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A Highly Effective and Reproducible Tissue Culture Method for Callus Induction and Multiple Shoot Regeneration in Groundnuts (*Arachis hypogaea* L.)

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ABSTRACT

The purpose of this study was to optimize the *in vitro* regeneration system for groundnuts using tissue culture technology, which can offer a favorable environment for food security. Using embryos as explant sources, an efficient *in vitro* regeneration technique was developed to select the most responsive type of groundnut (GJG20 and GJG22) for tissue culture. The highest callus induction frequencies (86 & 78%) in GJG20 and GJG22, respectively, were produced by MS medium containing 5.5 mg/LBAP and 1.5 mg/L NAA, which were determined to be the best. Likewise, 4 mg/LBAP, 1 mg/L NAA, and 1.1 mg/L TDZ were applied to produce the greatest number of multiple shoots/plant (9 & 6) and the ideal length of shoots (4.8 & 4.1 cm)in GJG20 and GJG22, in that order. Regarding root initiation, GJG20 and GJG22 varieties on MS media containing 1 mg/^{L} phytohormone IBA had the maximum root frequency (90 & 76%) with optimal roots/shoots (8.3 & 6.2) and significant root length (8.1 & 7.0 cm). The callus and multiple shoot induction as well as *in vitro* rooting varied according to the genotype, which may have been caused by genetic differences between the kinds and variable hormone combinations in the media. This work provides a foundation for effective *in vitro* multiplication and genetic modification of elite groundnut genotypes to achieve desired traits.

Keywords : Callus induction, Embryo slices, Genetic transformation, Multiple shoots, Plant growth regulators

INTRODUCTION

The most common oilseed crop and the third-ranked crop in the world for oil production is groundnut (*Arachis hypogaea* L.) (Upadhyaya *et al*., 2003). Around the world, groundnuts are mostly grown in tropical, subtropical, and temperate climates (Cuc *et al*., 2008). Because of their small genetic base characteristics and self-pollination, groundnut plants are very difficult to breed. It takes a lot of time and highly skilled person to undertake emasculation, crossing, and selection (Pasupuleti *et al*., 2013; Moretzsohn *et al*., 2005). According to Tiwari *et al*. (2008) and Lemaux (2008), gene transformation eliminates allof these obstacles and adds significant genes with beneficial agronomic traits. However, a genetic transformation strategy requires an effective regeneration technique. Thus, the development of a groundnut tissue culture

system that is adaptable will be very useful for improved production with good seed quality (Sharma and Anjaiah, 2000).

Plant growth regulators, medium composition, genotype, explants, photoperiod temperature, and environment are just a few of the variables that affect the effectiveness of a plant regeneration system (Ishag*et al*., 2009; Shah *et al*., 2013; Shah *et al*., 2014 a; Shah *et al*., 2014 b). While TDZ and BAP were observed to initiate numerous shoot cultured explants, BAP and NAA facilitated callus induction (Hutchinson *et al*., 1994). Previously, mature dry seeds (Palanivel*et al*., (2002): Baker *et al*., 1995), leaflets (Chengalrayan*et al*., 2001; Tiwari and Tuli, 2009), hypocotyls (Venkatachalam & Jayabalan, 1997; Cheng*et al*.,1992; Venkatachalam *etal*., 1996;)whole immature reported (Shan *etal*.,2009). However, it is difficult to obtain more planting material in short time due to the larger size of these explants.

Plant breeders have traditionally used the tissue culture technique to create somaclonal diversity (Kaeppler*et al*., 2000). In an effort to increase crop yield, oil contents, and the development of *Arachis hypogaea* cultivars that are resistant to a variety of diseases, plant species are selected through somaclonal variations, which are used to create genetic variability among crop plants (Robinson *et al*., 2011; Lacroix *et al*., 2003) Unfortunately, traditional breeding has only had patchy success in improving groundnut production and quality, as well as developing resistant types against pests, drought, fungi, cold, and salt (Banerjee *et al*., 2007). Plant breeders have been working for a long time to achieve these goals. While conventional plant breeding takes longer, genetic transformation has become a popular approach for quickly and barrier-free transfer of desired genes into crops (Taji *et al*., 2002). However, the achievement of genetic change requires a good tissue culture methodology. Thus, the goal of this work was to develop a reliable and effective tissue culture protocol for groundnut callus induction, multiple shoot regeneration, and in vitro root induction.

