the results obtained by Nair (1988). The percent of plant establishment (85-90%) in sterilized soil: sand mixture is much greater than the percent of established plants (60-70%) in unsterilized soil : sand mixture (**Table 3 and 4**).

4. Morphological Characterization and Biochemical Studies.

Morphological characters of the tubers of the regenerants and the mother plant were found to be similar. Yield of *in vitro* raised plants were also observed to be as good as source plants. Tuber qualities like, dry matter and starch content were also at par with the source plant. RAPD profiles of the source and *in vitro* raised plants revealed homology indicating true to type. Thus the results indicate that the growth regulators combinations that induced shoots/plantlets do not bring about significant changes in the plant characteristics of the source genotypes. Hence the results of the present study can successfully be employed to bring about efficient and faster proliferation in yams.

Table 1: Growth response of different yam species in different media

Species	*% of explant response*	*Mean shoots/explant in different growth media (MS + GR mg/L)*			
		BA(0.5)+NAA(0.5)+GA_3 (2.0)+ Ascorbic acid (100)	BA(1)+NAA(0.5)+GA_3 (2.0)+ Ascorbic acid (100)	BA(2.0)+NAA(0.25) +GA_3(1)+ 2,4-D(0.5)	BA(2.5)+NAA(0.25)+ GA_3(1)+ 2,4-D(0.5)
D. esculenta	84.2 ± 2.0	5.2 ± 0.68	7.8 ± 0.82	2.9 ± 0.62	3.2 ± 0.52
D. alata	68.5 ± 1.6	3.2 ± 0.44	3.3 ± 0.63	5.2 ± 0.66	7.4 ± 0.78
D. rotunda	60.5 ± 1.8	4.6 ± 0.65	6.8 ± 0.80	3.0 ± 0.42	3.5 ± 0.65

± Standard error

Table 2: Induction of callus in different treatment media

TreatmentsMS + growth regulators (mg/L)	*% of callusing*		
	D. esculenta	*D. alata*	*D. rotundata*
T_0- MS (control)	-	-	-
T_1- MS + BA(0.5) + NAA (0.25) + GA_3 (0.5) + 2,4-D(1)	65-70	58-66	48-54
T_2- MS + BA(2) + NAA (0.25) + GA_3 (1) + 2,4-D(0.5)	52-60	51-56	41-46
T_3- MS + BA(2.5) + NAA (0.25) + GA_3 (1) + 2,4-D(0.5)	45-54	38-47	33-37
T_4- MS + BA(0.5) + NAA (0.5) + GA_3 (2)	36-41	32-38	27-34
T_5- MS + BA(1) + NAA (0.5) + GA_3 (2)	28-35	22-28	19-24
T_6- MS + BA(1) + NAA (0.5) + GA_3 (4)	21-27	18-24	10-14

Table 3: Callusing and shoot organogenesis

Species	*Mean shoot/ explant (50 mg callus) in different media (MS + growth regulators mg/L)*	
	BA(2.0) + NAA(0.25)+GA_3(1) + 2,4-D(0.5)	BA(2.5) + NAA(0.25) + GA_3(1) + 2,4-D(0.5)
D. esculenta	15.5 ± 1.3	24.4 ± 1.8
D. alata	14.6 ± 1.3	23.2 ± 1.6
D. rotunda	14.6 ± 0.9	20.5 ± 0.5

± Standar error

Table 4: Hardening of *in vitro* plantlets

Species	*Percentage response*		
	Hoagland's mixture	*Sterilized soil:sand*	*Unsterilized soil: sand*
D. esculenta	92-96	89-91	67-70
D. alata	90-92	86-88	62-64
D. rotunda	86-88	85-88	63-66

