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IMPROVED AND EFFICIENT PROTOCOL FOR DIRECT REGENERATION FROM COTYLEDONARY NODE OF GROUNDNUT (*ARACHIS HYPOGAEA* L.)

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ABSTRACT
Protocol has been developed for micropropagation of *Arachis hypogaea* L. under *in vitro* conditions. Cotyledonary node explants gave rise to multiple shoots when cultured on MS (Murashige and Skoog) medium supplemented with different concentrations of TDZ, BAP and Kin (Kinetin) singly along with auxin. The highest response of shoot multiplication was obtained in MS containing 1.0 mg/l TDZ and 0.5 mg/l NAA. Multiple shoots were transfer to elongation medium augmented with Gibberellic acid (GA_3) + Indole butyric acid (IBA). The regenerated shoot lets were rooted on MS basal medium with different concentrations of IBA (Indole butyric acid) and NAA (Naphthalene acetic acid). The maximum frequency of rooting and highest number of roots were produced on medium containing IBA 0.5 mg/l. The plantlets, thus successfully established in soil and later to field.

KEYWORDS: Groundnut, Cotyledonary node, Shoot organogenesis, Thidiazuron, Plant micropropagation.

INTRODUCTION
Groundnut (*Arachis hypogaea* L.) is an economically important oil and protein rich crop whose seeds contain about 40 to 50% oil and 26 to 28% protein that has a significant impact in tropical and sub-tropical regions of Asia, Africa, North and South America. It is being cultivated on over 25.2million ha worldwide with a total production of 41.2 million tons with an average yield of 1.67 tons/ha. India is the second largest producer of groundnut accounting 8 million tons from 6 million ha (FAOSTAT, 2014) which needs to be increased up to 15.8 million tons by 2025 to meet the growing demand. Productivity of ground nut has been low due to many biotic and abiotic stresses. There are several common diseases of groundnut crops viz., bacterial wilt and bacterial scab. Fungal diseases have resulted in decreased trend of its yield/acre. Conventional breeding approaches seem to be inadequate due to restricted direct gene pool and sexual incompatibility amongst the wild relatives (Sing and Sing 1989). In order to exploit direct gene transformation, an efficient regeneration system is pre-requisite.

Direct organogenesis from pre-existing meristem may provide a reproducible high frequency regeneration system. Though several protocols were developed. These protocols were specific for some genotypes and often not reproducible. So there is a need for further improvement in shoot induction. Except cotyledonary node root, leaf and stem it is noticeably one of the least exploited tissues in groundnut tissue culture and genetic engineering, primarily, because of its reticence and lower responses for adventitious plant formation *in vitro* compared to other tissues, such as (McKently *et al*. 1990; Banerjee *et al*. 2007).

The present study exploits cotyledonary node potential to form reliable shoots *in vitro*. It reports the most efficient protocol that applies different treatments to achieve greater results for abundant multiple-shoot formation that outperforms all existing improved groundnut protocols for shoot induction in cotyledon node (Tiwari and Tuli 2011; Aina *et al*. 2012). The present success could increase the usage of these tissues in tissue culture for *in vitro* commercial and scientific micro-propagation as well as genetic engineering for crop improvement purposes.

MATERIAL AND METHODS
Seeds of groundnut cultivars GPBD-4 were surface sterilized with 0.1% (m/v) mercuric chloride for 4 - 5 min. Seeds were rinsed with sterile distilled water about 4 - 5 times and germinated on filter paper bridges containing sterile distilled water. Cotyledonary node

were excised aseptically from 4 to 5-days-old germinating seeds and inoculated on to Murashige and Skoog's (1962) medium supplemented with different concentrations of N^6-benzyl-aminopurine (BAP), thidiazuron (TDZ) and kinetin (KIN) alone or in combination with NAA or indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA). Observations were recorded on a weekly basis. After a month, shoots more than 3 cm in length were transferred onto rooting medium. Those less than 3 cm long were cultured on shoot elongation medium containing MS salts and supplemented with various concentrations and combinations of GA 3, TDZ and NAA. After 30 days, shoots (4 - 5 cm long) with at least 3 - 4 internodes were excised from shoot clumps and transferred to half-strength MS medium containing different concentration of NAA and IBA. All the media contained 3% (m/v) sucrose, pH adjusted to 5.8 and solidified with 0.8% agar before autoclaving at 121^0C for 20 min. All the cultures were maintained at a temperature of 25 $\pm 2^0$C, 16-h photo-period with irradiance of 50 - 100 µmol m^{-2} s^{-1} provided by cool white, fluorescent lamps. Rooted plantlets were transferred to medium without hormones for two weeks for pre hardening. Finally plantlets were transferred to pots containing sterilized soil and sand in the ratio 3:1.

