Chapter 2

TISSUE CULTURE STUDIES FOR DEVELOPMENT OF REGENERATION PROTOCOL FOR GROUNDNUT

2.0 Introduction

Groundnut (*Arachis hypogea* L.) is an important oil, food and fodder crop and plays an important role in the agricultural economies of countries of the semi-arid tropics. It contributes significantly to food security and alleviates poverty (Naidu et al., 1999) and as a legume, improves soil fertility by fixing nitrogen and increases productivity simultaneously.

It is native to South America and forms an important legume in Asia and Africa. It is mainly grown under rain-fed, irrigated, and residual moisture conditions. The crop is cultivated on 24.8 million hectares with an average productivity of 1.32 tonnes/hectare.

It is also one of the major cash crops of the state of Gujarat with highest production and productivity in the country.

There are several constraints to the productivity of the groundnut crop that result in great economic losses annually (Sharma et al., 2000). Conventional breeding has contributed towards the improvement of groundnut (Reddy et al., 1996; Garcia et al., 2006) but strong interspecific barriers, low recovery of hybrids and linkage of undesirable traits limit the introgression of variability from wild species (Halward et al., 1993; Tallury et al., 2005). Genetic transformation approach allows for introducing novel genes that are not accessible normally by conventional cross-breeding, i.e., limited by sexual incompatibility.

Genetic transformation overcomes these limitations and allows introduction of agronomically important genes across taxa (Li et al., 1997; Singsit et al., 1997; Yang et al., 1998; Magbanua et al., 2000; Sharma

and Anjaiah, 2000; Livingstone et al., 2005; Tiwari et al., 2008). The availability of an efficient regeneration system is however an essential prerequisite for utilizing this approach.

For successful genetic modification, production of transgenic plants, effective regeneration system is imperative. Although several reports on efficient regeneration from diverse explants of groundnut have been published, but not much success with genetic transformation of *Arachis* species has been achieved and many of them are genotype dependent (Cheng et al., 1992). This is due to the lack of efficient protocols to regenerate whole plants through *in vitro* regeneration of adventitious shoot buds from the transformed tissues (Lacorte et al., 1991).

2.1 Literature studies

Various regeneration systems reported so far from different explants in groundnut are as given below.

Immature leaflets from young seedlings of groundnut had been utilized to achieve organogenesis (Mroginski et al., 1981; McKently et al., 1991; Cheng et al., 1992; Sukumar and Sree Rangasamy, 1984; Narasimhulu and Reddy, 1983; Akasaka et al., 2000; Chengalrayan et al., 2001). However, among these reports some had shown low regeneration efficiency and excessive time required for the development of the regenerants. For instance, Mroginski et al., (1981) and Cheng et al., (1992) reported bud primordia development that failed to regenerate normal plants. Sukumar and Sree Rangasamy (1984) reported calli development in seven *Arachis* spp. but none of them formed shoots. Narasimhulu and Reddy (1983) reported callus mediated plant development in only 19 % of the explants. Akasaka et al., (2000) reported various abnormalities in shoot development and low conversion rate (34.7%) from shoot buds to shoots. Mature zygotic embryo derived leaflet explants have been deployed by Chengalrayan et al., (2001) to examine the effect of phytohormones on organogenesis and somatic

embryogenesis. Sharma and Anjaiah (2000) have reported efficient regeneration in groundnut from cotyledon explants.

From above it is clear that a perfect protocol for regeneration of groundnut is still elusive. It is also a requirement for the production of transgenic plants.

2.2 Materials and Methods

The pods of peanut cultivar var. GG20 was obtained from the Agriculture University, Junagadh, Gujarat. The methods employed are discussed in subsequent discussion.

2.2.1 Standardization of surface sterilization treatment

A successful tissue culture protocol starts with effective explant sterilization (Dodds and Roberts, 1985). Optimization of the surface sterilization protocol is an important aspect in Tissue Culture study and this ensures that large number of clean explants survive sterilization.

