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Studies on Factors Influencing Callus Induction in elite Groundnut (*Arachis hypogaea*) Cultivar TMV (Gn) 13

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ABSTRACT: Groundnut (*Arachis hypogaea* L.), a major food grain legumes is an important economic crop that yields vegetable oil and serves as a rich protein source. Crop improvement through modern biotechnological approaches necessitates establishment of cell cultures for multiple uses. Groundnut like other legumes are recalcitrant to tissue culture and any attempt in optimizing protocol for cell cultures is considered to be a boon for plant genetic manipulation. The present study was conducted to evaluate various factors influencing callus initiation for subsequent cell cultures establishment from various explants under *in vitro* condition in an elite groundnut cultivar TMV (Gn) 13. Leaf and DEC explants are considered as the best choice of explants for callus induction in TMV (Gn) 13 with callus induction frequency of 97.22% when cultured in MS medium supplemented with 5.0 mg l $^{-1}$ 2, 4-D. The effect of 2, 4-D alone to stimulate better callus induction in various explants of groundnut is demonstrated.

Keywords: Auxin, callus, cell cultures, Arachis, Groundnut, tissue culture.

INTRODUCTION

Legumes are third largest family of higher plants, wherein groundnut (*Arachis hypogaea* L.) is one of the most important species. It is among the world choicest agriculturally important economic crop not only in vegetable oil but also provide a source of protein, calcium, iron and vitamin B complex such as thiamine, riboflavin, niacin and vitamin-A. Attempts to develop groundnut cultivars resistant to biotic and abiotic stresses by conventional breeding methods had limited success due to narrow genetic variability among the germplasm accessions. Association studies for the yield contributing traits in *Arachis hypogaea* has been done for several genotypes, which shall be rewarding for yield improvement in groundnut (Roy *et al*., 2021). Among various approaches, crop improvement through genetic engineering needs a high frequency plant tissue culture protocol, of which cell cultures is a prerequisite.

The extensive efforts to improve legumes conferring desirable traits through *in vitro* manipulation have been a focal subject of research in recent years. Legumes exhibit a diverse response when cultured *in vitro*. Depending on several factors, regeneration occurs *via* organogenesis or embryogenesis, either directly from explanted tissue or indirectly after an intervening callus phase. With few exceptions, legumes were commonly described as recalcitrant with regard to tissue culture of the large seeded legumes.

Trivedi, (2014) has stated that in most of the legumes including groundnut, regeneration is sporadic and transient, due to their recalcitrant nature. Successful regeneration of legume species has been significantly supported by species specific determination of critical parameters, such as explant source, genotype and media constituents. Tiwari and Tuli (2008) reported the importance of orientation of placing the de embryonated explant on the above media was critical for *in vitro* regeneration. Nazir *et al*., (2011) studied the effect of longitudinally halved deembryonated cotyledons on various combinations of BAP and NAA and found that 4 and 0.1 mg l^{-1} of BAP and NAA respectively to be the best for regeneration. The variety BARI-2000 was the best responsive for *in vitro* regeneration.

Earlier reports for the establishment of *in vitro* regeneration system through indirect organogenesis used whole immature cotyledon Robinson *et al*., (2011), cotyledonary nodes and mature embryos Lacroix *et al*., (2003), mature dry seeds Baker *et al*., (1995), leaflets Chengalrayan *et al*., (2001); Tiwari and Tuli, (2009), hypocotyls Venkatachalam *et al*., (1997), mature epicotyl Shan *et al*., (2009).

However, owing to the large size of the explants it's often difficult to obtain more planting material in short time. Effect of pre and post treatment of explants like zygotic embryos, plumular apices and embryonic axis in various hormonal combinations were found to be effective in peanut cultivar NC-7 (Ozkan and Aasim 2019).

Venkatachalam *et al*. (1996) made a thorough study of callus induction and morphogenesis of different groundnut explants. Immature leaf was identified as the most efficient explant in producing callus in medium containing NAA (3 mg 1^{-1}) and Kn (0.5 mg 1^{-1}). Palanivel *et al.* (2002) evaluated the efficiency of callus induction and plantlet regeneration from mature cotyledonary segments of groundnut cultivars VRI (Gn) 2 and VRI (Gn) 3. Ganesan *et al*. (2011) reported method to regenerate *A.hypogaea* from embryo explants. Recently, efficient in vitro systems for whole plant regeneration have been reported in *Arachis glabrata*, a rhizomal perennial peanut (Dolce *et al.*, 2018).

The present study was conducted to evaluate various factorsinfluencing somatic embryogenesis in cell suspension cultures and the morphogenetic ability of various explants under *in vitro* condition in a elite groundnut cultivar TMV (Gn) 13.

