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IN VITRO DIRECT ORGANOGENESIS AND PLANTLET FORMATION FROM DE-EMBRYONATED COTYLEDON EXPLANTS OF PEANUT (*ARACHIS HYPOGAEA* L.)

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ABSTRACT

Peanut/Groundnut (*Arachis hypogaea* L.) is economically the most important species of the Legume family. It is cultivated for its oil and proteins. The present study reports on the direct induction of multiple shoots from de-embryonated cotyledon explants of peanut cvs ICG 7827 and ICG 13942 which is prerequisite for genetic transformation experiments. Longitudinally halved cotyledons were inoculated on shoot induction medium (SIM) containing MS salts with B5 vitamins supplemented with different concentrations and combination of plant growth regulators (BAP/TDZ, NAA/IAA+BAP/TDZ). Maximum number of direct multiple shoots/explant (45.67±0.23) with 94% of shoot conversion was found at 0.5 mg/L IAA+15 mg/L TDZ in cv ICG 13942 followed by 37.82±0.11 multiple shoots/explant with 85% of conversion at 0.5 mg/L NAA+20 mg/L BAP in cv ICG 7827. The individual shoots were elongated on shoot elongation medium (SEM) fortified with 0.5 mg/L BAP. For *in vitro* rooting, these elongated micro-shoots were transferred on to root induction medium (RIM) supplemented with 1 mg/l NAA. *In vitro* rooted plantlets were transferred to soil: sand: vermicompost (1:1:1) mixture and subsequently these were acclimatized in the green house. Thus, the regeneration protocol developed in the present investigation can be used for *Agrobacterium* mediated genetic transformation to transfer fungal resistant genes.

KEYWORDS: ***Arachis hypogaea*, cotyledons, multiple shoots, *in vitro* rooting, plantlet establishment.**

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INTRODUCTION

Peanut/Groundnut (*Arachis hypogaea* L.) is the 13th most important food crop of the world. It is the world's 4th most important source of edible oil and the 3rd important source of vegetable protein [1]. The seeds are mostly used to supply vegetable oil (50%), carbohydrates (5-10%) and proteins (25-30%) for human as well as animal consumption [2]. Peanuts are also a safe alternative to reduce hunger in Asia, Africa and Latin America. Peanut yields are substantially reduced because of the damage caused by subterranean insects, bacterial and fungal diseases [3]. In India, losses in yield due to leaf spots have been estimated to be in the range of 15-59% [4]. There is a lack of resistant varieties against early leaf spot and late leaf spot disease commonly called as *Tikka disease* and it is caused by *Cercospora arachidicola* and *Cercosporidium personatum*s respectively. These are two important constraints to decrease the yield of groundnut. Several exogenous genes have been introduced into peanuts by particle bombardment [5] or *Agrobacterium*-mediated transformation[6]. The successful exploitation of *in vitro* techniques in peanuts depends on the establishment of efficient regeneration protocol. Leaflets and cotyledons are the most widely used explants in peanut tissue culture. Direct organogenesis from various types of explants in peanut reported such as cotyledonary node [7, 8, 9], cotyledon [6, 10, 11, 12, 13], leaflet[14, 15, 16, 17], epicotyl, hypocotyl [18] and axillary meristems [19]. Our aim is to develop a reproducible regeneration protocol for developing fungal resistance in peanut by using *Agrobacterium* mediated genetic transformation from de-embryonated cotyledonary explants of peanut, which is a prerequisite for transferring novel genes into peanut.

MATERIALS AND METHODS

Plant material

Mature seeds of groundnut cvs ICG 7827 and 13942 obtained from the germplasm bank of ICRISAT, Patancheru, Hyderabad, were used. The seeds were rinsed under running tap water for 10-15 min followed by liquid detergent Tween-20 (5%-v/v) for 5 min and it was repeated twice. Later these were washed with sterile distilled water thoroughly. The seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 8 min and followed by rinsing in sterilized distilled water for 3-4 times. These sterilized seeds were dried on sterile tissue paper and the cotyledons were separated from the zygotic embryo.

