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Full Length Research Paper

In vitro regeneration of Pakistani peanut (*Arachis hypogea* L.) varieties using de-embryonated coteledonary explants

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Peanut (*Arachis hypogaea* L.) belongs to the family leguminosae and is one of the world's largest oilseed crops. This study is the first report on peanut regeneration from Pakistan using four commercially released peanut varieties, that is, Golden, BARI-2000, BARD-479 and BARD-92. Longitudinally, halved cotyledons with removed embryos were employed as explants. Among various tested combinations of BAP and NAA, the best combination was 4 and 0.1 mg/L respectively. BARI-2000 proved to be the best responsive variety for *in vitro* regeneration in terms of number of shoots/explant (133.3%) and number of rooted plants/explant (124.99%). The optimized protocol, which is the first one in the country, would be used to incorporate important traits in future breeding programmes of peanut in Pakistan.

Key words: *Arachis hypogea*, de-embryonated cotyledons, BAP, NAA, *in vitro* regeneration, Pakistan.

INTRODUCTION

The oil seed crop *Arachis hypogea* L. (groundnut/peanut) belongs to family the *leguminosae* and sub family *Papillionacea*. The seed containing 50% oil and 25 to 30% proteins can be used as meal for food and feed (Ahmad and Rahim, 2007). In Pakistan, about 85% of total groundnut is grown in Potowar tract of Punjab, 13% in NWFP and 2% in Sindh (Government of Pakistian, 2008). Weeds are one of the major problems causing reduction in yield (Anderson, 1983). Groundnut yield can be reduced up to 80% due to the weed competition with the major loss at early stages of crop development (Gill et al., 1986; Reddy, 1984). Most of the weeds have broad leaves as the groundnut plants so the application of broad leave herbicides becomes impossible. Therefore, it is necessary to incorporate herbicide resistance genes in groundnut so that herbicide can be applied safely. There is also lack of resistant varieties against early leaf spot disease commonly called as Tikka disease caused by *Fusarium* spp. These are two important constraints (leaf spot disease and weeds) to increase yield of groundnut in the Potohar region of northern Punjab, Pakistan. The introduction of foreign genes from broad resistant sources is possible through genetic transformation. Extensive research has been done in recent years to develop the methods for *in vitro* regeneration and somatic embryogenesis of peanut crop using various explants (cotyledonary nodes, de-embryonated cotyledons) and media combinations in different countries (Little et al., 2000; Radhakrishnan et al., 2001; Tiwari and Tuli, 2007). Recently, Tiwari and Tulli (2009) developed a protocol using leaflet explants and obtained a high shoot regeneration efficiency (80%). However, in Pakistan the peanut crop remained neglected and currently there are no reports on its genetic improvement through innovative approaches. In this study, we report for the first time, an efficient protocol for regeneration of Pakistani peanut cultivars from de-embryonated cotyledonary explants and role of different growth regulator combinations resulting in high frequency of shoot regeneration. These findings will be utilized for genetic manipulation of groundnut for

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Abbreviations: BAP, NAA, etc

herbicide and disease tolerance.

MATERIALS AND METHODS

Plant material

Mature seeds of four different peanut varieties, that is, Golden, BARI-2000, BARD-479 and BARD-92 were obtained from Barani Agriculture Research Institute (BARI) Chakwal, Pakistan. Seeds were removed from mature pods and sterilized by soaking into 70% (v/v) ethanol for one minute followed by the treatment with 20% commercial bleach (colorax) for 10 min. Then, several washing were made with double distilled autoclaved water and soaked for 3 h in double distilled water at room temperature. The seed coat and embryos were removed surgically with full care, the cotyledon were cut longitudinally into two halves to obtain the four cotyledonary explants per seed.

The explants were cultured on MS macro and micro salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 30 g/L sucrose and supplemented with 12 different hormone combinations (Table 1). 8 g/L agar was added after adjusting the pH to 5.8 at 25°C. The de-embryonated coteledonary explants were placed with their cut edges in contact with the medium for 3 weeks at 25± 2°C with a photoperiod of 16/8 h. The experiment was conducted in completely randomized design and was repeated in three batches, each batch consisting of 30 explants for each treatment. The data on number of shoots/explants, gain of chlorophyll by explants, callus percentage and increase in explant size were recorded after two weeks of culture.

