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Research Article

Optimized In-Vitro Regeneration of Peanut (*Arachis hypogaea* L.) via Callus Induction and Embryogenesis

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ABSTRACT

To investigate the effect of the growth regulators of auxin plants (NAA) and cytokinin (BAP) on the regeneration potential of two varieties of Bard-479 and Potohar peanuts in-vitro culture. As explants, 22-day-old seedlings derived from callus and the embryonic axis was used. The explant as cotyledonary nodes were cultured in basal medium (MS) of medium resistance supplemented with different combinations and concentrations of auxin (NAA) and cytokinin (BAP) for the primary proliferation of the shoots. For the culture of embryos, Picloram (19mg/l), BAP (0.1, 0.2, 0.3, 0.4 mg / l) in combination with NAA (0.1, 0.2, 0.5mg/l) and 2, 4 - D (0.1-0.3 mg/l) together with BAP and NAA used with the same combinations and concentrations were used for callus. The whole plant's shoots, leaves, and regeneration were scored after four weeks according to the percentage of explants that produced shoots, calluses, embryos, and roots per explants. The best shoots were observed at 0.4 mg / l BAP with 0.1 mg / l NAA; 87% were observed in BARD-479, and POTOHAR showed 75% regeneration by embryogenesis. Callus showed 73% recovery in BARD-479 and 65%in POTOHAR. Similarly, some media could produce structures similar to the roots at less than 10% frequency. For Bard-479, the best rate for roots were found with 0.3 mg / l IBA and 0.2 mg / l NAA, i.e. (95%), and for Potohar (87%) is recorded. ANOVA (analysis of variance) reported in Table 1-8 showed that callus and embryogenesis were of great importance.

Keywords: *Arachis hypogaea* L., Embryogenesis and Callus, Auxin (NAA) and Cytokinin (BA).

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INTRODUCTION

Oilseed crops play a vital role in global food production, providing nutrition, edible oils and protein (Begum et al., 2022; Bangash[a] et al., 2012; Gul et al., 2011). Among these, groundnut (*Arachis hypogaea* L.), belongs to the *Leguminaceae* family, also known as peanut, holds significant importance due to its high oil content, nutritional value, and versatility in food and industrial applications. As a leguminous crop, groundnut not only contributes to edible oil production but also enhances soil fertility through biological nitrogen fixation (Bangash[b] et al., 2013). Its adaptability to diverse agro-climatic conditions and economic value make it a crucial component of sustainable agricultural systems, particularly in tropical and subtropical regions.

It is one of the economically essential legumes and is profoundly grown throughout the world in a wide range of climatic conditions (Rehman et al., 2020).

Peanuts are major edible oil and a significant source of dietary proteins and fats and nearly 30% protein (Aslam et al., 2021; Gohari & Niyaki, 2010). Asia, Africa, and North Central America are the large producers of peanuts with 71.7, 18.6, and 7.5%, respectively (ICRISAT, 2009).75% peanuts is used for extracting oil, while 24% is for food after cereals legumes are used to intractable to tissue culture regeneration. Therefore, to develop an efficient *in-vitro* regeneration system, many efforts have been devoted (Ahmed et al., 2021; Anuradha *et al*., 2006).In the Potohar region of Rawalpindi, Pakistan 500 ha were under cultivation in 1949-1950 (Mehmood[a] et al., 2021; Khalil & Jan 2005). The annual production of peanuts for 2021-22 was 90 thousand metric tons and area harvested 138 thousand hectares (Anonymous, 2021). Transgenic crops could be successfully generated, and their efficacy depends on the development of proper protocols (Mehmood[b] et al., 2021; Ahmed et al., 2012; Misra & Misra, 1993).