Culture media and culture conditions

Longitudinally halved de-embryonated cotyledon explants were cultured on shoot induction medium (SIM) containing MS salts [20] with B_5 vitamins [21] containing 3% sucrose, supplemented with varying concentrations (1-50 mg/L) of 6-Benzylaminopurin (BAP) and Thidiazuron (TDZ) alone and also in combination with 0.5 mg/L NAA/IAA (Tables 1, 2). The pH of the medium was adjusted to 5.7±0.02 with either 0.1N NaOH or 0.1N HCl before the addition of Difco bacto-agar. The medium was solidified with 0.8% (w/v) Difco bacto-agar and autoclaved at 121ºC under 15 psi for 15-20 min. All the cultures were incubated at 25±2ºC under 16 h day exposure to white light for 4-5 weeks for multiple shoots induction.

Shoot elongation and in vitro rooting

The regenerated shoots from de-embryonated cotyledons were transferred on to shoot elongation medium (SEM) containing MS salts+B_5 vitamins containing 3% (w/v) sucrose supplemented with 0.5 mg/L BAP for two to three passages of four weeks each. The elongated shoots (5-6cm long) were rooted in root induction medium (RIM) containing MS salts+B_5 vitamins with auxins NAA/ IBA/IBA+NAA.

Acclimatization and plantlet establishment

The *in vitro* regenerated plantlets were taken out from the culture vessels and washed with sterile distilled water to remove the remains of agar. Later shifted to plastic cups containing sterilized soilrite and kept in the plant growth chamber for acclimatization. These were covered with polythene bag to maintain the RH (85-90%). After 4 weeks, the plantlets were transferred to plastic cups

containing soil mixture of soil: sand: vermicompost (1:1:1) and maintained in the greenhouse. Subsequently, these *in vitro* regenerated plantlets were transferred to the field.

Data analysis

Data were collected periodically on number of primordia/explant, number of multiple shoots conversion/explant and root a number per micro-shoot. 20 replicates were maintained for each experiment and each experiment was repeated atleast twice.

RESULTS AND DISCUSSION

Longitudinally halved de-embryonated cotyledon explants of peanut cvs ICG 7827 and 13942 were cultured on shoot induction medium (SIM) augmented with varying concentrations (1-50mg/L) of BAP/TDZ alone and also in combination with 0.5 mg/L NAA/IAA. The results are presented in Tables 1, 2; Figures. 2, 3, 4, 5. The explants were turned into green within a week of incubation and shoot bud induction was confined to the proximal portion of each explant (Figure. 1b). Shoot buds developing on the proximal end were small and too numerous to count and they subsequently grew faster (Figure. 1c). Whereas SIM free from PGRs did not induce any morphogenesis from the explants (Figure. 1a). This indicates that rapid shoot regeneration was promoted by adding exogenous PGRs. The shoot regeneration rate varied significantly after 2nd subculture (10-12weeks) (Tables 1, 2). In the present investigation, all the concentrations and combinations of PGRs have induced the development of adventitious shoots, but the response was found to be varied. Maximum percentage of response (93%) from the de-embryonated explants was observed at 0.5 mg/L IAA+15 mg/L TDZ in peanut cv ICG 13942 and followed by ICG 7827 (91%) at 0.5 mg/L NAA+20 mg/L TDZ. Highest shoot primordia/explant with more number of shoots formation (45.67±0.23 in ICG 13942 & 36.10±0.23 in ICG 7827) and maximum percentage of conversion from shoot buds to shoots (94% in ICG 13942& 88% in ICG 7827) were found at the same concentration and combination of PGRs in comparison to other PGRs used in both the cvs. Lili Geng *et al.* [17] reported 40.90% of leaf disc explants developed multiple shoot buds on MS medium supplemented with 0.5 mg/L NAA + 0.5 mg/L TDZ. While Akasaka *et al.* [22] reported the highest frequency of induced shoots was 34.70%, this was obtained from growing peanut leaves on TDZ containing medium. Percentage of response was recorded less on SIM supplemented with TDZ alone compared to TDZ in combination with NAA/IAA in both the cvs used. Whereas the percentage of response was found to be more when BAP used as a sole PGR. Number of adventitious shoots was also recorded more on SIM supplemented with all the concentrations of TDZ/BAP in combination with 0.5 mg/L NAA/IAA in both the cvs studied. Thus, among all the concentrations of BAP/TDZ alone and in combination with NAA/IAA the two genotypes exhibited a tendency to develop an enhanced number of multiple shoots. These multiple adventitious shoots were elongated on SIM supplemented with 0.5 mg/L BAP. Venkatachalam *et al.* [23] reported that the maximum number of multiple shoots (14shoots/explant) was observed in MS medium supplemented with 0.5 mg/L NAA+ 5.0 mg/L BAP. The percentage of shoot bud differentiation and the mean number of shoots per culture increased at higher concentration of cytokinin in combination with auxin [24]. The results of this study indicate that the addition of NAA increased the frequency of shoot bud proliferation.