MATERIALS AND METHODS

Plant material, disinfection, and explants preparation

Two groundnut cultivars, GJG20 and GJG22, were obtained from the Junagadh Agriculture University located in Junagadh, Gujarat, for seed. For the purpose of surface sterilizing healthy, ripe seeds, 70% ethanol was utilized. For the surface sterilization were used70% ethanol for 2 min, and then after 15 min wash of 6% sodium hypochlorite (NaOCl) by constant shaking. After that, double-distilled water was used to wash the seeds to get rid of any remaining ethanol and NaOCl. To aid in the excision of zygotic embryos, these seeds were immersed in double-distilled water for a duration of fifteen minutes. Slices of embryos were aseptically extracted from their seeds by tearing off the seed coat to use them as a source of explants. By carefully radicle cutting these seeds on both sides,and plumule were excised and then their embryos were cut into several pieces.

Plant growth regulators and culture conditions

The Amitya University in Rajkot served as the site of this research investigation. For all the cultures, Murashige and Skoog's (1962) basal medium containing different types of vitamins (Myo-inositol, Nicotinic Acid, pyridoxine. HCl, Thiamine HCl and Glycine) at pH 5.8 was supplemented with 30 g/^{L} sucrose and 3 g/^{L} gum powder. To achieve callus and the regeneration of numerous shoots, different concentrations of auxins (NAA, TDZ, and IAA) and cytokinins (BAP, KIN) were added to MS medium (Tables 1 and 2). Using post-autoclaved IBA in MS media allowed for the induction of roots *in vitro*.

Effect of growth regulators on call us and multiple shoot induction

To induce callus, different concentrations of BAP, IAA, and NAA were present in MS basal medium (Table 1). After that, explants were positioned beneath tissue paper so that they were shielded from the intense light at 28 °C. After ten days, the explants were enlarged five times to their original size and the calli were induced. The explants were then subculture on the same media for an additional ten days in order to promote continued regeneration. The restored calli kept multiplying and eventually subculture on multiple shoot induction medium (MSM) containing BAP, KIN, and TDZ hormone combinations (Table 2) for ten to fifteen days in less than sixteen hours photoperiod having fluorescence light intensity of 50 $\mu molm^{-2}s^{-1}$ and 65-70% relative humidity at 25 ± 2 °C (Shah *et al*., 2015; Shah *et al*., 2020).

***In vitro* rooting and plant let acclimatization:**

Aseptically removed from many shoots, individual shoots (2–5 cm in length) were placed on growth regulator-free MS medium fortified by IBA for two to three weeks, and this process was repeated three times (Table 3). Four weeks later, the number of roots/explants, root length, and rooted plants were measured reported. After that, these were moved to a glass house with hydroponic conditions and Yoshida solution (Yoshida *et al*., 1976) to allow the roots to continue developing for ten days. For fourteen days following extensive root extension (approximately 3 cm) with secondary roots, plantlets should be kept in a glass house with a mixture of Compost Manure and sand (1:3) at a constant temperature of 30 °C for acclimation.

Statistical analysis

The experimental trials were using a completely randomized design, and ANOVA was employed to identify significant difference at P≤ 0.05, with this three replication of each experiment was imply to this study. The Statistix v. 8.1 statistical program was utilized for this purpose (Analytical Software, 2005).