Advanced techniques such as high-throughput phenotyping, nanotechnology; PCR, genome sequencing, protein sequencing, and real-time PCR have revolutionized agricultural research, enabling precise genetic analysis, trait selection, and disease resistance studies (Amanat et al., 2024; Jamil et., 2022; Farrukh et al., 2022; Khattak et al., 2020; Ur-Rehman et al., 2020; Waqar et al., 2018). In peanut (*Arachis hypogaea*), tissue culture combined with molecular tools facilitates rapid propagation, genetic transformation, and stress tolerance improvement (Saleem et al., 2024; Sebiani-Calvo et al., 2024). Real-time PCR aids in detecting gene expression patterns, while genome sequencing accelerates trait identification for breeding programs (Khattak et al., 2022; Qaiser et., 2022). These innovations enhance tissue culture efficiency, ensuring the development of high-yielding, resilient peanut varieties.

Peanut is resistant to tissue culture, and techniques like in vitro propagation can help with mass propagation. (Ahmed[a] et al., 2019; Akasaka *et al*. 2000; Heatley and Smith 1996).Different explant sources were used to build efficient and effective methods. In peanut, in vitro regeneration has been documented using a variety of explant sources, including epicotyl, leaf segment, immature leaflet, hypocotyl, cotyledon, somatic embryos, cotyledonary node, and seed. (Mehmood[c] et al., 2021; Limbua et al. 2019; Venkatachalam et al., 1999; McKently *et al*. 1990).The frequency of regeneration varied depending on the explant source and the depending on the concentration of hormone utilized. ICGV12991, CG7, and Red Valencia Kenyan peanut genotypes were successfully regenerated using the cotyledonary node, with a regeneration rate of 80 to 81 percent (Limbua et al. 2019).On the other hand, at varying concentrations of BAP, shooting rates of 86 and 98 percent were reported, with no significant difference (Hsieh *et al*. 2017). Immature embryonic axes and cotyledonary node (Pathak *et al*. 2017), pigeon pea cotyledonary node, chickpea plumular apices (Aasim et al. 2013), and mung bean cotyledonary node have all been described as legume crops regenerated in in vitro culture using various explant sources (Ahmed[a] et al., 2011; Mojumder *et al*. 2015).Tissue culture procedures, in addition to shoot buds regeneration or growing roots utilize different explant sources, which are crucial in the growth of transgenic plants. In crop breeding projects, for example, effective plant regeneration methods and suitable explant are useful in transforming genes utilizing *Agrobacterium-mediated* gene transformation. The availability of a suitable genetic transformation methodology that is well-suited to the in vitro propagation regeneration technology of the chosen and targeted plant species is the most critical and primary stage for efficiently gene transfer to plant species (Ahmed[b] et al., 2019; Ahmed[b] et al., 2011; Kar *et al*. 1996). In peanuts, certain scientific research discoveries have been documented (Aslam et al., 2018; Sharma and Anjaiah 2000; Anuradha et al. 2006; Bhatnagar et al. 2010). Using CNs for in vitro regeneration of diverse plant species confirmed that it is the best method for the development and production of a large number of independently converted plants, according to these researchers. Similarly, Hsieh et al. (2017) found that direct regeneration with CN reduces the time needed to generate healthy, repeatable plants in tissue culture systems and is acceptable for genetic transformation.

In vitro regeneration of peanut plants have been achieved from different tissues both through organogenesis and embryogenesis (Hoa et al., 2021; Siddiqui et al., 2017). Somatic embryogenesis is more efficient and valuable than organogenesis for plant regeneration (Aslam[a] et al., 2017; Bhanumathi *et al*., 2005). As peanut is a very complex crop, minimal success has been achieved in-vitro through tissue (Heatley & Smith, 1996). Peanut is a distinct crop that requires specific protocols for each type, so the micro-propagation is tedious (Ahmed[a] et al., 2010). The rate of proliferation and transformation is high, and therefore it is preferred (Begum et al., 2019; Snedecor, 1998). Tissue culture that includes direct embryoid induction and callus induction has been reported by Palanivel et al., (2002), and regeneration is very squat. Micropropagation in peanuts is very tedious as it is a specific genotype. Plant multiplication is not efficient because of high transformation potential and proliferation through somatic embryogenesis (Zahid et al., 2022; Ahmed et al., 2017; Ahmed[b] et al., 2010; Snedecor, 1998).