The maximum number of multiple shoots/explant (37.82±0.11) with 85% of shoot conversion were found at 0.5 mg/L NAA+20 mg/L BAP in cv ICG 7827 followed by ICG 13942 (34.54±0.24) with 83% of shoot conversion at 0.5 mg/L IAA+15 mg/L BAP (Table 2). But earlier Mc Kently *et al.* [25] reported that de-embryonated cotyledon explants incubated on MS medium supplemented with 25 mg/L BAP showed a 15% response for multiple shoot bud formation with only 3.7 elongated shoots per responding explant. The use of de-embryonated cotyledons as explant was in agreement with Swathi *et al.* [26] who

suggested that if cotyledon is cut vertically and its adaxial side is in direct contact with medium the regeneration efficiency was found improvement. Further supporting this observation of cotyledon gradient competence, using serial sections of peanut cotyledons. Victor *et al.* [27] observed an increase in meristematic conversion in the epidermal and subepidermal cell layers as the sections approached the hypocotyledonary notch region when exposed to TDZ and BAP. In comparison with BAP induced regeneration, the use of TDZ of our study resulted in considerably higher number of shoots per explant (45.67±0.23 shoots/ explant) (Table 1). In addition, the period required to achieve shoot induction with BAP is longer, more than 30days while the TDZ induced shoot primordial appeared within 15 days of culture with de-embryonated cotyledons of two cultivar varieties.

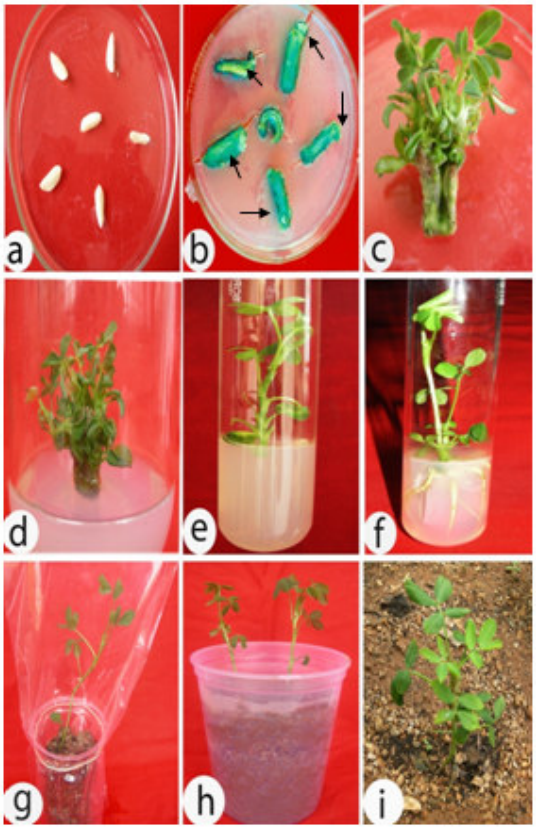

Figure 1(a-i)
In vitro regeneration and plantlet establishment from de-embryonated cotyledon explants in two cvs of peanut.

a) De-embryonated cotyledon explants cultured on SIM without PGRs (control).
b) Explants were turned into green after 7th day of incubation (Note the enlargement and shoot primordia induction)
c-d) Induction of multiple shoots on SIM fortified with 15 mg/L TDZ+0.5 mg/L IAA in cv. ICG 13942 & 20 mg/L BAP+0.5 NAA in cv. ICG 7827 after 4 weeks of inoculation respectively.
e) Elongation of multiple shoots on SEM supplemented with 0.5 mg/L BAP.
f) *In vitro* rooting of shoots on RIM augmented with 1.0 mg/L NAA (Note profuse rhizogenesis).
g) Acclimatization of plantlet in a plastic pot containing sterilized soilrite.
h) Plants are shifted to plastic pot containing soil mix and maintained in green house.
i) Plants growing in the research field (two weeks after transplantation.