The experimental design was random and factorial with auxins and cytokinins as independent variable. The data pertaining to number of shoot and root formation were subjected to analysis of variance (*ANOVA*). Mean separation was done using Duncan's new multiple range test. Thirty cultures were raised for each treatment and all experiments were repeated thrice.

RESULTS AND DISCUSSION

Groundnut is a legume of economic importance, whose improvement could greatly benefit from the integration of both classical and modern techniques. Although biotechnology techniques such as tissue culture and gene transformation have been reported in groundnut.

Naturally, Cotyledonary node is a meristamatic tissue and is different, from other standard organs, such as, leaf, stem, and root. Hence, the expectation for morphogenetic responses is generally high. The results of this study demonstrate that the groundnut cotyledon node is potentially as efficient as other standard organs for *in vitro* plant formation. The study also resulted in the successful, direct adventitious shoot formation in groundnut to avoid chimeric plants in plant transformation. The age of the explants has been reported to be an important factor in obtaining regeneration in chickpea (Polisetty *et al*. 1997). Fruitful results have been obtained using cotyledonary node taken from 6 to 7-d-old seedlings (Chakraborti *et al*. 2006; Kiran *et al* 2009).

In our preliminary experiments shoot-bud were elicited from cotyledonary node explants, cultured on MS medium supplemented with different concentration of TDZ (0.25 - 2.0 mg/L) BAP (0.25 - 2.0 mg/L) and KIN (0.25 - 2.0 mg/L). Among the different cytokinine, TDZ was found to be good compare to other hormone tested. The best performing treatment of the studies was the TDZ concentration that formed the large number of shoots (41) and per explant and rate of explants that formed shoots (100%) (Table 1). Although high performances have generally been associated with low concentrations of TDZ in tissue culture across plant species. (Mok *et al.* 1987, Akasaka *et al.* 2000, Anuradha *et al*. 2006, Karami and Kordestani 2007) which justifies related common practices, we have consistently associated greatest responses with greatest concentrations of TDZ in groundnut (Mat and Prakash 2007) and (Matand *et al*. 1994).

Cotyledonary node explants as the best source of multiple shoot induction have been suggested in chickpea (Sanyal *et al.* 2005: Kiran *et al* 2009) and bean (Gang and Wei 2009). In earlier reports, IBA was the auxin most frequently used for achieving regeneration in chickpea (Sharma and Amla 1998). In the present study however, the response to IBA was poorer as it induced callusing along with shoot buds which hindered further development. Consequently, the shoot buds remained as a crumpled mass or distorted leafy structures which is a well-known problem encountered during regeneration in legumes (Batra *et al.* 2002). Conversely, when NAA was added along with TDZ into the primary induction medium much better results were obtained. There was no callusing and a large number of shoot buds were induced (Table 2). NAA at all concentrations induced multiple shoots with varied frequency, but at 0.5 mg/l along with TDZ (1.0 mg/L) yielded the best results (100% frequency and 47 shoots per explant; Fig. 1). Multiple shoots were induced better in the presence of NAA followed by IBA; such a response of NAA was also observed by (Kiran *et al*. 2009). The direct formation of buds became visible on the proximal end of cotyledonary node after 6 - 8 d of culture and after 25 - 30 d these buds were proliferates. Between the two cultivars, GPBD-4, produced more shoots per culture as compared to TMV-2 (Table 1 and 2), suggesting genotypic dependency on shoot regeneration as earlier reported in other legumes (Ma and Wu 2008).

Our studies revealed that the morphogenic response of cotyledonary node was asynchronous. A buds large number of shoot differentiated within 3 - 4 weeks in the primary shoot-bud induction medium (NAA + TDZ). Three types of shoot formed in the primary culture. Those that were 3 cm or longer and could be directly transferred on the rooting medium.

Our studies revealed that the morphogenic response of cotyledonary node was asynchronous. A large number of shoot buds differentiated within 3 - 4 weeks in the primary shoot-bud induction medium (Ga3 + IBA). Three types of shoot formed in the primary culture. Those that were 3 cm or longer and could be directly

transferred on the rooting medium. The shoot buds that were 1 cm or longer elongated best on medium supplemented with 1.0 mg/l GA_3 in the first subculture (Table 2). The presence of GA_3 in the medium also prevented hyper-hydricity in elongated shoots (Fiore *et al*. 1997). The third category of buds (smaller than 1 cm), were elongated on IBA plus GA_3 (Table 3). On medium supplemented with 0.5 mg/l IBA + 1.0 mg/l GA_3, larger numbers of shoots elongated as compared to medium containing 1.0 mg/l GA_3 (Table 3).