The regenerated shoots were excised and incubated on the same medium on which shoots were induced. On attaining a height of 3.0 cm, these shoots were shifted to rooting medium.

The rooting medium consisted of MS salts, B5 vitamins, 30 g/L sucrose and was supplemented with 2 mg/L NAA. The rooted plants were transplanted in pots containing peat moss kept in greenhouse. For the first week the pots were covered with polythene sheet to maximise the humidity. The aforementioned data for number of shoots induced per explant and rooting efficiency were recorded for the four genotypes used in the experiment. The whole procedure has been shown pictographically in Figure 1.

Data analysis

The experimental data were subjected to analysis of variance (ANOVA). Significance of means was computed by Duncan's Multiple Range Test (at P = 0.05) using M-STATC software.

RESULTS AND DISCUSSION

Effect of hormone combinations and genotype on *in vitro* regeneration

To see whether various treatments and varieties make some significant difference or not, analysis of variance (ANOVA) is performed. The ANOVA in this case showed that the difference among varieties as well as hormone combinations was highly significant for all the traits under study namely: number of shoots/explants, gain of chlorophyll by explants, callus percentage and increase in explant size (Table 2). Plant growth regulators have been regarded as important parameter in determining the success of regeneration system. A deviation from an optimum level of hormone will have a significant impact. The combination of cytokinin and auxin, particularly the concentration of cytokinin, in the regeneration medium has been described as more critical to affect the regeneration. As for the treatments (hormone combinations) concerned in our experimentation, T3 (BAP 4 mg/L and NAA 0.1 mg/L) was the best treatment, followed by T4 (BAP 6 mg/L and NAA 0.1 mg/L) for number of shoots/explant (Figure 2A) and number of rooted plants/explants (Figure 2D).

The highest number of shoots/explants (133.3%) and number of rooted plants/explants (124.99%) were observed in BARI-2000 at T3. The second highest figure (120%) for number of shoots/explants was observed again in BARI-2000 at T4, while the number of rooted plants/explants BARD-479 took the second position (115.52%) (Figure 2A and D), the highest chlorophyll (96.67%) formation was seen in BARD-479 at T3 and T11 while same was the result in Golden at T3 (Figure 2B). BARD-479 topped in callus formation (89.67%) at T9.

The selection and identification of suitable genotype is the primary requirement before the optimization of a genetic transformation protocol. Ranking of varieties on the basis of overall mean values by Duncun's Multiple Range Test (DMRT) revealed that BARI-2000 outclassed all others in number of shoots/explants (133.3%) and number of rooted plants/explant (124.99%) (Table 3). So, there seemed to be genotypic differences regarding *in vitro* response.

The use of de-embryonated cotyledons as explant was in agreement with Swathi et al. (2006) who suggested that if cotyledon is cut vertically and its adaxial side is in direct contact with medium, its regeneration efficiency is improved.

It is evident from the result that in general by increasing BAP concentration in medium, number of shoots/explants increased upto 4 mg/L and then it dropped. While on other hand, the callus formation was reduced by increasing BAP concentration. Explant enlargement and chlorophyll accumulation remained almost unaffected with change in hormone combination. BARI-2000 produced highest shoots and rooted plants which were followed by BARD-479. However, in chlorophyll gain and explant enlargement both the bold seeded varieties namely: BARI-2000 and Golden, performed better than the small seeded varieties namely: BARD-479 and BARD-92.

McKently et al. (1995) emphasized the use of zygotic embryo axes as explants but cotyledons have several comparative benefits like robustness, time saving and cost effectiveness. Venkatachalam et al. (2000) also used cotyledons as explants to produce fertile plants via embryogenesis which involves much more tissue culture (too many media, culture conditions and time) as

Table 1. Treatments (Hormone combination used).