Table 1
Effect of TDZ/NAA/IAA+TDZ on multiple shoot induction from de-embryonated cotyledon explants in two cvs of peanut.

Concn. of PGRs (mg/L)		cv ICG 7827 % of response	Average. no. of shoot primordia/ explant±(SE[a])	Average no. of shoots/ explant±(SE[a])	% of conversion	cv ICG 13942 % of response	Average. no. of shoot primordia/ explant±(SE[a])	Average no. of shoots/ explant±(SE[a])	% of conversion
TDZ									
1		45	22.61±0.02	15.38±0.12	68	55	22.25±0.14	14.02±0.23	63
5		54	30.00±0.10	22.52±0.05	75	78	24.97±0.04	17.24±0.12	68
10		69	36.13±0.21	28.21±0.12	78	88	32.32±0.16	24.24±0.21	75
15		75	39.20±0.13	34.03±0.23	87	82	33.30±0.22	26.00 ±0.04	78
20		80	35.71±0.03	25.02±0.19	70	76	37.16±0.23	30.10±0.15	81
25		64	28.95±0.07	19.12±0.15	56	66	29.35±0.08	19.10±0.03	65
50		51	19.40±0.12	12.03±0.16	48	59	22.69±0.13	12.03±0.10	53
NAA+TDZ									
0.5	1	58	25.19±0.10	15.38±0.05	60	45	25.39±0.17	16.00±0.20	63
0.5	5	63	34.34±0.06	21.30±0.14	62	54	38.22±0.05	25.25±0.11	66
0.5	10	67	39.53±0.16	26.11±0.09	65	59	41.10±0.28	32.44±0.02	77
0.5	15	72	40.06±0.35	32.05± 0.07	80	68	42.85±0.09	34.32±0.14	80
0.5	20	91	41.02±0.23	36.10±0.16	88	71	38.45±0.16	30.41±0.15	77
0.5	25	77	40.05±0.09	28.04±0.10	70	80	32.93± 0.20	24.05± 0.10	73
0.5	50	61	26.96±0.12	17.00±0.21	63	53	29.52±0.16	19.50±0.12	66
IAA+TDZ									
0.5	1	49	23.77±0.12	14.03±0.05	59	56	28.02±0.03	17.10±0.15	61
0.5	5	57	35.15±0.05	21.10±0.23	60	68	35.21±0.13	26.06±0.09	74
0.5	10	72	39.62±0.08	25.00±0.30	63	83	46.24±0.31	37.00±0.15	80
0.5	15	78	40.60±0.21	33.13±0.04	84	93	48.58±0.24	45.67±0.23	94
0.5	20	82	40.76±0.30	35.06±0.12	86	80	39.24±0.03	32.18±0.06	82
0.5	25	67	33.46±0.17	25.10±0.03	75	61	35.97±0.18	28.06±0.13	78
0.5	50	55	25.70±0.10	14.14±0.10	54	56	34.66±0.26	21.84±0.30	63

[a]*SE=Mean±Standard Error*

Table 2
Effect of BAP/NAA/IAA+BAP on multiple shoot induction from de-embryonated cotyledon explants in two cvs of peanut.