In present experiments, elongated shoots were transferred to ½ strength MS medium supplemented with IBA or NAA or PAA at different concentration (0.25 - 2.0 mg/l). The highest rooting frequency and number of roots were observed on medium containing 0.5 mg/l NAA followed by 1.0 mg/l NAA (Table 3). From the results it is evident that NAA is a very effective plant growth regulator for production of normal, elongated whitish roots in groundnut whereas IAA and IBA showed a marked tendency towards poor rhizo-genesis (Table 4).

Regenerates were successfully transferred to polycups containing a mixture of soil and sand in a ratio of 3:1 (Fig.1 e.) and later to the field with 74% survival rate. Tissue culture regenerated plants produced normal flowers and set the seeds and no morphological variations were observed.

This protocol has several distinct advantages over the earlier published protocols. They are initial explants cotyledonary node is available irrespective of season in direct organogenesis time period is reduced by avoiding callus phase and IBA is a less expensive than IAA which offers great economy for routine regeneration. This protocol can be also used in transformation experiments.

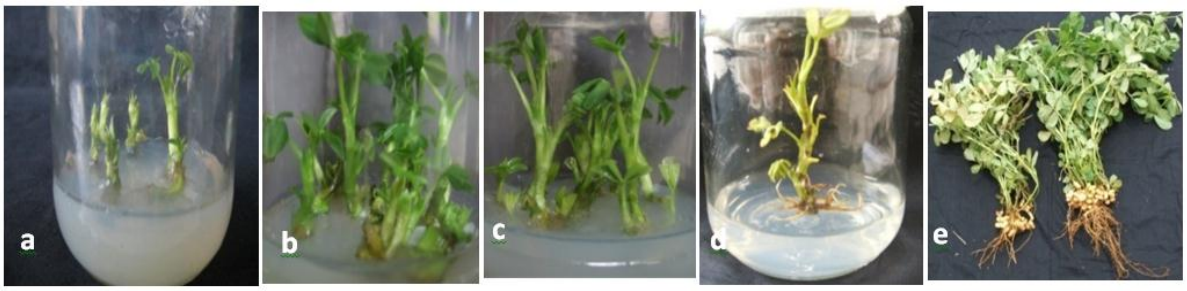

Fig. 1: Cotyledonary node explants showing multiple shoot formation.

a) Large number of shoot buds differentiated within 3 - 4 weeks in the primary shoot-bud induction medium with TDZ+ NAA.
b) Proliferation of multiple shoot in primary culture after 4 weeks on medium supplemented with TDZ 2.0 mg/l BAP + 1.0 mg/l NAA.
c) Elongated shoots in first subculture on MS medium + 0.4 mg/l GA_3 + 0.2 mg/l IBA
d) Elongated shoots in half strength MS medium with IBA or NAA at different concentration (0.25 - 2.0 mg/l).
e) Tissue culture raised plants harvested after 4 months.

Table 1: Bud organogenesis in cultured cotyledonary node of. GPBD-4 on MS medium supplemented with BAP, TDZ and Kinetin.

PGR	Concentration	TMV-2		GPBD-4	
	mg/ l	Frequency (%)	Shoot number (explant-1)	Frequency (%)	Shoot number (explant-1)
TDZ	2.0 + 0.25	80	35 ± 0.57d	82	34± 0.57c
	2.0 + 0.50	85	41.3± 0.88b	90	44± 1.20b
	2.0 + 1.00	95	44.5± 0.57a	100	47± 0.33a
	2.0 + 1.50	74	38± 1.20c	76	42.3± 0.88b
	2.0 + 2.00	61	21.5± 0.68g	50	14.2± 0.44f
	2.2 + 2.50	32	0.5± 0.86j	40	7± 0.44h
BAP	2.0 + 0.25	69	15.9± 0.96h	75	19.7± 0.38e
	2.0 + 0.50	62	31± 0.57e	66	36± 0.78c
	2.0 + 1.00	46	27± 0.33f	62	29.5± 0.48d
	2.0 + 1.50	21	14.6± 0.65h	28	10± 0.49g
	2.0 + 2.00	18	4.4± 0.56j	22	5± 0.68i
Kinetin	2.0 + 0.25	28	13.4± 1.14h	26	11± 0.65g
	2.0 + 0.50	55	22.6± 0.88g	67	21.6± 0.69e
	2.0 + 1.00	20	15.6± 1.20h	29	14± 0.98f
	2.0 + 1.50	13	8.± 0.95i6	11	3± 0.59j
	2.0 + 2.00	13	5.2± 1.25j	8	2.9± 0.62j

Table 2: Bud organogenesis in cultured cotyledonary node of. GPBD-4 on MS medium supplemented with BAP along with PAA, IBA or NAA.