This study is concerned with working on plant growth regulators to find a suitable combination for callusing and

embryogenesis (somatic) cotyledonary node (CN). This study aimed to develop an efficient and productive micro-propagation protocol specifically for two promising varieties, i.e., BARD-479 and Pohotor in vitro multiplication.

MATERIALS AND METHODS

Seeds of high production cultivar POTOHAR and BARD-479 were selected and requested from Barani Agricultural Research Institute, Chakwal. The seeds were washed with 70% ethanol for 1 min followed by surface sterilization in 10% sodium hypochlorite (Commercial bleach) or 50 % Clorox with two drops of Tween-20 for 20 min. Seeds were then washed 4-5 times with deionized water and soaked for three h or overnight (Afzal et al., 2017; Owonubi *et al.,* 2011).

Callusinduction and plant regeneration:

Cotyledons were plated on a basal medium supplemented with hormones for callus induction. The explants obtained from cotyledons used for direct embryo induction were cultured on the medium with vitamins B5, 2.8g phytagel, 30 g/L sucrose, 2, 4- D, and NAA (Gamborg et al., 1968). These explants were then cultured Petri-plates. After 3-4 subcultures of 12 to 14 days each, 48 days old calli with white creamy colors were transferred to MS medium supplemented with vitamins B5, 3% sucrose, BAP (0.1-0.4 mg/l) and NAA (0.1-0.5 mg/l), 2, 4- D (0.1-0.3mg/l) for regeneration.

Direct somatic embryogenesis and plant regeneration:

On MS media supplemented with 3% (w/v) sucrose and 2.8g (w/v) phytagel (Gamborg et al., 1968), seeds incubated for round about 22 days at 26±1°C after the cotyledons were cut from proximal ends with PH 5.8. They were kept consistently under 16-h photoperiod with 60 l E/m2/s light intensity at 25±1o C. Explants were sub-cultured after 25 days of embryoid induction. The Mature embryoids, shifted to regeneration media (B5 vitamins and with different concentrations of BAP (0.2-0.4 mg/l) and NAA (0.1-0.5 mg/l). Then it will be transferred to 1:1:1 soil, sand, and manure after 35- 45 days of root development; for complete adaptation, the plants will be shifted to plastic pots.

Statistical analysis:

Combination of media suitable for callusing and embryogenesis will be subjected to Mini-tab for analysis.

RESULTS

The ANOVA (analysis of variance) as mentioned in Table 1-8 showed that combination of callus induction and embryogenesis was highly significant.

Callus induction:

Callus induction was done by using explant as cotyledonary nodes on MS media with the addition of vitamins B5 (Gamborg et al., 1968), growth hormones BAP (0.1-0.4 mg/l), and NAA (0.1-0.5 mg/l) were used for culture. (Table: 1-2). 73% was recorded as highest for inducing callus with 0.3 mg/l of 2, 4-D concentration (Fig. 1). For callus induction, Bard-479 gave maximum callus induction, i.e., 73%, which was attained with BAP (0.4 mg/l) and NAA with an amount of (0.1 mg/l). Potohar genotype showed maximum of 65% induction rate obtained with BAP (0.4 mg/l) with NAA (0.2 mg/l). The combination of BAP and NAA proved to be the best in callus induction than any NAA and BAP used individually. Similarly, increasing or decreasing concentration was affecting the results.

Table 1: Callus induction media of BARD 479 using different harmones.

Replication	200 mg/ml BAP, 100 mg/ml IAA& 2-4 D 300 mg/ml		400 mg/ml BAP, 100 mg/ml IAA		300 mg/ml BAP, 100 mg/ml IAA		100 mg/ml BAP& 500 mg/ml IAA		300 mg/l BAP&IAA300 mg/ml		400 mg/l BAP&IAA200 mg/ml	
	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq
1	18	72	14	56	6	24	3	12	1	4	2	8
2	17	68	16	64	6	24	4	16	3	12	0	0
3	18	72	15	60	5	20	5	20	3	12	0	0
4	20	80	16	64	7	28	3	12	2	8	1	4
Total	73		61		24		15		9		3	

When NAA concentration was increased from lower to higher, i.e., 0.1 to 0.5 mg/l, enhancement in callus induction was observed, but the optimum and best concentrations amount of both the hormone (NAA & BAP) that induced

the highest rate of callus was obtained with (0.1 mg/l, 0.2 mg/l and 0.3 mg/l, and 0.4 mg/l), respectively. After the end of about one month of culture, none of those as mentioned above hormonal combinations showed any bud induction. Similarly, some of the media could produce root-like structures with less than 10% frequency.