MATERIALS AND METHODS

Seeds of groundnut cultivar TMV (Gn) 13 were obtained from the Oilseeds Research Station, Tindivanam. TMV (Gn) 13 is a red kernel variety preferred for its high oil recovery by oil mills. Explants of TMV (Gn) 13 were collected from the *in vitro* germinated seedlings *viz*., leaf, de-embryonated cotyledon, hypocotyl and epicotyl

Surface sterilization of seeds

Seeds of groundnut cultivar TMV (Gn) 13 was washed for few minutes under continuous stream of running tap water and then treated with 2-3 drops of Tween-20 for 5 min. and instantly rinsed with tap water followed by rinsing twice with distilled water. Seeds were treated with 0.1% (w/v) of $HgCl_2$ for 10 min. followed by rinsing 3-4 times with sterile distilled water under aseptic condition in laminar air flow chamber. Subsequently, the seeds were treated with 70% ethanol for 30 sec. followed by rinsing with sterile double distilled water. Sterilized seeds were blot dried on a sterile tissue paper and inoculated for germination in half strength MS media and incubated at 25±2 °C and 60 % relative humidity inside the culture room.

Callus induction

Callus induction medium

Callus induction medium (CIM) was prepared using MS nutrients supplemented with varied concentrations of the auxin, 2,4-D. For the induction of callus in TMV (Gn) 13 Leaf, de-embryonated cotyledon (DEC), hypocotyl and epicotyl explants from the *in vitro* germinated seedlings were used for callus induction and incubated at 25±2 °C in dark conditions with 60-70% relative humidity inside the culture room.

Effect of auxin on callus induction in TMV (Gn) 13

For callus induction from the different explants of TMV (Gn) 13, MS medium supplemented with 2, 4- D at six different concentrations (5, 10, 15, 20, 25, 30 mg l^{-1}) were used.

Sub-culturing of calli

The calli was subcultured to the fresh medium of same concentration in which callus was induced, at an interval of 14 days. The subcultured plates were maintained in dark condition. The calli from second subculture was used for establishment of cell suspensions.

Callus induction frequency

The best treatment concentration of the media was decided based on the callus induction frequency (expressed in percentage) observed after 30 days of inoculation. Callus induction frequency was calculated using the following formula given below.

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of explants showing initiation}}{\text{Total no. of explants inoculated}} \times 100$$

Determination of growth of cells in callus cultures

Growth of cells in callus was determined after 30 days of inoculation by calculating fresh weight and dry weight. Each time before subculturing, the calli were collected in a sterile filter paper and the fresh weight of callus (10 nos.) was measured using an electronic balance. Fresh callus weight was expressed in g $calli^{-1}$. For dry weight measurement, the calli was dried in hot air oven at 60 °C overnight and the weight was measured. The difference in weights shows the growth of callus for each treatment combinations and the best treatment was assessed (Dung *et al.*, 1981).

$$\text{Dry weight percentage (\%)} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

Statistical analysis: The experiments were carried out in three replications with 12 explants per replication in completely randomized design for callus induction studies. The observations were tabulated and statistical analysis were carried out using the statistical software AGRES and the results were interpreted.

RESULTS

A. Callus induction studies

***In vitro* germination and collection of explants**

Surface sterilized seeds of TMV (Gn) 13 were inoculated in the half strength MS media to induce *in vitro* germination. The germinated seedlings (14 days after inoculation) were collected and used as explants for callus induction. Epicotyl, hypocotyl, leaves and de-embryonated cotyledon (DEC) explants (Fig. 1) were chosen for identifying the best choice of explant for callus induction in TMV (Gn) 13 whereas leaf explant from the field grown groundnut cultivar.

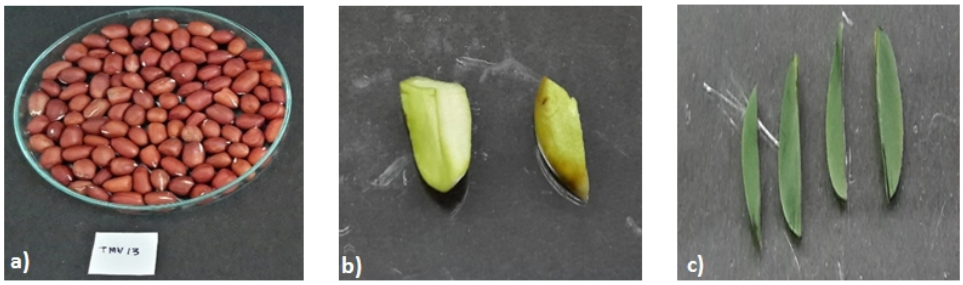


Fig. 1. Explants for *in vitro* studies in Groundnut (*Arachis hypogaea*)
a) Seeds of TMV (Gn) 13
(b) DEC explant of TMV (Gn) 13 from in vitro germinated seedling
(c) Leaf explants of TMV (Gn) 13 from in vitro germinated seedling.

