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Rapid protocol for callus induction and differentiation of roots and shoots in *Dioscorea alata*—a medicinal plant

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ABSTRACT

The present study was carried out to investigate the effect of different concentrations of growth regulators on *in vitro* differentiation of *Dioscorea* through nodal explants which provide a fast, reliable and alternate method of multiplication and facilitate *ex situ* conservation of this valuable plant species. Nodal explants excised from *in vivo* grown plants were cultured on Murashige and Skoog (MS) medium supplemented with different combinations of plant growth substances. The maximum (100 per cent) callus induction as well as growth was obtained on NAA (2.5 mg/l). The induced callus was transferred onto the nutrient media supplemented with different combinations of growth regulators for the differentiation of roots and shoots. Roots were developed on MS medium + NAA (2.0 mg/l) + IBA (0.5 mg/l), shoots on MS + BAP (2.0 mg/l) + NAA (0.5 mg/l) and roots-shoots on MS + NAA (2.5 mg/l) + 2, 4-D (2.0 mg/l) from the callus.

Key words : Callus induction, Differentiation, NAA, Yam

The greater yam (*Dioscorea alata* L.) is the most widely distributed species of the genus *Dioscorea* in the tropics. It is an important food source for millions of poor in the Asian, African and American tropics. Despite huge economic importance, its commercial cultivation is constrained due to the limited supply of elite planting stock (Lebot *et al*. 1998). Traditionally, yams are propagated vegetatively by seed tubers or setts. However, these methods are inefficient for the large-scale multiplication of superior clones and also increase the risks of transfer of diseases in the international trade (Otto *et al*. 2005). The plant tissue culture (PTC) techniques provide an efficient alternative for the production of elite plants and desirable medicinal compounds from the plants (Rao and Ravishankar 2002). Besides, PTC technology has great potential for the conservation of genetic diversity. Plant tissue culture techniques have been successfully used for a rapid clonal multiplication of high yielding genotypes or for the production of specific virus-free plants (Mantell *et al*. 1978).

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There are few reports on *in vitro* multiplication of *Dioscorea* through explants (Shu *et al*. 2005). However, this technique has not been routinely applied due to the problems of tissue oxidation and necrosis (Belarmino and Gonzales 2008). Reports on *de novo* regeneration in *D. alata* are limited to callus derived from petiole explants (Fautret *et al*. 1985) and somatic embryos derived from the root cells (Twyford and Mantell 1996).

The differentiation and the formation of roots and shoots from callus have been known in plant tissue culture of various origins for a long time. Callus cultures have been found useful for nonconventional techniques of crop improvement like genetic transformation. Development of new variants following irradiation of callus and its subsequent organogenesis has been found very effective technique in crop improvement (Peer *et al*. 2012). Nutrients and type of hormones supplemented to the medium regulate the formation of roots and shoots in cultured explants depending upon their concentrations and interactions (White 1939). Keeping in view the importance of callus organogenesis, present studies was to initiate and maintain the calli produced from nodal explant and to define the nutritional conditions for their regeneration.

MATERIALS AND METHODS

The nodal explants obtained from field grown plants were washed thoroughly in the running tap water. Subsequently, the explants were washed with 1.0 % teepol

solution for 10 min by vigorous stirring. Further operations were carried out aseptically under a laminar air flow cabinet. The explants were surface sterilized with 0.1% mercuric chloride (2 min) followed by 70% ethanol (1 min) and thereafter washed 4-5 times with sterile distilled water to remove traces of mercuric chloride. The MS basal medium (Murashige and Skoog 1962) containing 3% sucrose and 0.8% agar along with growth regulator was poured into 150 ml flasks, having 30-40 ml of the medium. The pH of the medium was adjusted to 5.8 with 1N sodium hydroxide (NaOH) or 1N hydrochloric acid (HCl), prior to addition of agar and autoclaving at 121ºC, 1.2 kg/cm^2 pressure for 15 min. All the chemicals used were of analytical grade. All the cultures were incubated at 26 ± 2 ºC under white fluorescent light with a photoperiod of 16 hr light (intensity of 2000 lux) and 8 hr of darkness. The surface sterilized explants were inoculated on MS medium supplemented with auxins (NAA, 2, 4-D, IBA) alone or in combination with cytokinin (BAP) for callus initiation. After 4-weeks, the callus obtained from nodal explants was transferred on different combinations of plant growth substances for differentiation of roots and shoots. All experiments were repeated at least twice, using 8-10 replicates (flasks) each containing three explants. The data were analyzed statistically using completely randomized design and the significance was tested at 5% level of critical difference using the table 'ANOVA'.