RESULTS AND DISCUSSION

Morphogenetic response of explants on callus induction media:

Embryo slices were positioned horizontally in contact with the surface of callus induction media (CIM) containing different combinations of PGRs in order to create callus cultures (Table 4; Figure A). Following

ten days of culture, the explants either turned yellow and friable (GJG20) (Figure D) or brown and compact (GJG22) (Figure C), expanding five times their initial size (Figure B). In the first experiment, embryo slices were placed on MS media supplemented alone with varying concentrations of BAP to test the explants' response (Table 4).where the callus induction frequency (CIF) in GJG20 and GJG22 was no more than 19% and 13%, respectively. Different PGR levels (BAP + IAA and BAP + NAA) on MS medium were employed to increase callus induction frequency (Table 4). For these two kinds, optimal CIF (86% & 78%) was achieved on CIM 13 (Table 4). No any calli formed in control. Furthermore, at the higher levels of BAP and NAA (CIM 10) callus inhibition was seen in both varieties.

BAP in combination with TDZ and KIN promotes multiple shoot induction:

The research verified that transferring healthy callus to multiple shoot regeneration media (MSM) containing lowered hormone levels resulted in the regeneration of multiple shoots. Multiple shoot induction was made easier by BAP, either by itself or in conjunction with TDZ and KIN. After being cut away, brown and dead callus portions removed and weresub cultured healthy cell on MSM for 15 days while using plant growth regulators (PGRs). Calli's apical meristem stretched and grew considerably more than others on hormone-free medium. Ten to twenty buds per explant developed after 15 days on MSM; in contrast, no buds emerged in the control experiment. There was a noticeable difference in the quantity of shoots between the kinds. In both the GJG20 and GJG22 types, MSM9 enabled multiple shoot induction with significant shoot length and leaf counts, however MSM1–MSM5 were unable to induce maximum shoots. With the most shoots, shoot length, and leaves produced, MSM13 was the most productive. Callus formed on the lower shoot surfaces as a result of higher hormone concentrations in MSM10 and MSM15 inhibiting shoot formation. New shoots were generated on fresh MSM after older shoots were removed. Every two weeks, buds and shoots were sub-cultured on shoot elongation medium (SEM) before being returned to MSM for more regeneration.

Root induction needs no PGR application

Longer shoots, ranging from 2 to 4 cm, broke off from several callus-formed shoot clusters and moved to RIM on average. When IBA concentrations were added to MS medium, a significant rooting system was seen. In both kinds, callus induction caused regenerated shoots to grow roots after two to three weeks. RIM1–RIM5 shoots did not encourage roots. On RIM8, 90% of regenerated shoots formed roots with the largest root length (8.1 cm) and the highest number of roots per plant (8.3), indicating the highest rooting efficiency was attained by the GJG20 variety. 76% of the shoots rooted in the GJG22 variety had shorter roots (7.0 cm) and fewer roots per plant (6.3).While IBA (>1.5 mg/L) impeded rooting and caused callus formation at the basal region of plantlets, resulting in thicker and shorter roots, the roots on RIM8 were healthy and normal. After that, the plants were moved to hydroponic settings and given Yoshida solution to lengthen their roots. Both kinds showed notable root development after two weeks. After a week of successful adaptation in the glasshouse, the plantlets

were moved to pots with a 1:3 cocopeat and sand mix, where they survived with an 85% success rate. These plants resembled the donor plants morphologically and were uniform and healthy.

Figure 1: Steps involved in *in vitro* regeneration of groundnut (A) Embryo slices used as explants (B) Enlarged explants on Callus induction media (C) Yellow and friable texture of callus in GJG20, (D) Brown andcompact texture of callus in Golden with multiple shoots (E) Multiple shoot regeneration from callus inGJG20 (F) Multiple shoot inhibition on higher levels of hormones (G) Explants with roots on rootinductionmedia (H) Root inhibition on higher levels of IBA (I) Plantsin pot.



Table 1 : Callus induction media used for calluspro liferation in groundnut.