PGR	Concentration	GPBD-4		TMV-2	
	mg/ l	Frequency (%)	Shoot number (explant-1)	Frequency (%)	Shoot number (explant-1)
TDZ +NAA	2.0 + 0.25	80	35± 0.57d	78	25±0.57^{c}
	2.0 + 0.50	89	41.3± 0.88b	86	35±1.10^{b}
	2.0 + 1.00	100	44.5± 0.57a	87	31± 0.33^{a}
	2.0 + 1.50	78	38± 1.20c	78	20±0.88^{b}
	2.0 + 2.00	60	21.5± 0.68g	53	21±0.44^{f}
	2.2 + 2.50	33	5± 0.86j	38	15±0.54^{h}
TDZ +IBA	2.0 + 0.25	78	15.9± 0.96h	76	12.9±0.35^{e}
	2.0 + 0.50	65	31± 0.57e	68	26±0.88^{c}
	2.0 + 1.00	43	27± 0.33f	69	19±0.58^{d}
	2.0 + 1.50	29	14.6± 0.65h	21	10.2±0.59^{g}
	2.0 + 2.00	15	4.4± 0.56j	20	3.0±0.78^{i}
TDZ+PAA	2.0 + 0.25	30	13.4 ± 1.14h	26	11±0.75^{g}
	2.0 + 0.50	53	22.6± 0.88g	62	15±0.69^{e}
	2.0 + 1.00	18	15.6± 1.20h	28	35±0.88^{f}
	2.0 + 1.50	16	8.6± 0.95i	12	35±0.59^{j}
	2.0 + 2.00	14	5.2± 1.25j	8	35±0.62^{j}

The experiment was repeated thrice.
Means ± SE, n = 25. Means followed by same superscript in a column are not significantly different at P = 0.05.

Table 3: Elongation of shoot buds (formed in primary cultures) upon sub-culturing on MS medium supplemented with different plant growth regulators. The experiment was repeated thrice.

Longer than 1 cm			Shorter than 1 cm					
GA_3	Frequency	shoot number	GA_3+PAA	Frequency	shoot number	GA_3+IBA	Frequency	shoot number
mg/ l	frequency %	explant^{-1}	mg/l	frequency %	explant^{-1}	mg/ l	frequency %	explant^{-1}
0.25	78	5± 0.57d	0.2 + 0.1	52	5± 0.45d	0.2 + 0.1	12	3.8 ± 0.59b
0.5	89	8.5± 0.64c	0.2 + 0.2	73	11.5± 0.96c	0.2 + 0.2	13	3.4 ± 0.45c
0.75	98	16.6± 0.83a	0.2 + 0.3	88	14± 0.89b	0.2 + 0.4	10	4.9 ± 0.53a
1	82	11.5± 0.66b	0.2 + 0.5	99	17.5± 0.65a	0.2 + 0.6	0	0
1.25	74	4± 0.23d	0.2 + 0.8	73	10± 0.76c	0.2 + 0.8	0	0
1.5	52	1± 0.43e	0.2 + 1.0	54	4.5 0.63d	0.2 + 1.0	0	0

Means ± SE, n = 25. Means followed by same superscript in a column are not significantly different at P = 0.05

Table 4: In vitro rooting of elongated shoots supplemented with different concentration of IBA, NAA and PAA.

PGR	Concentration	GPBD-4		TMV-2	
	mg/ l	frequency %	root number (explant –I)	frequency %	root number (explant-1)
PAA	0.25	82	4.3± 0.48d	92	5.30.+68c
	0.5	98	9± 0.58a	100	15.5 ± 0.46a
	1	79	7.3± 0.64b	100	13± 0.66b
	2	72	3± 0.66d	74	4.5± 0.98d
IBA	0.5	62	6.4± 0.63c	54	4.3± 0.86d
	1	32	3± 0.83e	38	2± 0.64e
NAA	0.5	24	2± 1.00f	32	1.5± 0.58f
	1	32	2.9± 1.10e	24	2.5± 1.20e

The experiment was repeated thrice. Means ± SE, n = 25. Means followed by same superscript in a column are not significantly different at P = 0.05

CONCLUSSION

Cotyledonary node explant responded better than any explant was observed in both NAA and TDZ. Large number of shoot differentiated explants with TDZ and NAA. Maximum rooting frequency and number of roots were observed on medium containing 0.5 mg/l NAA followed by 1.0 mg/l NAA. This study is a baseline to

carry further research on this groundnut variety for improvement by using gene transfer technology.

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