Treatment (T)	Hormonal combination	
	NAA mg L^{-1}	BAP mg L^{-1}
1	0.1	0
2	0.1	2
3	0.1	4
4	0.1	6
5	0.5	0
6	0.5	2
7	0.5	4
8	0.5	6
9	1	0
10	1	2
11	1	4
12	1	6

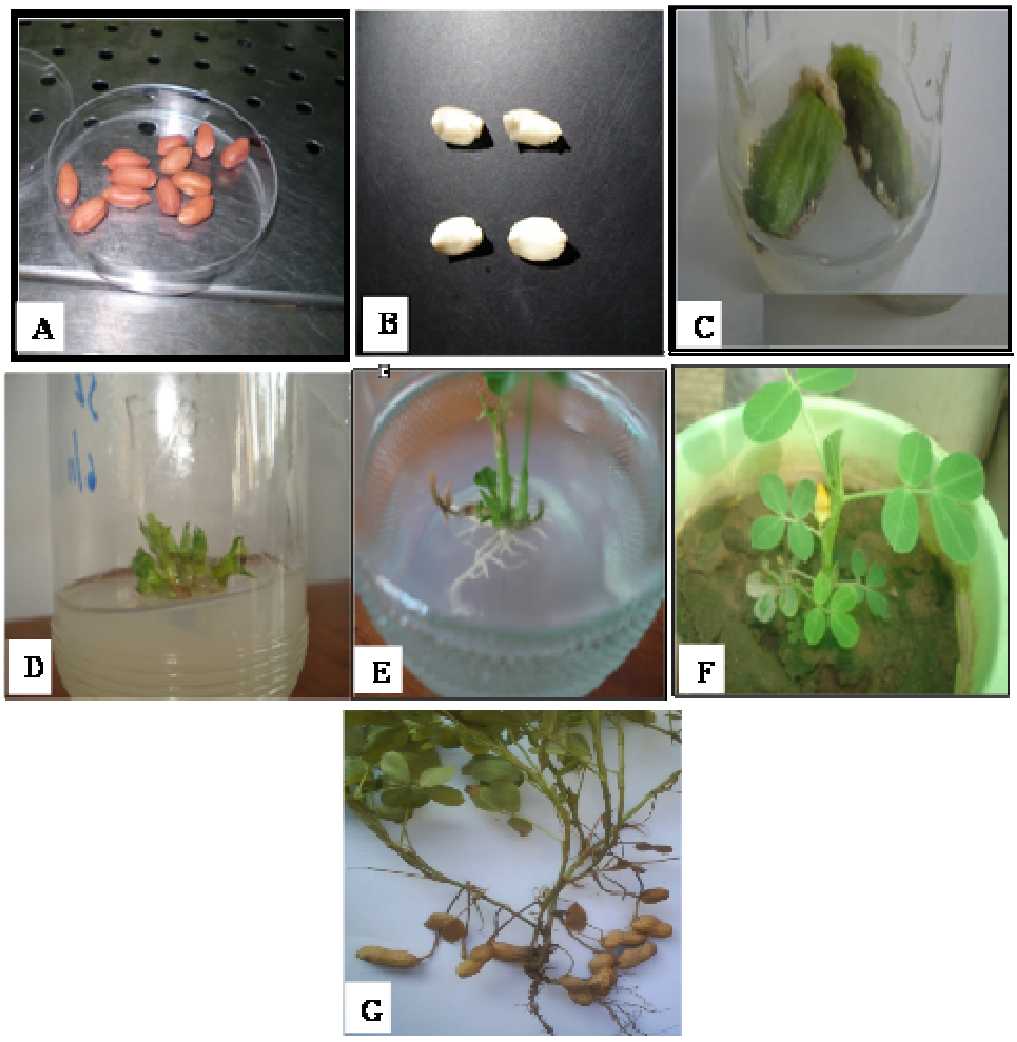
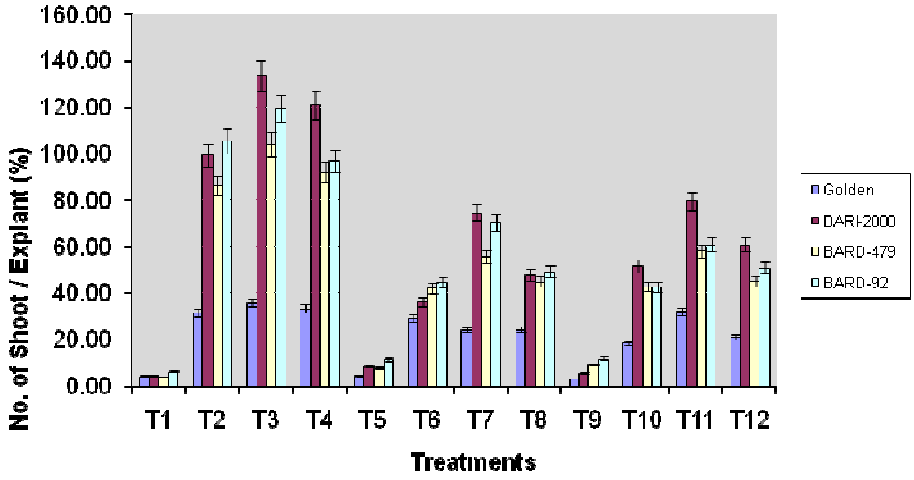