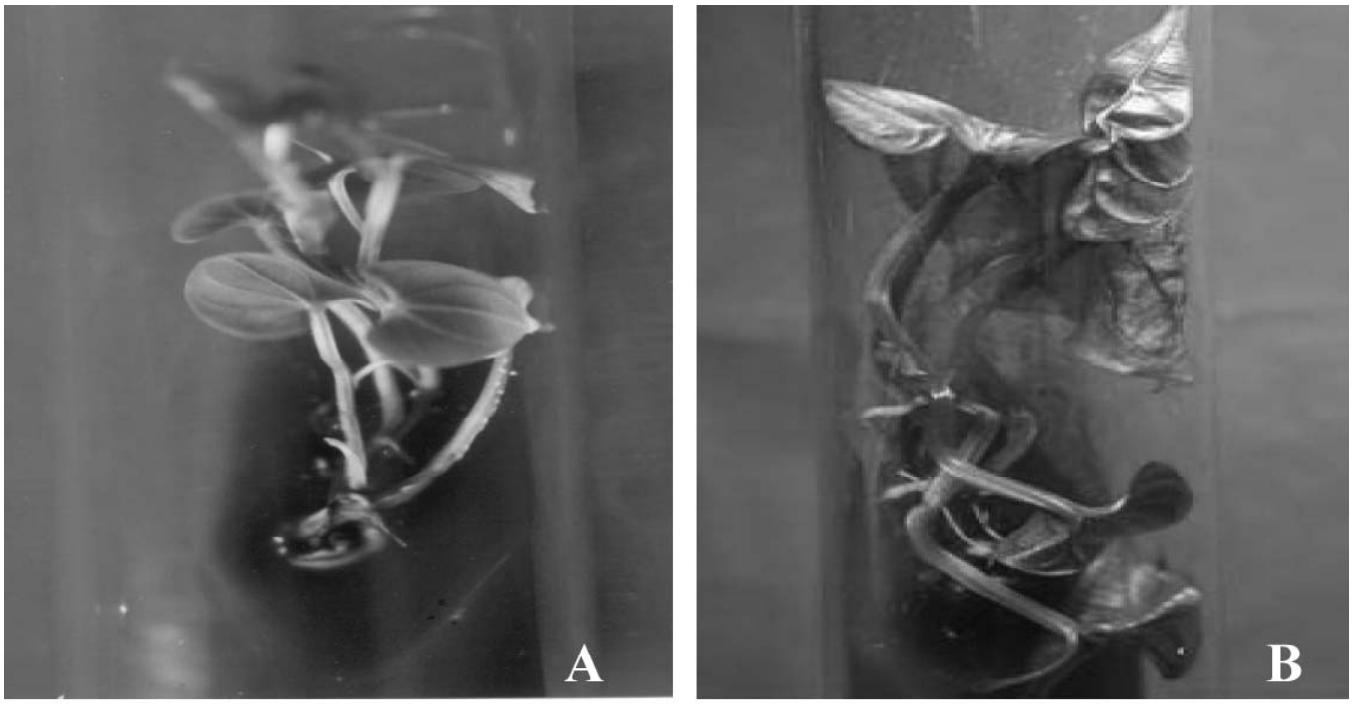

Fig. 1: A & B. Axillary shoot proliferation in yam.

Fig. 2: Callusing and regeneration in *D. alata.*

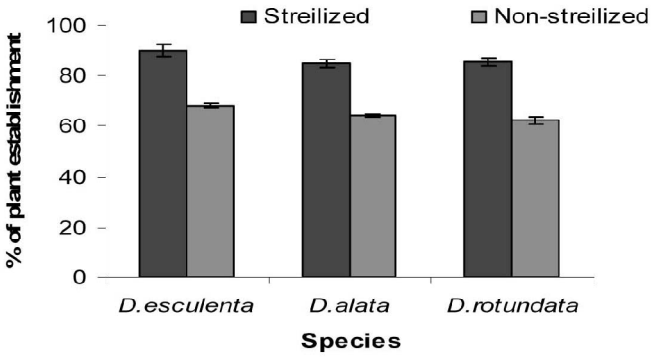

Fig. 3: Plant establishment (%) after 1-2 weeks pre hardening in liquid nutrient media.

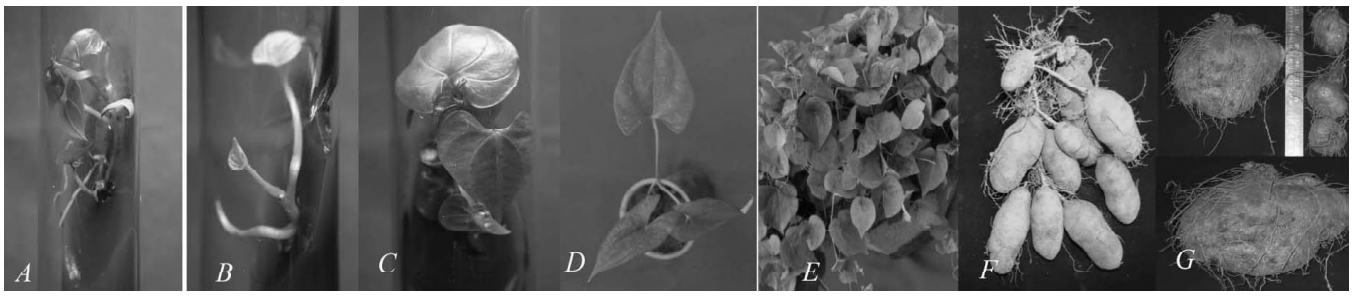

Fig. 4: Micropropagation in yams (Hardening and field establishment).

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