Surface sterilization was followed as per standard procedure with 0.1 % mercuric chloride($Hgcl_2$) for 10 minutes however all seeds turned brown and no growth was observed in the cultures. Reducing the concentration and/or duration of exposure resulted in heavy fungal contamination. Since seeds/pods are underground parts of the plants heavy fungal/bacterial contamination is obvious and careful elimination of this is required for raising the axenic cultures. Various duration & concentrations of $Hgcl_2$ as well as sodium hypochlorite were tried to obtain sterile explants.

The mature seeds were washed under running tap water for 25 min. It was then subjected to, treatment with a solution of detergent Extran for 10 min, treatment of antifungal agent Bavistin for 10 min and finally washed thoroughly in sterile double distilled water.

Further procedure was carried out in Laminar Air Flow under sterile condition. Seeds were rinsed in 70% iso propyl alcohol (1 min) followed by 3 to 4 washes with distilled water.

Treatment	**Survival (%) after one week of inoculation**	**Contamination (%)**	**Response pattern**
0.525% NaOCl; 10 min	20	75	Heavy fungal & bacterial contamination
1.050% NaOCl; 10 min	35	60	
0.525% NaOCl; 20 min	30	45	
1.050%NaOCl; 20 min	40	35	Best among NaOCl treatment
0.05% $HgCl_2$, 5 min	40	40	Better survival compared to NaOCl treatment.
0.1% $HgCl_2$, 5 min	55	25	
0.05% $HgCl_2$, 7.5 min	50	35	
0.1% $HgCl_2$, 7.5 min	80	20	Best response with healthy explants
0.05% $HgCl_2$, 10 min	55	45	
0.1% $HgCl_2$, 10 min	95	0	Least contamination Explants turned brown and dead

Table 2.1: Explant contamination and survival rates following surface sterilization treatments with NaOCl as well as $HgCl_2$

Using NaOCl, explants response was better. However it was accompanied with heavy fungal contamination and hence could not be used in further experiments. The best treatments with NaOCl was 1.05% of NaOCl for 20 min indicating efficient sterilization without excessive damage to the explants but it could give only 40 % survival of the explants. In comparison, explant survival rates were generally higher following $HgCl_2$ sterilization

ranging between 80-90 % with least contamination. Among $HgCl_2$ treatment also survival and contamination control was directly related to the concentration of $HgCl_2$ as increased exposure with lower concentration did not result in controlling contamination. Similarly in all the NaOCl treatments also the explants survived after one week developed contamination gradually later during 2nd and 3rd week of incubation. Higher concentration with more exposure to $HgCl_2$ lead to death of the explants which was apparent as they turned brown. However $HgCl_2$ allowed better initiation of regeneration than NaOCl. The optimum treatment for best explant survival and initiation of regeneration was 0.1% $HgCl_2$ for 7.5 min in which around 80% explants could be initiated sterile and there was no aberrant effect.

2.2.2 Culture Medium and growth conditions

In all the experiments, MS (Murashige and Skoog, 1962) medium was used which was fortified with B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol and 30 g/l sucrose. The cultures were maintained at 28 ± 1°C under a continuous 16/8 h (light/dark) photoperiod with light supplied by cool white fluorescent lamps at an intensity of about 1600 lux. The media were solidified with 0.65% agar (colloids). The pH was adjusted to 5.8 before autoclaving at 121°C for 20 min.

All the experiments were performed using Petri dishes (94 mm diameter), test tubes (25 mm diameter) and glass bottles (300 ml). About 4-5 explants were inoculated per Petri dish and bottles, while 2 explants were inoculated per test tube. All experiments were performed in triplicate.

The response was measured in terms of (1). No reaction (explants showing no physical change damage (2). Survival-explants turning green, producing callus or developing shoots. (3) Pathogen contamination.

2.3 Results and Discussion

2.3.1 Direct Organogenesis from young leaflets.

Groundnut leaves are pinnately compound and new emerging leaflets are conduplicate, i.e., folded lengthwise along the middle. The leaflet lamina and leaf rachis elongated extensively before leaflet unfolding.

The development and nodal position of the leaflets used for explanting are noted. Nodes are numbered beginning with the most proximal leaves of the shoot beyond the cotyledons.