Figure 1. Different steps in peanut tissue culture. (A) peanut seeds having seed coat, (B) cotyledonary explants placed on media, (C) explants size increase four times the original size, (D) multiple shoot formation, (E) rooting in *in vitro* plants, (F) mature peanut plant in pot in green house, (G) plant after harvesting.

Table 2. F values as calculated by two-factor factorial ANOVA.

Numberof shoot/ explant (%)		Chlorophyll content (%)		Number of explant/ with callus (%)		Size of explant/ original size		Number of rooted plant/ explant (%)	
Treatments	319.54**	Treatments	23.03**	Treatments	254.22**	Treatments	2.68**	Treatments	197.82**
Varieties	315.42**	Varieties	9.54**	Varieties	9.55**	Varieties	265.02**	Varieties	106.82**

** Treatments/varieties have highly significant differences.



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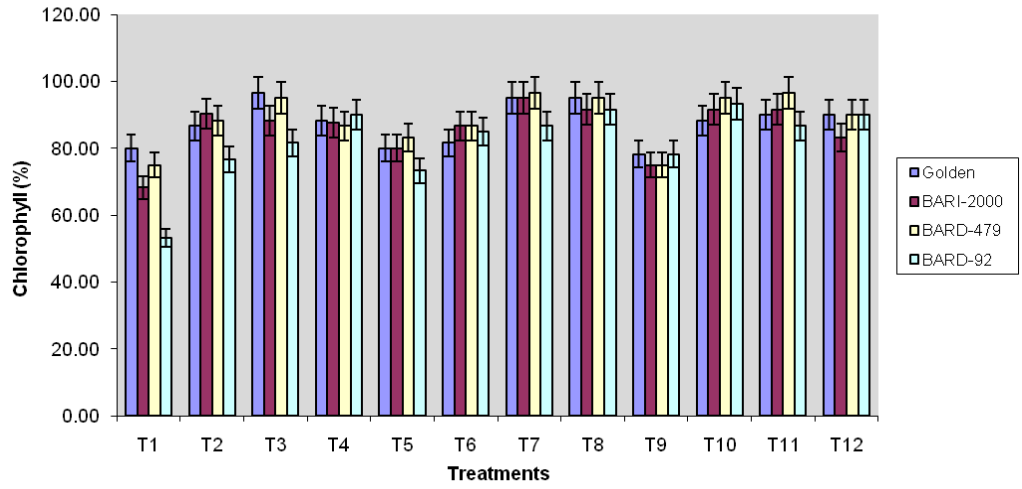
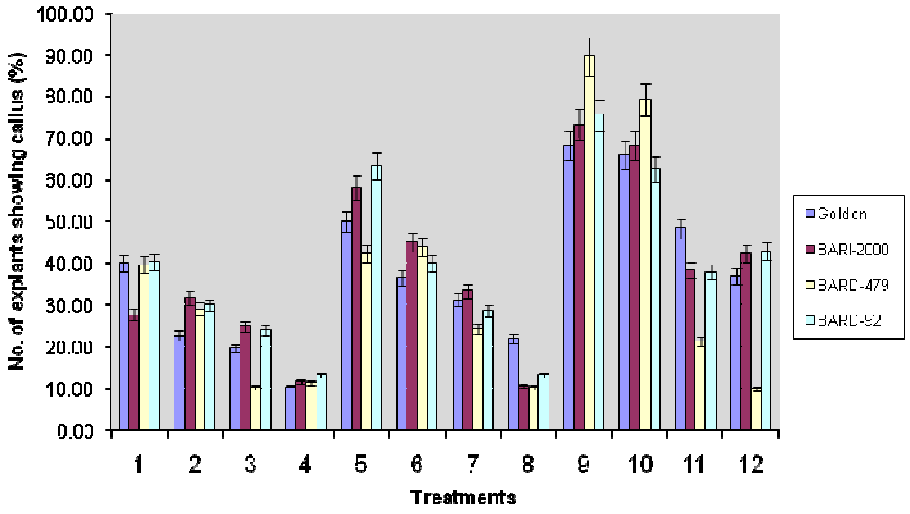