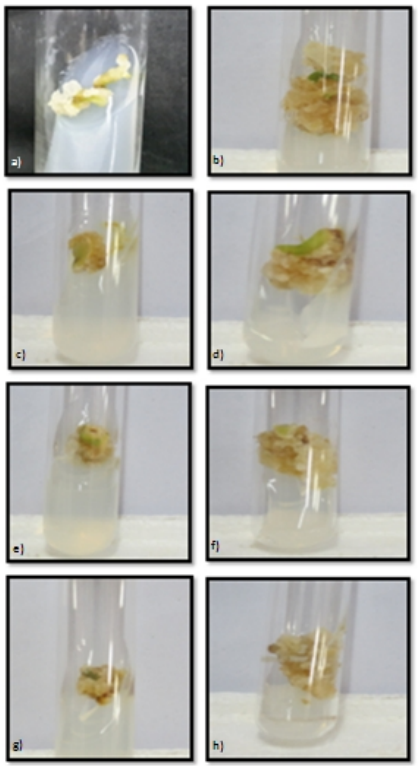
B. Callus initiation from explants

Effect of explants on callus induction

In case of TMV (Gn) 13 cultivar callus was induced from explants collected from *in vitro* germinated seedlings. Among the different explants *viz*. leaf, DEC, epicotyl and hypocotyl; leaf and DEC explants gave maximum callus induction frequency (97.22 %) when cultured in the MS medium supplemented with 5.0 mg l^{-1} 2,4-D. The concentration of 2,4-D for callus induction was optimized for various explants (Table 1). Further increase in the concentration of 2, 4-D, decreased the induction of friable calli as observed in hypocotyl (94.44 %) and epicotyl explants (44.44 %). This observation suggests that 2, 4-D has an effect on callus induction. Leaf and DEC explants are considered as the best choice of explants for callus induction in TMV (Gn) 13 (Fig. 2, Table 2).

Table 1: Effect of Growth regulator concentration on various explants for callus induction.

Conc. of 2,4-D (mg/L)	Explant	No. of explants inoculated	Callus induction from explants	Callusing frequency (%)
2	Leaf	10	5	50
5		10	10	100
10		10	9	90
2	Hypocotyl	10	6	60
5		10	10	100
10		10	7	70
2	Epicotyl	10	4	40
5		10	5	50
10		10	5	50
2	Cotyledon	10	5	50
5		10	10	100
10		10	9	90
5	Leaf	10	10	100
10		10	9	90
15		10	8	80
20		10	8	80
25		10	7	70
30		10	6	60
5	De-embryonated cotyledon (DEC)	10	10	100
10		10	9	90
15		10	8	80
20		10	7	70
25		10	7	70
30		10	5	50



(a) Leaf derived calli one week after inoculation (B5+5 mg l^{-1} 2, 4-D), (b) Leaf derived calli two weeks after inoculation (B5+5 mg l^{-1} 2, 4-D) (c) DEC derived calli one week after inoculation (B5+5 mg l^{-1} 2, 4-D), (d) DEC derived calli two weeks after inoculation (B5+5 mg l^{-1} 2, 4-D), (e) Hypocotyl derived calli one week after inoculation (B5+5 mg l^{-1} 2, 4-D), (f) Hypocotyl derived calli two weeks after inoculation (B5+5 mg l^{-1} 2, 4-D), (g) Epicotyl derived calli one week after inoculation (B5+5 mg l^{-1} 2, 4-D), (h) Epicotyl derived calli two week after inoculation (B5+5 mg l^{-1} 2, 4-D)

Fig. 2. Callus induction from different explants of Groundnut cv. TMV (Gn) 13.

Table 2: Effect of explants in callus induction of TMV (Gn) 13.

S.No.	Treatment	Concentration of 2, 4-D (mg l^{-1})	Explant	Callus induction frequency (%) Mean±SE*
1.	CIM I	5.0	Leaf	97.22±2.780[a]
2.			DEC	97.22±2.780[a]
3.			Epicotyl	44.44 ±5.55683[b]
4.			Hypocotyl	94.44±2.780[a]

* Mean is average of 3 replicates, with 12 explants per replicate; CIM: Callus Induction Media
Completely Randomized Design was used for statistical analysis
Treatments following same alphabets are on par
Group a ->Leaf, DEC, Hypocotyl, Group b-> Epicotyl
Group a, has the best treatments and Group b has the poorest performing treatments.