RESULTS AND DISCUSSION

We noted variations in the morphology, colour and texture of the callus cultures with different plant growth regulators and their concentrations. Interestingly, there was no response for callusing from the leaves, internodal explants taken *in vitro* and *in vivo* (root tuber) grown plants in the MS medium without growth regulators except nodal explants. The callogenic potential of the explants depended upon the growth regulator supplements. This showed that exogenous supply of plant growth regulators was required to disturb the established polarity of auxins in the explants.

The data on callusing and callus growth are furnished in Table 1. It was noted that the MS medium supplemented with 2, 4-D at different concentrations (0.5, 1.0, 1.5 and 2.0 mg/l) gave 15-25% callusing within 13 days but the growth of callus was poor. It was also observed that NAA (2.0 and 2.5 mg/l) alone and in combination with IBA (0.25 and 0.5 mg/l) gave good callus growth even up to 4 weeks after inoculation (Fig 1-4). However, on the MS medium supplemented with NAA (1.0 mg/l and 2.0 mg/l) along with BAP (0.5 and 1.0 mg/l), no callusing or poor callus induction was observed. The earlier experiments have proved that the plant explants require an optimum concentration of growth regulators for the proliferation into unorganized callus. Our results are consistent with the earlier reports on *Dioscorea* opposite (Araki *et al*. 1992), *Lycopersicon esculentum* (Magdoleen *et al*. 2010) and *Solanum xanthocarpum* (Sundar and Jawahar 2011) where NAA alone has been used for callus induction.

Table 1 *In vitro* response of nodal explant of *D. alata* on MS medium supplemented with different growth regulators after 6-weeks.

Medium + growth regulators (mg/l)		Callus induction		
		% explants callusing	Days for callus induction	Visual growth of callus after 4-weeks*
Control	0			
2,4-D	(0.5)	0		
	(1.0)	15	13	+ (Yellow, fragile)
	(1.5)	25	13	+ (Yellow, fragile)
	(2.0)	0		+ (Yellow, fragile)
NAA	(0.15)	0		
	(0.5)	0		
	(1.0)	25	10	++ (Brown, compact)
	(1.5)	67	10	++ (Brown, compact)
	(2.0)	75	10	+++ (Brown, compact)
	(2.5)	100	8	+++ (Pinkish brown, compact)
	(3.0)	0		
	(4.0)	0		
NAA	(1.0)			
+ IBA	(0.5)	33	24	++ (Brown, fragile)
NAA	(2.0)			
+ IBA	(0.25)	50	38	+++ (Brown, compact)
	(0.5)	66	38	+++ (Brown, compact)
	(1.0)	33	38	++ (Brown, compact)
	(2.0)	15	38	+ (Brown, compact)
NAA	(1.0)			
+ BAP	(0.5)	0		
	(1.0)	0		
NAA	(2.0)			
+ BAP	(0.5)	15	37	+ (Green, fragile)
	(1.0)	0	—	—
	(2.0)	0	—	—

*– No callus, + Poor callus, ++ Moderate callus, +++ Good callus

Based on these insights, we subsequently selected different combinations of NAA and IBA (Table 2) to find out the high growth value of callus in terms of fresh and dry weight. For this purpose, the callus derived from the nodal explants were subcultured for 4-weeks on MS medium supplemented with NAA (2.0 and 2.5 mg/l) alone and in combination with IBA (0.25 and 0.5 mg/l). The best growth of callus was observed on MS + NAA (2.5 mg/l) with pinkish brown, compact callus in 8 days (Table 2 and Fig 1).

Visual observations like number of days required for induction of callus, per cent explant callus induction, colour, shape and texture of callus were recorded periodically and used to select best callus induction medium. The number of replicates was 10-15. Based on these observations, this