B

Figure 2. Variation in response of four varieties under different treatments (T_1 to T_{12}), (A) Number of shoots/ explants in all varieties, (B) Chlorophil percentage gained by an explants, (C) Number of explants showing callus, (D) Number of rooted plants/ explants in all varieties at different treatments, (E) increase in size of explants in different varieties at different treatments.

compared to direct regeneration from cotyledons shown in this study. This explant is easy to use as sterilization is simple and does not need too much care. Moreover, it also saves time and sources needed for *in vitro* germination which is a pre-requisite for protocols involving epicotyls or leaves as explants.

BARI-2000 is a commercial cultivar released to Potowar region of Pakistan. The successful establishment of regeneration of this variety will have a number of benefits in future. Use of de-embryonated cotyledon as explant



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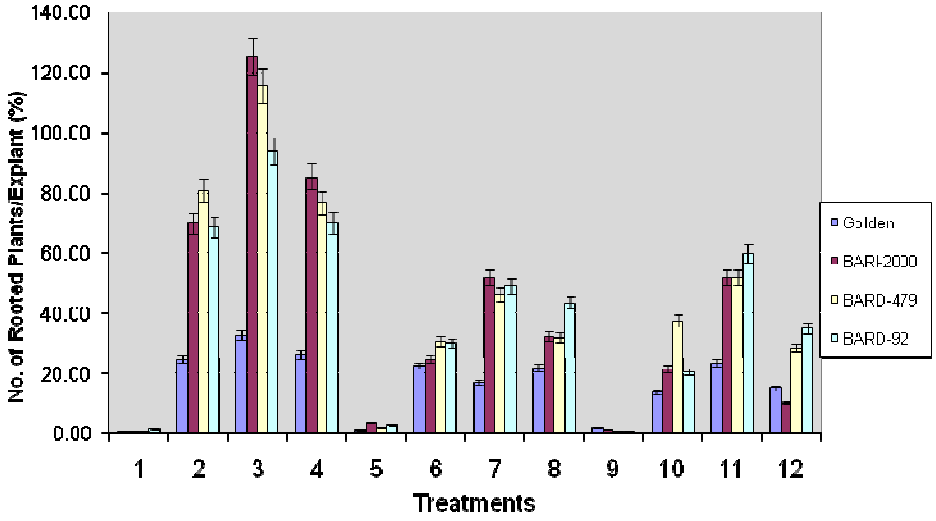

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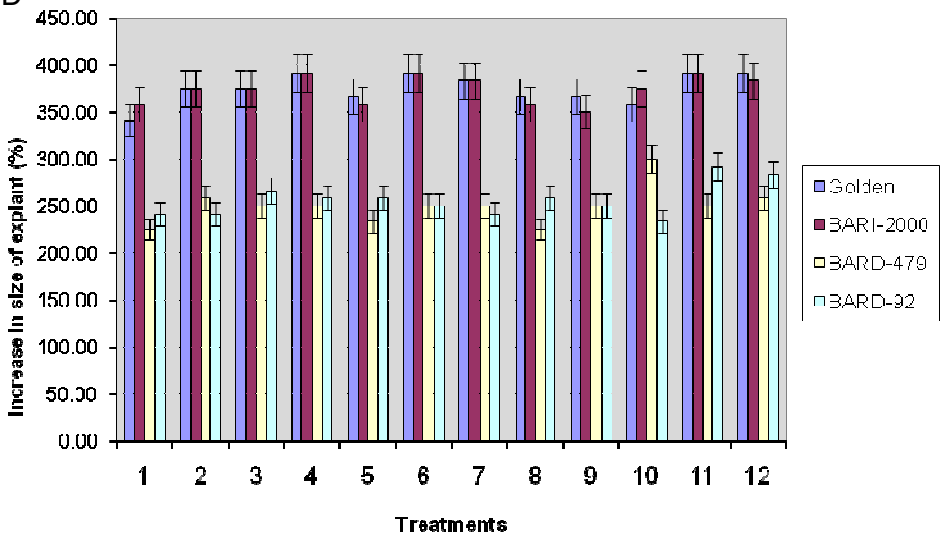

E

Figure 2. Continue.