DISCUSSION

Evaluation of factors influencing callus induction in groundnut

Tissue culture is a prerequisite for improvement of the crops through genetic engineering. Hence it is inevitably important to develop a reproducible protocol for the *in vitro* studies, multiplication and efficient transformation of elite groundnut cultivars. Trivedi (2014) has stated that in most of the legumes including groundnut, regeneration is sporadic and transient, due to their recalcitrant nature. Successful regeneration of legume species has been significantly supported by species specific determination of critical parameters, such as explant source, genotype and media constituents. *In vitro* regeneration in groundnut is genotype specific and involves optimization of protocol for individual cultivars considering various other factors that influence callus induction and plant regeneration. The plant regeneration system generally relies on several factors such as genotype, explants, plants growth regulators, media composition including the physical factors photoperiod, temperature and the environment (Ishag *et al*., 2009). Hutchinson *et al*., (1994) reported the role of BAP and NAA in callus induction from the mature zygotic embryos of a tetraploid *Alstroemeria* whereas the TDZ and BAP showed multiple shoot initiation from the cultured explants.

Callus, an undifferentiated mass of proliferating parenchymatous cells can be induced in plant organs and tissues. Usually callus develops in response to injury in the plants. Auxins and cytokinins either alone and in combinations could be used to promote callus induction, proliferation or accelerated growth. Callus induction was reported earlier by Verma and Huystee (1969) from cotyledon explants of *A. hypogaea* in MS medium supplemented with NAA (2 mg 1^{-1}) and Kinetin (0.5 mg 1^{-1}) along with amino acids.

The present study has evaluated the choice of explants for induction of callus from a promising cultivar TMV (Gn) 13 using the auxin, 2,4-D at varied concentration levels. In case of TMV (Gn) 13, among various explants used, the leaf and DEC explants gave maximum callus induction frequency (97.22 %) when cultured in the MS medium with 5.0 mg l^{-1} 2,4-D. Furtherincrease in 2, 4-D levels, decreased the induction of friable calli. Leaf and DEC explants were shown to the best choice of explants for callus induction in TMV (Gn) 13. This observation in the present study suggests that 2,4-D alone has significant effect on callus induction and is similar to the reports of Narasimhulu and Reddy (1983), where 2,4-D as a single potent auxin was used to stimulate callus induction in groundnut.

In an effort to choose the best responsive variety between Golden and BARI 2001, Ahmad *et al*., (2020) reported that MS media having 5.5 mg/l BAP and 1.5 mg/l NAA was found to be best for producing the highest callus induction frequencies (86 & 78%) in Golden and BARI 2001, respectively.

Sukumar and Rangasamy (1984) obtained callus and root formation in other relatives of cultivated groundnut *viz*., *A. diogoi*, *A. duranensis*, *A. marginata*, *A. monticola*, *A. hagenbeckii*, *A. glabrata* and *A. villosulicarpa* utilizing petiole, pinna and stem as explants in MS media containing 2, 4-D (2 mg l^{-1}). Narasimhulu and Reddy (1983) has also reported the formation of roots at a concentration of 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin in addition to the formation of callus from the cut ends. Similar observations were made in the present study, when de-embryonated cotyledons of TMV (Gn) 13 were inoculated in MS media supplemented with B5 vitamins and 2,4-D at 5.0 mg l^{-1} and 10 mg l^{-1} concentrations. Rhizogenesis, in this instance could be attributed to some unknown substances within the tissue which may be altering the effectiveness of auxin.

Plant regeneration through somatic embryogenesis comprises a series of developmental stages, which require the proper execution of each step for successful completion of the plant regeneration (Ramakrishnan *et al.,* 2005). Xu *et al*., (2016) established a new method for peanut regeneration through somatic embryogenesis. In MS media containing 2,4-D 20.0 mg 1^{-1} culturedindark, all the inoculated cotyledon explants formed BLBs and spontaneously developed into 3–5 shoots per BLB. Thus, future research thrusts must focus on enhancing conversion frequency of somatic embryos after transformation into normal plantlets regeneration. Many protocols reported earlier are quite often not reproducible and obtained with limited success in terms of plantlet conversion from somatic embryos.

CONCLUSION

The present study has made efforts to optimize protocol for callus induction in groundnut. Leaf and DEC explants are considered as the best choice of explants for callus induction in TMV (Gn) 13 with callus induction frequency of 97.22 % when cultured in the MS medium supplemented with 5.0 mg l^{-1} 2,4-D. The effect of 2,4-D alone to stimulate better callus induction in various explants of groundnut is demonstrated. Further, *in vitro* studies through indirect organogenesis can be attempted with various explants and hormonal combinations for successful plant regeneration in groundnut.

Conflict of Interest. The authors declare no conflict of interest.

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