Nodes 1 and 2 are usually separated by short internodes, in which case the first two true leaves appear almost oppositely oriented, in contrast to the wider-spaced, alternating leaf pattern at subsequent nodes. Leaflets at the first two nodes are smaller and usually dark green in color than later-formed leaves. The effect of leaflet size from leaves at nodes 1 and 2 was evaluated. To obtain these leaflets, after surface sterilization, seeds were soaked in sterile double distilled water over night and inoculated on MS medium with 2.22 µM BAP for pre-incubation.

After seven days of pre-incubation, the enlarged seeds were taken out aseptically, seed coat was removed, elongated radical was cut apart and the green young leaflets were separated (Fig. 2.1A-2.1B) and used as explants for direct organogenesis.

These leaflets were given superficial cut on the edges and further placed on MS medium with various combinations containing different concentrations of N ^{6}-benzylaminopurene (BAP; 2.22-22.2 µM) and α-naphthalene acetic acid (NAA-2.68 -5.36 µM).

Percentage of explants showing shoot development response was noted. Percent response, mean and standard deviation in each experiment were

calculated from data generated at this stage. Subsequently, shoot clusters were sub-cultured on similar medium every three weeks for enhancing shoot elongation. After four cycles, the number of elongated shoots was recorded. Shoots (3-4 cm) derived from the shoot clusters were excised and rooted on MS medium supplemented with NAA (4.95 µM).

The young, green, folded leaflets from 7 days pre-incubated seeds when used as explant and inoculated on MS medium containing different combinations of BAP and NAA gave direct shoot bud initiation within 3 weeks. The response of shoot initiation started with up ward curling of leaf cut edges. Few explants did not respond and turned transparent. The shoot bud cluster formation started and was restricted at the basal end of the leaves. MS medium with 13.32 µM BAP and 2.68 µM NAA gave 70 % direct shoot formation within 30 days of incubation.

Maximum of 3 elongated shoots were observed per explants directly within a month however, numerous shoot forming buds were found which when transferred to same medium showed further development and elongation. The immature leaflet explants are reported by many researchers to be most responsive in giving direct organogenic response.

The results obtained correlates with Seitz et al. (1987) who reported that immature leaves induced up to 30% shoot formation on the MS medium containing NAA and BAP.

Cheng et al. (1992) and Akasaka et al. (2000) also reported that MS medium containing NAA and BAP was the most promising combination for shoot bud formation from immature leaflet explants.

Fig.-2.1A: Seed coat peeled off from pre-soaked sterilized seed.

Figure-2.1B: Embryonated cotyledon with immature leaflets intact in pre-soaked sterilized seed.

Fig.2.2A: Young green folded leaflets developed during seed germination within 7 day incubation

Fig.2.2B: Young green folded leaflet lamina separated from seeds and used as explant.

Tiwari and Tuli (2009) also reported 81.5 % shoot bud formation on the MS medium with combination of NAA and BAP.

However, the findings differs from such earlier reports in a way that we used green young leaflets and obtained direct shoot formation within one month of incubation or at the most after 2 subculture. Direct shoot formation with least possible time is mandatory prerequisite for genetic transformation using biolistic method. The findings are also especially important for targeting

chloroplast DNA transformation where chlorophyll containing tissues like leaves are required to be the explants.

BAP (µM)	**NAA (µM)**	**Response after 3 weeks of incubation**	**Percentage of explants showing shoot buds**	**Average number of shoots per explants**
2.22	2.68	No change to explant, gradually drying up	-	-
4.44			-	-
6.66		Curling of edges Shoot bud initiation	45	2
8.88			60	3
13.32			70	4
17.76		Thickening of explants, curling of edges, no further development	-	-
22.2			-	-
2.22	5.36	No change to explant, gradually drying up	-	-
4.44	5.36		-	-
6.66	5.36	Curling of edges Shoot bud initiation	50	2
8.88	5.36		40	1
13.32	5.36		40	3
17.76	5.36	Thickening of explants, callus initiation no further development	-	-
22.2	5.36		-	-

Table 2.2: Effect of different concentrations of BAP and NAA on direct organogenic response from young leaves of *A. hypogaea* cv GG-20.