Table 3. Mean values of all the varieties for different parameters as ranked by Duncun's Multiple Range Test.

Number of shoot/ explant (%)		Chlorophyll content (%)		Number of explant with callus (%)		Size of explant/ original size (%)		Number of rooted plant/ explant (%)	
BARI 2000	53.10^{A}	BARD-479	88.61^{A}	BARD-92	39.36^{A}	Golden	375.0 ^{A}	BARD-479	41.87 ^{A}
BARD-92	39.30^{B}	Golden	87.50^{AB}	BARI-2000	38.81 ^{A}	BARI-2000	374.3 ^{A}	BARI-2000	39.96 ^{A}
BARD-479	29.22^{C}	BARI-2000	85.81^{B}	Golden	37.69 ^{A}	BARD-92	259.0 ^{B}	BARD-92	39.56 ^{A}
Golden	21.75^{D}	BARD-92	82.22 ^{C}	BARD-479	34.31^{B}	BARD-479	250.0 ^{B}	Golden	16.75 ^{B}

Values with similar letters do not differ significantly while values with different letters show significant differences.

makes this protocol more attractive for *Agrobacterium tumefacien* transformation. It is strongly hoped that traits like disease resistance, herbicide resistance, drought tolerance which are lacking in prevailing germplasm, would be transformed through genetic engineering into BARI-2000 using our optimized protocol. By incorporating these traits, the researcher would be able to enhance the productivity and area of peanut crop. Production of useful peanut variants through somaclonal propagation would provide another application for its genetic improvement.

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REFERENCES

Ahmad N, Rahim M (2007). Evaluation of promising groundnut *Arachis hypogaea* L. varieties for yield and other characters. J. Agric. Res., 45(3): 185-189.

Anderson WP (1983). Weed Science Principles. 2nd edition, West Pub. Co. St. Paul. Minn, USA., pp. 33-42.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.

Gill BS, Samra JS, Brar HS, SP Mehra (1986). Studies on the critical period of weed competition in groundnut. J. Res. Punjab Agric. Univ. India, 23: 394-397.

Government of Pakistan (2008). http://www.pakistan.gov.pk/divisions/food-division/media/ Statistical Tables2008/Table-30.

Little LI, Magbanua ZV, Parrott WA (2000). A protocol for repetitive somatic embryogenesis from mature peanut epicotyls. Plant Cell Rep., 19: 351-357.

McKently AH, Moore GA, Doostdar H, Niedz RP (1995). Agrobacterium-mediated transformation of peanut (*Arachis hypogaea* L.) embryo axes and the development of transgenic plants. Plant Cell Rep., 14: 699-703.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant, 15(3): 473-497.

Radhakrishnan T, Murthy TGK, Chandran K, Bandyopadhyay A (2001). Somatic embryogenesis in *Arachis hypogaea*: revisited. Aust. J. Bot., 49: 753–759.

Reddy PS (1984). Groundnut in India present status and strategy. Proc. Meeting of Asian Reg. Res. on grain legumes ICRISAT., pp. 32-35.

Swathi TA, Jami SK, Dalta RS, Kirti PB (2006). Genetic transformation of peanut (*Arachis hypogea* L.) using cotyledonary node as explant and a promoterless *gus::npt*II fusion gene based vector. J. Biosci., 31(2): 235-246.

Tiwari S, Rakesh T (2009). Multiple shoot regeneration in seed-derived immature leaflet explants of peanut (*Arachis hypogaea* L.). Sci. Hortic., 121: 223-227.

Tiwari S, Rakesh T (2008). Factors promoting efficient *in vitro* regeneration from de-embryonated cotyledon explants of *Arachis hypogaea* L. Plant Cell Tissue Organ. Cult., 92: 15-24.

Venkatachalam P, Geetha N, Abha K, Shaila MS, Sita GL (2000). *Agrobacterium*-mediated genetic transformation and regeneration of transgenic plants from cotyledon explants of groundnut via somatic embryogenesis. Curr. Sci., 78: 1130-1136.