Concn. of PGRs (mg/L)		cv ICG 7827 % of response	Average. no. of shoot primordia/ explant±(SE[a])	Average no. of shoots/ explant±(SE[a])	% of conversion	cv ICG 13942 % of response	Average. no. of shoot primordia/ explant±(SE[a])	Average no. of shoots/ explant±(SE[a])	% of conversion
BAP									
1		58	11.32±0.04	06.23±0.10	55	51	18.04±0.13	08.12±0.24	45
5		63	14.80±0.12	09.03±0.21	61	56	22.98±0.20	11.04±0.04	48
10		74	27.64±0.09	18.80± 0.08	68	61	27.14±0.05	14.12±0.13	52
15		82	38.32±0.14	31.43±0.16	82	65	32.92 ±0.13	20.10±0.19	61
20		70	33.09±0.20	21.84±0.07	66	80	36.82± 0.32	28.02±0.24	76
25		68	29.54±0.16	18.02±0.24	61	69	29.90±0.21	16.15±0.26	54
50		51	17.36±0.09	09.03±0.16	52	60	19.26±0.17	08.09±0.16	42
NAA+BAP									
0.5	1	51	21.96±0.13	11.00±0.14	50	48	17.05±0.09	09.04±0.12	53
0.5	5	58	29.51±0.20	15.35±0.13	52	56	25.83±0.16	14.21±0.04	55
0.5	10	63	30.90±0.31	21.03±0.10	68	66	28.37±0.23	17.03±0.12	60
0.5	15	74	32.46±0.04	24.04±0.23	74	72	40.91±0.02	32.32±0.21	79
0.5	20	78	44.48±0.10	37.82±0.11	85	75	39.87±0.14	23.13±0.18	58
0.5	25	75	33.98±0.09	23.13±0.09	67	61	35.55±0.32	19.20±0.10	54
0.5	50	61	13.54±0.10	08.13±0.12	60	54	25.00± 0.05	12.00±0.08	48
IAA+BAP									
0.5	1	48	29.06±0.12	13.08±0.13	45	50	22.17±0.16	10.20±0.08	46
0.5	5	50	38.40±0.31	19.21±0.10	50	56	32.71±0.21	16.03±0.12	49
0.5	10	65	37.66±0.09	23.36±0.24	62	65	32.16±0.10	17.05±0.34	53
0.5	15	69	28.23±0.02	18.07±0.03	64	79	40.60±0.09	34.54± 0.24	83
0.5	20	72	39.09±0.12	32.06±0.09	82	61	37.93±0.13	22.00±0.09	58
0.5	25	59	21.19±0.18	11.02±0.14	52	57	32.71±0.20	16.03±0.12	49
0.5	50	52	20.07±0.10	08.03±0.12	40	48	26.82±0.10	11.00±0.09	41

[a]*SE=Mean±Standard Error*

Table 3
Effect of NAA/IBA/NAA+IBA/IBA+NAA on in vitro root induction from elongated shoots of peanut.

Concn. of PGRs (mg/L)		% of response	Average no. of roots /shoot ±(SE[a])
NAA			
0.5		70.03	14.23±0.21
1.0		94.00	20.42±0.12
1.5		78.23	16.31±0.27
2.0		71.67	09.50±0.09
IBA			
0.5		61.00	10.24±0.13
1.0		74.54	17.37±0.08
1.5		82.78	14.44±0.10
2.0		64.08	09.39±0.12
NAA+IBA			
0.5	0.5	54.56	09.31±0.10
0.5	1.0	71.78	15.25±0.21
0.5	1.5	68.00	11.66±0.34
0.5	2.0	60.54	08.09±0.29
IBA+NAA			
0.5	0.5	62.45	10.54±0.08
0.5	1.0	76.08	16.47±0.30
0.5	1.5	68.05	09.83±0.12
0.5	2.0	51.54	08.60±0.09

[a]*SE=Mean±Standard Error*

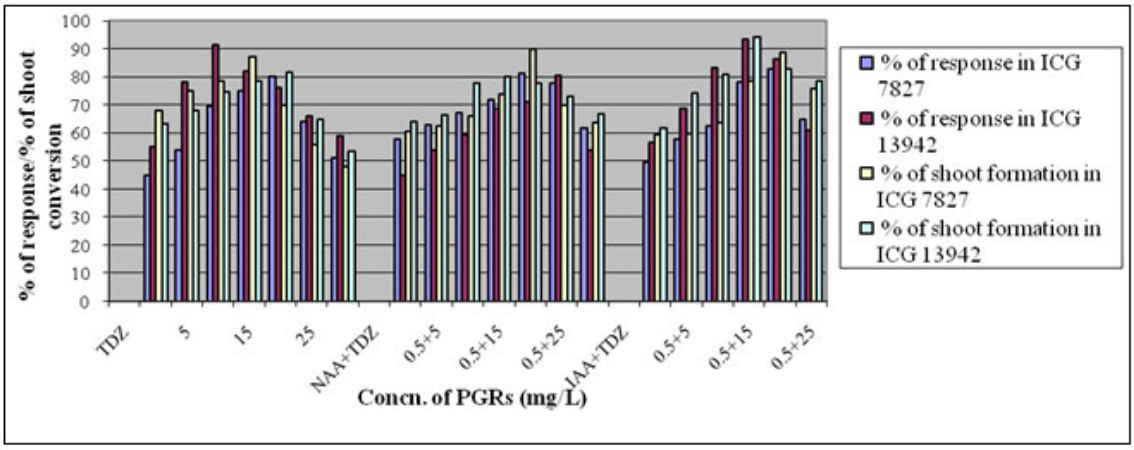

Figure 2
Effect of TDZ/NAA/IAA+TDZ on percentage of shoot primordia induction and percentage of shoot conversion from de-embryonated cotyledon explants in two cvs (ICG 7827 & 13942) of peanut.