Media	Composition
CIM1	MS+BAP(1.0mg/L)
CIM2	MS+BAP(2.0mg/L)
CIM3	MS+BAP(3.0mg/L)
CIM4	MS+BAP(4.0mg/L)
CIM5	MS+BAP(5.0mg/L)
CIM6	MS + IAA (2.0 mg/L) + BAP (3.5 mg/L)
CIM7	MS + IAA (2.5 mg/L) + BAP (4.5 mg/L)
CIM8	MS + IAA (3.0mg/L) + BAP (5.5 mg/L)
CIM9	MS + IAA (3.5 mg/L) + BAP (6.5 mg/L)
CIM10	MS + IAA (4.0 mg/L) + BAP (7.0 mg/L)
CIM11	MS+NAA(0.5mg/L)+BAP(3.5mg/L)
CIM12	MS+NAA(1.0mg/L)+BAP(4.5mg/L)
CIM13	MS+NAA(1.5mg/L)+BAP(5.5mg/L)
CIM14	MS+NAA(2.0mg/L)+BAP(6.5mg/L)
CIM15	MS+NAA(5.5mg/L)+BAP(7.5mg/L)

MS: 4.3g/L MS basalsalts and vitamins (Murashige and Skoog, 1962),NAA:1-naphthaleneacetic acid,IAA:Indole-3-acetic acid,BAP: 6-benzylaminopurine

Table 2 : Multiple shoot induction media used for multiple shoot formation in groundnut.

Media	Composition
RIM1	MS + IBA (0.5 mg/L) (IBA used after autoclaving)
RIM2	MS + IBA (1.0 mg/L) (IBA used after autoclaving)
RIM3	MS + IBA (1.5 mg/L) (IBA used after autoclaving)
RIM4	MS + IBA (2.0 mg/L) (IBA used after autoclaving)
RIM5	MS + IBA (2.5 mg/L) (IBA used after autoclaving)
RIM6	MS + IBA (0.5 mg/L) (IBA used after filter sterilization)
RIM7	MS + IBA (1.0 mg/L) (IBA used after filter sterilization)
RIM8	MS + IBA (1.5 mg/L) (IBA used after filter sterilization)
RIM9	MS + IBA (2.0 mg/L) (IBA used after filter sterilization)
RIM10	MS + IBA (2.5 mg/L) (IBA used after filter sterilization)

Kin : Kinetin,TDZ : Thidiazuron

Table 3 : Root induction media used for root formation in groundnut.

Media	Composition
MSM1	MS+BAP(4.0mg/L)
MSM2	MS+BAP(4.5mg/L)
MSM3	MS+BAP(5.0mg/L)
MSM4	MS+BAP(5.5mg/L)
MSM5	MS+BAP(6.0mg/L)
MSM6	MS + BAP (4.0 mg/L) + Kin (0.5 mg/L)
MSM7	MS + BAP (4.5 mg/L) + Kin (1.5 mg/L)
MSM8	MS + BAP (5.0 mg/L) + Kin (2.0 mg/L)
MSM9	MS + BAP (5.5 mg/L) + Kin (2.5 mg/L)
MSM10	MS + BAP (6.0 mg/L) + Kin (3.0 mg/L)
MSM11	MS+BAP (4.0mg/L)+TDZ(0.5mg/L)
MSM12	MS+BAP (4.5mg/L)+TDZ(1.0mg/L)
MSM13	MS+BAP (5.0mg/L)+TDZ(1.5mg/L)
MSM14	MS+BAP (5.5mg/L)+TDZ(2.0 mg/L)
MSM15	MS+BAP (6.0mg/L)+TDZ(2.5mg/L)

IBA:Indole-3-butyric acid

Table 4 : Assessment of various combinations of PGR soncallus induction in groundnuts.