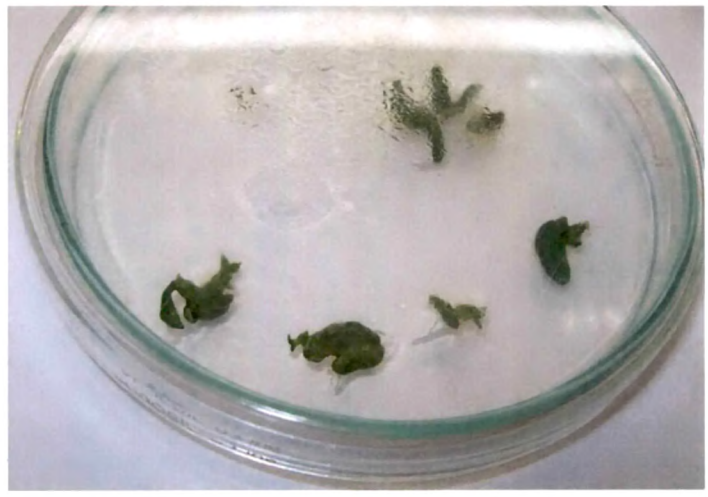

Fig. 2.3A: Shoot bud initiation after 15 days of inoculation

Fig.2.3B: Shoot elongation after 30 days of inoculation

2.3.2 Direct Organogenesis from embryo axis.

Intact zygotic embryo axis is a good source of highly merristematic cells. The connecting tissues of zygotic embryo axis with cotyledonary parts become more active when process of germination starts. To obtain this highly active and meristamatic tissue, seeds were imbibed in sterile double distilled water for 6 h after surface sterilization and kept for germination on autoclaved filter paper bridges in sterile double distilled water in test tubes. Explants were prepared from 3-7-days-old seedlings to evaluate the influence of explant age on regeneration efficiency. Based on the initial responses (data not shown) it was concluded that 3 days old seedlings gave best regeneration efficiency and hence in the subsequent experiments, 3-days-old seedlings were used. The seed coat and the radical were removed and the cotyledonary nodes were excised by cutting both epicotyls and hypocotyls approximately 2-3 mm above and below the nodal region and the embryo axis was bisected along the longitudinal plane. The meristematic region present in the nodal region was macerated by 6-8 diagonal shallow cuts with a sterile surgical blade and the cotyledons along with their nodes were embedded into the induction medium in such a way that the wounded meristematic nodal region and the adaxial surface of the cotyledon were in direct contact with the medium. From each seed, two explants were obtained.

Various combinations of growth regulators like 6-benzylaminopurine (BAP), kinetin (KN), 2,4-dichloro-phenoxy acetic acid (2,4-D) and α-napthaleneacetic acid (NAA) were tested with MS basal salts (Murashige and Skoog, 1962), A pH 5.6-5.8 was maintained before autoclaving and 3% sucrose was used as a carbon source. Explants were cultured on agar (0.65%) medium containing 17.76 µM BAP and 0.53 µM NAA that induced maximum number of shoots per explant. Subcultures were done at a15-days interval with 2-weeks duration each on induction media for the development of adventitious shoot buds. Explants producing multiple shoots were transferred to elongation medium comprising 8.88 µM BAP and 0.53 µM NAA. Elongated

shoots were cut at the inter nodal region and transferred to root induction medium comprising of 4.3 µM NAA with MS basal salts.

Three-days old seedlings responded well in terms of regeneration. The percentage regeneration and average number of shoots were highest on MS media fortified with BAP and NAA. Among the various BAP and NAA combinations tested, high frequency regeneration was obtained following culture of explants on MS medium supplemented with 13.32, 17.76 and 22.20 µM BAP along with 0.53 NAA (Table 2.3). BAP at 17.76 µM and 0.53 µM NAA was most effective for multiple shoot bud formation. Both the percent regeneration as well as the average number of shoot buds per explant was found to be higher (76% regeneration, 18 ± 1.5), when the adaxial side of the cotyledon was in direct contact with the medium compared to the abaxial side (58% regeneration, 8.75 ± 2.4). Elongated shoots when cultured on root induction medium developed adventitious roots within 15 days of culture. An average of 5 plants were recovered from each explant.