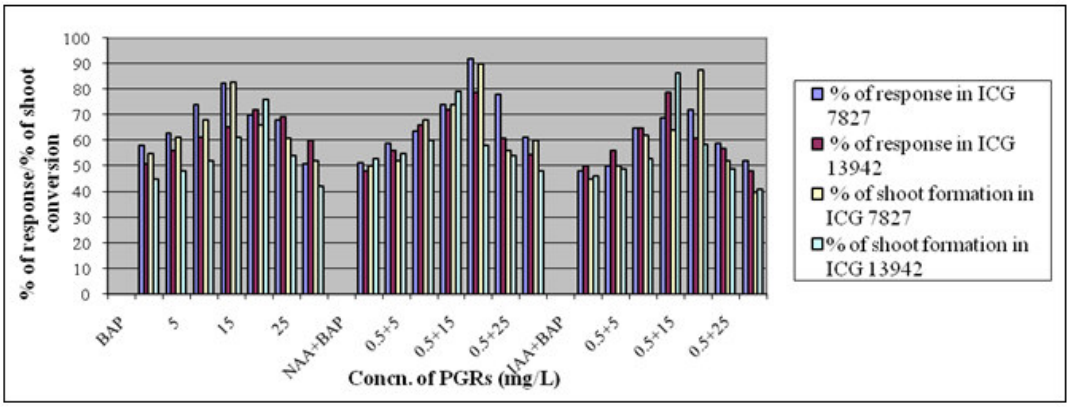

Figure 3

Effect of BAP/NAA/IAA+BAP on percentage of shoot primordia induction and percentage of shoot conversion from de-embryonated cotyledon explants in two cvs (ICG 7827 & 13942) of peanut.

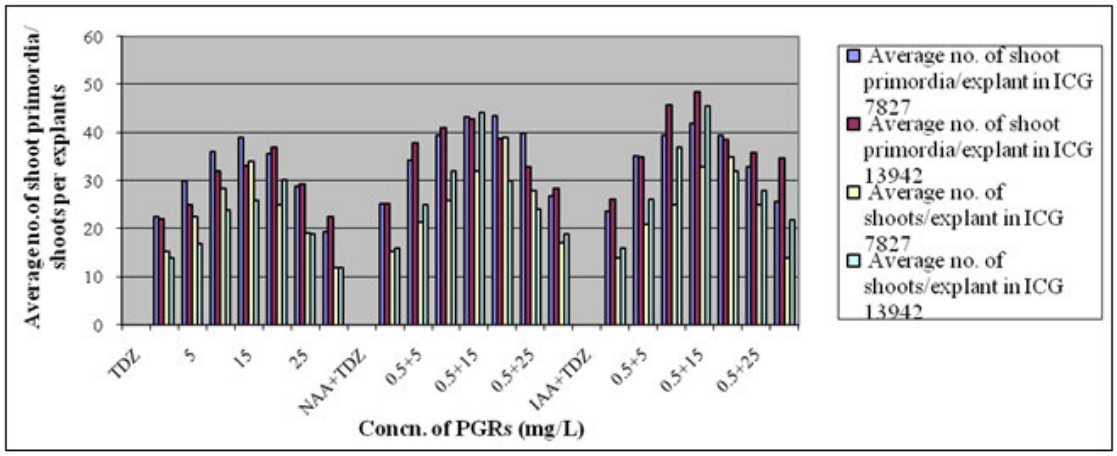

Figure 4

Effect of TDZ/NAA/IAA+TDZ on average no. of shoot primordia/explants and average no. of shoots/explant from de-embryonated cotyledon explants in two cvs (ICG 7827 & 13942) of peanut.