Media	GJG20		GJG22	
	No.of explants responded (%)	Callus induction (%)	No.of explants responded (%)	Callus induction (%)
CIM1	13.3±1.5	6.3±1.5	10.6±2.5	5.3±1.5
CIM2	23.5±3.0	10.3±1.7	17.3±2.8	8.0±2.2
CIM3	35.6±3.7	13.5±2.3	26.2±3.9	10.3±2.1

Media	GJG20		GJG22	
	No.of explants responded (%)	Callus induction (%)	No.of explants responded (%)	Callus induction (%)
CIM4	45.3±3.9	19.7±3.4	33.3±3.9	13.2±2.3
CIM5	38.1±4.1	15.5±3.1	26.6±3.1	9.0±1.8
CIM6	45.6±4.3	40.6±3.8	40.5±4.0	33.3±3.2
CIM7	57.3±5.3	51.3±4.0	49.9±4.4	38.5±4.1
CIM8	68.1±5.6	58.1±4.2	53.6±4.3	41.7±4.4
CIM9	61.0±5.2	43.7±3.9	56.4±5.2	39.3±4.5
CIM10	50.4±4.8	39.5±4.3	45.2±4.2	30.2±4.3
CIM11	58.8±4.9	45.3±4.7	46.7±4.7	38.8±3.2
CIM12	74.7±5.3	66.3±4.3	70.7±5.1	61.3±4.4
CIM13	**92.2±5.5**	**86.6±5.5**	**88.3±5.3**	**78.3±4.8**
CIM14	86.2±5.1	70.4±4.9	75.4±5.0	60.7±4.3
CIM15	73.7±4.8	61.3±4.7	61.7±4.4	49.5±4.9

The best results have been shown by bold letters.The values of standard deviation (n=3) have been given after ± symbol.

Table 5 : Assessment of various combinations of PGR son multiple shoot formation in groundnuts.

Multiple shoot inductionmedia	GJG20		GJG22	
	No.ofexplants responded(%)	No.of shoots/explants	No.ofexplants responded(%)	No.of shoots/explants
MSM1	44.7±3.7	1.4±0.5	40.4±3.3	1.3±0.5
MSM2	50.5±3.9	1.9±0.6	48.6±3.7	1.9±0.7
MSM3	71.3±5.5	2.7±0.7	64.6±5.3	2.5±0.9
MSM4	65.3±5.3	3.4±0.4	60.3±3.1	3.1±0.8
MSM5	59.6±4.2	3.1±0.3	49.7±4.2	1.9±0.4
MSM6	51.4±3.1	2.1±0.7	45.6±4.1	1.5±0.6
MSM7	63.7±4.4	3.3±0.8	56.3±4.3	3.0±0.8
MSM8	65.2±5.2	4.6±0.6	69.5±4.3	4.1±0.6
MSM9	80.1±5.8	4.1±0.3	62.7±4.7	3.6±0.5
MSM10	71.6±5.8	3.7±0.3	59.4±4.0	2.4±0.4
MSM11	59.9±4.2	3.2±0.6	53.3±4.3	3.3±0.8
MSM12	70.5±5.5	5.0±0.7	58.8±5.0	3.7±0.9
MSM13	**88.3±5.1**	**9.2±1.1**	**74.2±5.3**	**7.1±1.2**
MSM14	83.7±5.9	6.3±1.1	68.3±5.6	5.2±1.1
MSM15	71.5±5.5	3.7±0.5	56.5±5.1	3.2±0.9

The best results have been shown by bold letters.The values of standard deviation (n=3) have been given after ± symbol.

Table 6 : Assessment of various combinations of IBA on number of roots per shoot and average root length (cm) in groundnuts.