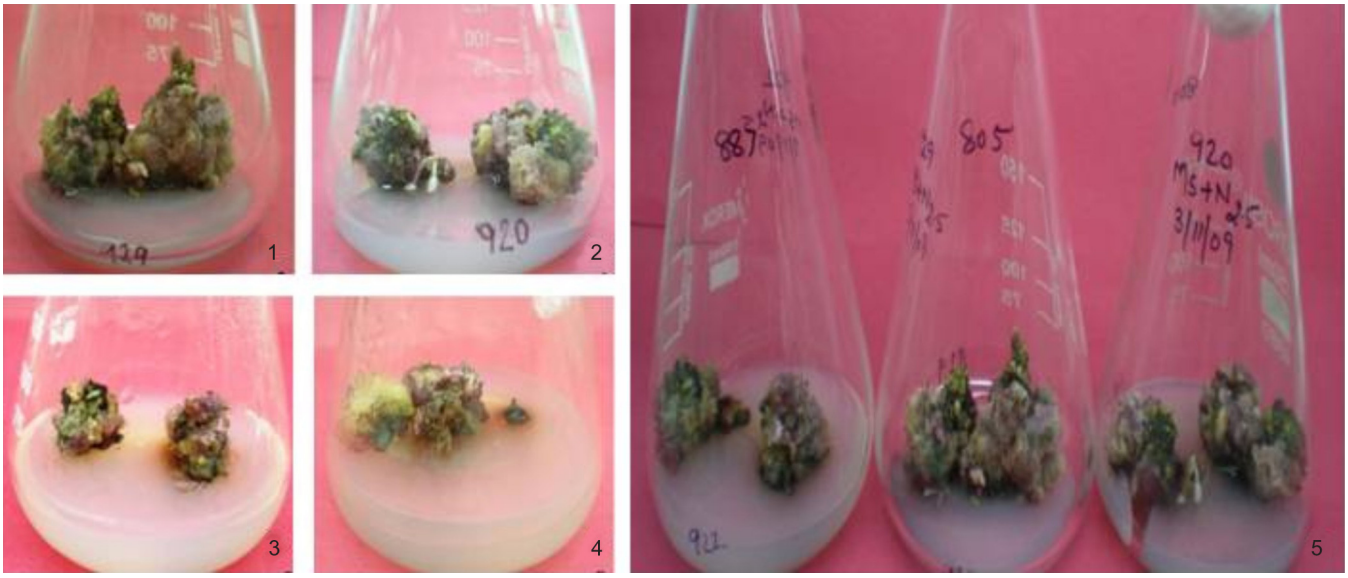

Fig 1 1-4 Depicting good callus growth from nodal explants on MS medium supplemented with (Fig. 1) NAA (2.0 mg/l), (Fig. 2) NAA (2.5 mg/l), (Fig. 3) NAA (2.0 mg/l) + IBA (0.25 mg/l) and (Fig. 4) NAA (2.0 mg/l) + IBA (0.5 mg/l) in *Dioscorea alata.* 1-5: Depicting high growth value callus (in terms of fresh and dry weight) from nodal explants on MS medium supplemented with NAA (2.5 mg/l) alone in *Dioscorea alata*.

Table 2 Callus growth on various concentrations of NAA alone or in combination with IBA supplemented to MS medium on fresh and dry weight of 4-week old calli derived from nodal explants of *Dioscorea alata* L.

Medium + Growth regulators (mg/l)	Weight of callus (g ± SE)	
	Fresh weight	Dry weight
MS + NAA (2.0)	3.225±0.059	0.239±0.019
MS + NAA (2.5)	3.45±0.107	0.432±0.019
MS + NAA (2.0) + IBA (0.25)	3.211±0.082	0.272±0.032
MS + NAA (2.0) + IBA (0.5)	3.121±0.248	0.227±0.039

CD – NS, SE – Standard error of mean

combination was selected for further callus maintenance and growth studies.

The undifferentiated callus when subcultured on MS medium without growth regulators failed to grow and there was no organogenesis. MS medium supplemented with 2, 4-D (0.5 and 1.0 mg/l) and NAA (0.5, 1.0, 2.0, 2.5 and 3.0 mg/l) alone showed poor to good callus growth but no organogenesis was observed. Differentiation process is complex in plant tissues and is controlled by various factors such as genotype, growing condition of donor plant, type and concentrations of hormones, nutrient medium and developmental stage of the explants during *in vitro* culture (Kumar *et al*. 2008). Auxins alone (NAA, IBA or IAA) gave