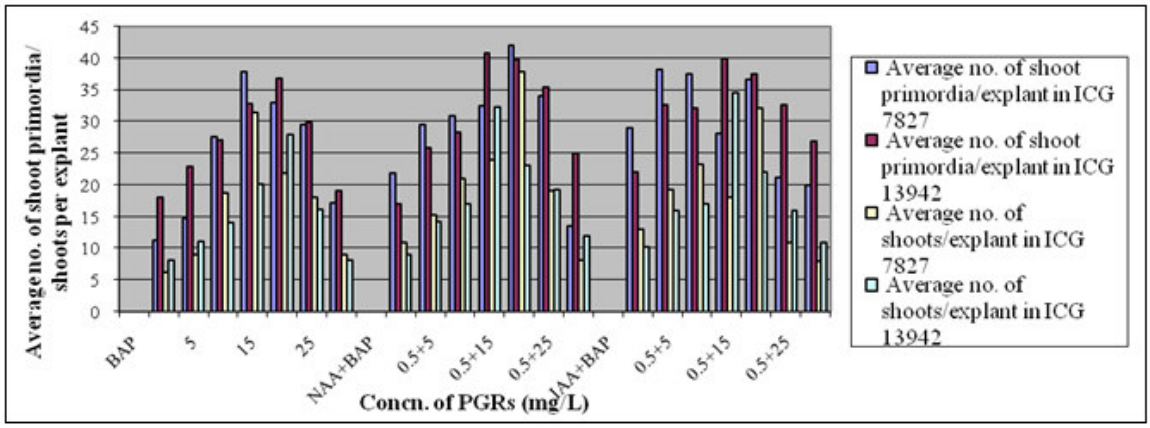

Figure 5

Effect of BAP/NAA/IAA+BAP on average no. of shoot primordia and average no. of shoots/explant from de-embryonated cotyledon explants in two cvs (ICG 7827 & 13942) of peanut.

***In vitro* rooting and plantlet establishment**

The elongated micro-shoots were cultured on RIM supplemented with different concentrations of NAA/IBA and in combinations of both PGRs (Table 3). All the concentrations and combinations of PGRs induced the rooting in two cvs of peanut. Roots were induced within 25-30 days in cv ICG 7827 and 30-35 days in cv ICG 13942. The percentage of response and the maximum number of roots/shoot increased up to 1.5 mg/L IBA/NAA alone. Among the different concentrations and combinations of NAA and IBA tested, maximum percentage of root induction (94%) was found at 1 mg/L NAA with maximum frequency no. of roots/shoot (20.4±0.12) (Table 3, Figure. 1f). As the concentration of IAA/NAA increased above the optimal level, the response of rooting was found to be poor. The micro-shoots developed from the both the cvs were cultured on RIM supplemented with 0.5 mg/L NAA+IBA (0.5-2.0 mg/L)/0.5 mg/L IBA+NAA (0.5-2.0 mg/L) (Table 3). It wa suppressed to record that less percentage of response was observed and the number of roots/shoot was also reduced on RIM containing both the auxins together. These results are in agreement with the earlier findings of Rajinikanth *et al.* [9]. *In vitro* rooting was established on RIM supplemented with 1 mg/L NAA in two cultivars of peanut. The *in vitro* regenerated plantlets were successfully acclimatized in plant growth chamber. Later these were shifted to the greenhouse. The survival percentage was found to be 91% before being transferred to the field. The *in vitro* regenerated plants were found normal with flowering and fruiting as that of parents (Figure. 1i).

CONCLUSION

In conclusion, among the PGRs TDZ alone and in combination with IAA was found to be more effective in cv ICG 13942 and BAP alone and the combination of NAA was found to be more effective in cv ICG 7827. Comparison of PGRS of TDZ and BAP, the TDZ with IAA was found to be more effective than TDZ, BAP alone and BAP+NAA/IAA, TDZ+NAA combination. It was also recorded that the cv ICG 13942 was more effective for multiple shoot formation than cv ICG 7827. The present protocol can be used for the gene transfer experiments in peanut ICG 7827 & 13942 which is a prerequisite for regeneration of antifungal resistance transgenic plants in peanut.

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ABBREVIATIONS

PGRs (plant growth regulators), BAP (6-benzylaminopurine), TDZ (thidiazuron), IAA (indole-3-acetic acid), NAA (α-naphthalene acetic acid), SIM (shoot induction medium), SEM (shoot elongation medium), RIM (Root induction medium).

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