Plant regeneration is usually a bottleneck for the development of highly reproducible transformation protocols. Adventitious shoot formation via organogenesis becomes more efficient, once a suitable explant has been identified. Previously, leaf discs and leaf-section explants were used to generate transgenic groundnut plants but with low frequencies (Eapen and George, 1994; Cheng et al., 1996).

BAP (µM)	NAA (µM)	Regeneration (%)	Average number of shoot buds / explant ± SE
4.44	0.53	55	14 ± 2.1
8.88	0.53	68	12 ± 2.5
13.32	0.53	66	13 ± 1.75
17.76	0.53	76	18 ± 1.5
22.20	0.53	60	16 ± 2
26.64	0.53	67	14 ± 3.4

Table 2.3. Effect of different concentrations of BAP and NAA on regeneration from 6-day-old CN explants of A. hypogaea cv GG-20.

Each mean value was an average calculated from three experiments ± SE.

In the studies by Venkatachalam et al. 2000, cotyledons were used as a source of explants for achieving high frequency of transformation in groundnut, more particularly in the cultivar JL-24 (Sharma and Anjaiah, 2000). However, the regeneration response from the cotyledonary nodes may be comparatively higher than the cotyledons and other explants reported so far. The junction of the cotyledon and the embryo axes contain axillary meristematic cells that are highly regenerable and hence could be useful as potential targets for gene delivery. Cotyledonary nodes from mature seeds have been proved to be most responsive for the induction of multiple shoots and to generate transgenic plants in other grain legumes studied; viz. Soybean (Olhoft et al., 2003), Pea (Bean et al., 1997), Pigeon pea (Geetha et al., 1999), Mungbean (Jaiwal et al., 2001), Black gram (Saini et al., 2003).

2.3.3 Direct Organogenesis from Cotyledon explants

If Cotyledons can be used as explants it would make them as choice source due to their round the year availability of the healthy explants therefore an efficient protocol for regeneration of plants from the cotyledon explants would be of great use.

After surface sterilization the seed coat were removed and the embryo axis were cut surgically. The seed was then cut vertically along its natural ribs to get the two individual cotyledon halves- Cotyledon node(CN) and the same were used as explants- Cotyledon explants.

The explants were placed on MS medium as said above with different combinations of BAP (4.44 17.76 µM) and NAA (2.68-5.36 µM) for getting direct shoot induction. About 20 explants were inoculated per combination of BAP and NAA.

After 4 weeks of incubation, the cultures were transferred to MS medium with BAP 8.77 µM where it elongated to form plantlets.

CN explants when inoculated on different combinations of BAP and NAA shown direct shoot formation response within one month of incubation.

Problem of bacterial contamination was encountered in case of the CN explants, however, it could be controlled with the inclusion of treatment of antibiotic combinations for about 5-6 minutes after surface sterilization as given below in the table 2.4.

Name of Antibiotic	**Concentration (mg/l)**
Ampicillin	100
Gentamycin	100
Ceftazedim	100
Ofloaxacin	100
Refmpicin	50
Total	**450**

Table 2.4 : Combination of antibiotics used to control bacterial contamination of explants

Explants turned green within 12-15 days of incubation on all the media combination used. After 4 weeks, shoot bud initiated at the proximal end of the CN explant (figure 2.4A) however not all combinations yielded shoot bud initiation. Maximum of 65 % of explants shown direct shoot bud formation from the halved CN explants.

Fig. 2.4A: Initiation and development of shoot buds at the proximal end of the de-embryonated CN explants.

Fig. 2.4B : Elongation of shoots after two sub culture.

Fig. 2.5 :Rooting of the elongated shoots

The best combination for direct shoot initiation was observed to be MS medium with BAP 17.76 µM and NAA 2.68 µM. As many as 6-8 shoot buds were observed which when transferred to MS medium with BAP 8.77 µM concentration developed and elongated.