Multiple shoot induction media	GJG20			GJG22		
	No.ofShoot producingroots(%)	No.of roots per shoot	Root length (cm)	No.of Shoot producing roots (%)	No.of roots pershoot	Root length (cm)
RIM1	15.5±2.3	0.0±0.0	0.0±0.0	11.3±2.3	0.0±0.0	0.0±0.0
RIM2	23.3±3.0	0.8±0.3	2.0±0.4	17.5±2.0	0.6±0.3	1.7±0.4
RIM3	35.4±4.7	3.1±0.5	2.8±0.7	26.8±3.3	2.3±0.7	2.0±0.6
RIM4	46.7±5.1	1.3±0.3	2.1±0.6	38.4±3.3	1.3±0.5	1.6±0.6
RIM5	39.3±4.1	0.8±0.3	1.7±0.3	27.3±2.9	0.6±0.3	1.3±0.3
RIM6	50.4±3.8	4.4±0.7	2.0±0.6	42.6±4.6	2.4±0.8	1.3±0.3
RIM7	58.8±3.0	6.3±1.1	5.8±0.8	50.7±5.1	4.2±1.0	3.6±0.8
RIM8	**90.5±5.8**	**8.3±1.4**	**8.1±1.5**	**76.3±5.8**	**6.3±1.3**	**7.0±1.1**
RIM9	79.6±5.5	4.8±0.8	6.3±1.3	70.8±4.8	3.4±0.9	4.3±0.7
RIM10	76.3±4.6	3.2±0.3	4.7±0.8	66.6±4.4	2.1±0.3	2.7±0.8

The best results have been shown by bold letters.The values of standard deviation (n=3) have been given after ± symbol.

Individual shoots were separated from the group of numerous shoots and placed in MS media containing IBA (0.5–2.5 mg/L) for two weeks to facilitate in vitro root regeneration. This study concluded that phytohormones are not required to produce roots (Asylin-Ozudogru*et al*., 2005). After being moved to root induction media, 2–5 cm shoots began to produce roots in both kinds 3–4 weeks later. According to Perveen *et al*. (2011), the greatest root induction frequency and maximum root length in both types were observed at 1.5 mg/L IBA, even though other dosages of IBA had previously been found to cause optimum root induction.Healthy roots first developed at the base of shoots at 1.5 mg/L of IBA; however, at higher IBA concentrations (> 1.5 mg/L), callus forms, causing roots to thicken and shorten and inhibiting the production of new roots. Following an increase of root production, these plantlets were moved to a hydroponic culture in Yoshida solution (Yoshida *et al*., 1976) for one to two weeks, with the addition of fresh Yoshida solution every four days. The plants were placed in plastic bags to acclimate them once they showed signs of lateral root development and considerable root elongation. After some time (2 weeks) these acclimatized, plants were again transferred to soil pots to maintain and grow in new environmental condition. Similar results were also reported by Venkatachalam &Jayabalan(1997); Banerjee *et al*. (2007); Verma *et al*. (2009); Radhakrishnan*etal*. (2000); by using mixture of hormones *i.e.* IBA, NAA and KIN for root induction in groundnut. Present technique provides a reliable and high frequency yielding process for obtaining morphologically normal peanut plants in a short time.

CONCLUSION

The current study offers a high frequency yielding method that is dependable and fast in producing peanut plants with normal morphology. The maximum callus induction frequency was achieved by combining BAP with NAA, which was the ideal hormonal combination. 5.5 mg/L BAP and 1.5 mg/L NAA in Golden produced the highest CIF when compared to other combinations. BAP encouraged multiple shoot induction in conjunction with TDZ and KIN. The highest callus induction rates (86 & 78%) in GJG20 and GJG22, respectively, were produced by MS media containing 5.5 mg/L BAP and 1.5 mg/L NAA, which were determined to be the best. Similarly, using 4 mg/L BAP together with 1 mg/L resulted in the maximum number of multiple shoots/plant (9 & 6) with the ideal length of shoots (4.8 & 4.1 cm).In GJG20 and GJG22, NAA and 1.1 mg/L TDZ, respectively. When IBA (>1.5 mg/L) was used, the rooting system was inhibited, but the roots on RIM8 [MS + IBA (1.5 mg/L)] appeared healthy and normal.

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