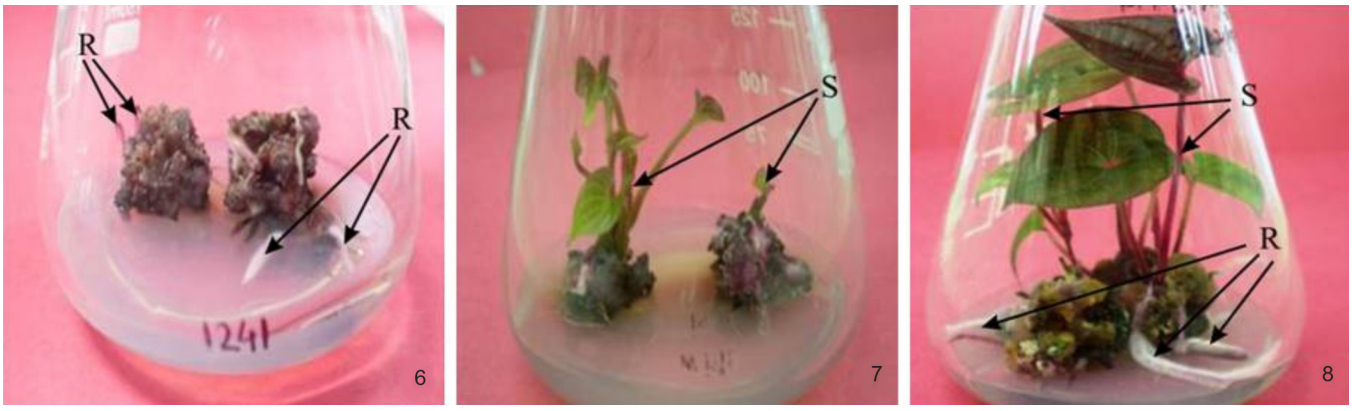

Fig 2 6-8 Formation of roots on MS medium supplemented with NAA (2.0 mg/l) + IBA (0.5 mg/l) (Fig. 6), shoots on MS medium supplemented with BAP (2.0 mg/l) + NAA (0.5 mg/l) (Fig. 7) and roots as well as shoots on MS medium supplemented with NAA (2.5 mg/l) + 2, 4-D (2.0 mg/l) (Fig. 2) from callus of nodal explants in *Dioscorea alata*. R – roots, S – shoots

Table 3 Effect of growth regulators supplemented to MS medium on nodal explants derived callus from *D. alata* for differentiation (observations recorded upto 6-weeks after inoculation)

Medium + growth regulator (mg/l)		Visual growth of callus after 4-weeks *	Colour and texture of callus	Differentiation (Days required)
Control		Brown, compact		
2, 4-D	(0.5)	+	Yellow, fragile	
	(1.0)	+	Yellow, fragile	
NAA	(0.5)	+	Black, compact	
	(1.0)	+	Black, compact	
	(2.0)	++	Brown, compact	
	(2.5)	+++	Pinkish brown, compact	
	(3.0)	++	Brown, compact	
NAA	(2.0)			
+ IBA	(0.1)	+	Brown, compact	
	(0.5)	+++	Pinkish brown, compact	Roots (15)
	(1.0)	++	Brown, compact	Roots (34)
NAA	(2.5)			
+ 2, 4-D	(1.0)	++	Brown, compact	
	(2.0)	+++	Brown, compact	Roots (28) & Shoots (31)
	(3.0)	++	Brown, compact	
BAP	(2.0)			
+ NAA	(0.5)	+++	Pinkish brown, compact	Shoots (16)
	(1.0)	+++	Pinkish brown, compact	
	(1.5)	++	Green, fragile	Abnormal shoots (34)
	(2.0)	+	Black, compact	
BAP	(2.0)			
+ IBA	(0.2)	+	Green, fragile	Shoots (42)
	(1.0)	++	Green, fragile	Shoots (36)

*– No callus, + Poor callus, ++ Moderate callus, +++ Good callus

rhizogenesis response from the callus. It was observed that MS medium supplemented with NAA (2.0 mg/l) along with IBA (0.5 and 1.0 mg/l) induced rooting in *D. alata* (Table 3 & Fig. 6) but 1.0 mg/l IAA respond in 34 days for rooting. Similar results have also been reported in *Chlorophytum borivilianum* (Joshi *et al*. 1998), *Momordica charantia* (Agarwal and Kamal 2004) and *Momordica cymbalaria* (Nikam *et al*. 2009).

In the present study, differentiation of shoots was found to be dependent on synergistic effect of auxin along with cytokinins in the medium which evoked good results for indirect shoot proliferation. It was observed that MS medium supplemented with BAP (2.0 mg/l) along with NAA (0.5 mg/l) resulted in shoot formation from the callus (Table 3 and Fig 2). Such variability in regeneration from callus has also been reported in *Dioscorea esculenta* (Nair and Chandrababu 1996), *Chlorophytum borivilianum* (Singh *et al*. 2006), *Cardiospermum halicacabum* (Kumar *et al*. 2008) and *Dioscorea* spp (Asha and Nair 2009). However, NAA (2.5 mg/l) along with 2, 4-D (2.0 mg/l), showed good growth of callus with the formation of roots and shoots within 28 and 31 days after inoculation respectively (Table 3, Fig 2).

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