NAA (µM)	BAP (µM)	Response Greening (%)	Average shoot buds after 30 days (no)
2.68	4.44	43.33	0.64 ± 0.81
	8.88	50.00	2.00 ± 1.00
	13.32	56.67	3.09 ± 1.51
	17.76	66.67	6.09 ± 1.30
5.36	4.44	60.00	0.45 ± 0.69
	8.88	53.33	2.91 ± 1.38
	13.32	33.33	1.45 ± 0.82
	17.76	60.00	2.27 ± 1.56

Table 2.5. Response of CN explant on MS medium supplemented with different combinations of NAA & BAP for direct shoot organogenesis.

The findings of direct shoot regeneration from cotyledon explants is in line with the number of other reports including Sharma et al. (2000), Maina et al. (2010). However they reported use of 2,4- D in combination with BAP. These results with further optimization of subculture stages can be effectively used for agrobacterium- mediated transformation.

2.3.4 Indirect organogenesis from mature leaves.

Regeneration in groundnut using mature leaf explants has not been reported successfully so far. However, an efficient regeneration through mature leaves could be of great use as availability of such explants would not be a limiting factor.

To get the mature green leaflets, seeds after surface sterilization were inoculated in MS medium with 2.22 µM BAP for germination. The seeds

germinated within 7-10 days of incubation and grown in to plantlets within 21-28 days of incubation. After 4 weeks, the grown plantlets were taken out aseptically and individual leaflets were used as explants for indirect organogenesis.

These mature leaflets were given superficial cut on the edges and further placed on MS medium with various combinations of plant growth hormones BAP (1.11 - 4.44 µM), kinetin (1.16 – 4.64 µM) in combinations with 2,4-D and NAA.

The mature leaf explants curled from the edges after 4 weeks of incubation and started showing growth of callus (green). The callus cultures were transferred to the same medium with same concentration of growth hormones where cultures developed different types of callus. White - Greenish friable callus was observed in medium containing 2,4-D while in case of medium containing NAA the callus type was brown and compact. The callus was subcultured further for 2-3 passage on the same medium for about 3-4 weeks.

After, 4^{th} subculture the green compact callus was transferred to MS basal medium where it showed gradual development of shoot buds which elongated to form defined shoots.

Both the types of callus were maintained on the same respective medium and on the MS medium with reduced concentrations for 3 subculture during which the callus mass proliferated. The brownish callus showed development of root initially however this was not accompanied by the shoot development. The white –brownish callus when transferred to MS plain medium, root initials developed in case of white friable callus while in case of green callus in MS medium containing 2.32 µM kinetin, it showed green spots at the center of hard compact callus within one week of transfer which enlarged in the size and showed initiation of shoots. The shoots elongated in the same medium within 4 weeks of incubation. As many as 6 defined shoots were observed

along with numerous developing shoot buds. Upon subculturing on same medium the culture with shoot buds developed in to further plantlets.

Fig. 2.6A: Initiation of callus at the cut edge of the leaf margin

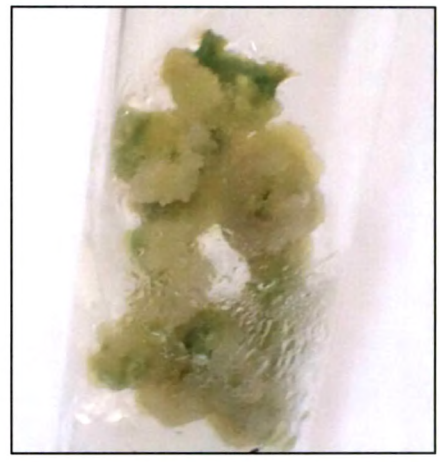

Fig. 2.6B: White-greenish friable callus growth after 3 sub culture on MS medium with BAP & 2,4,D

Fig.2.7A: Brownish-hard compact callus with development of root initial after 3 sub culture on MS medium with kinetin & NAA.

Fig.2.7B: Development of shoot buds and plantlets from the brownish hard callus on MS medium with reduced kinetin concentration after 5 subcultures.

This findings of shoots regeneration from hard compact greenish callus are in line with opinion of Maina et al. (2010).