Table 2: Callus induction media of POTOHAR using different harmones.

Replication	200 mg/ml BAP &300 mg/ ml IAA2-4 D, 3 mg/ml		400 mg/ml BAP & 100 mg/ml IAA		300 mg/ml BAP & 100 mg/ml IAA		100 mg/ml BAP & 500 mg/ ml IAA		300 mg/l BAP & IAA 300 mg/ml		200 mg/l BAP & IAA 100 mg/ml	
	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq
1	17	68	12	48	8	32	4	16	1	4	1	4
2	16	64	11	44	8	32	4	16	2	8	2	8
3	17	68	15	60	7	28	3	12	1	4	0	0
4	15	60	14	56	5	20	1	4	3	12	1	4
Total	65		52		28		12		7		4	

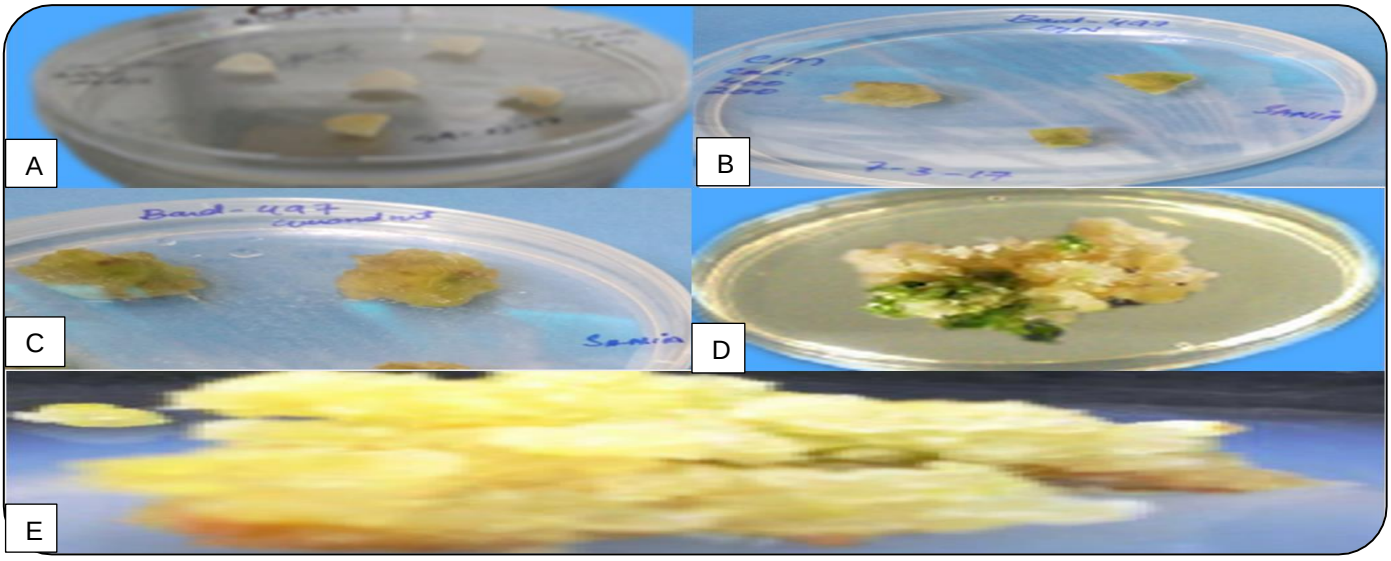

Figure 1. Callus induction with different hormonal combination A) cleaned seed put on media with different conc. B) growth/sprouting on MS media. C) semi callus development of seed. D and E) fully callus development (Close up with flash)

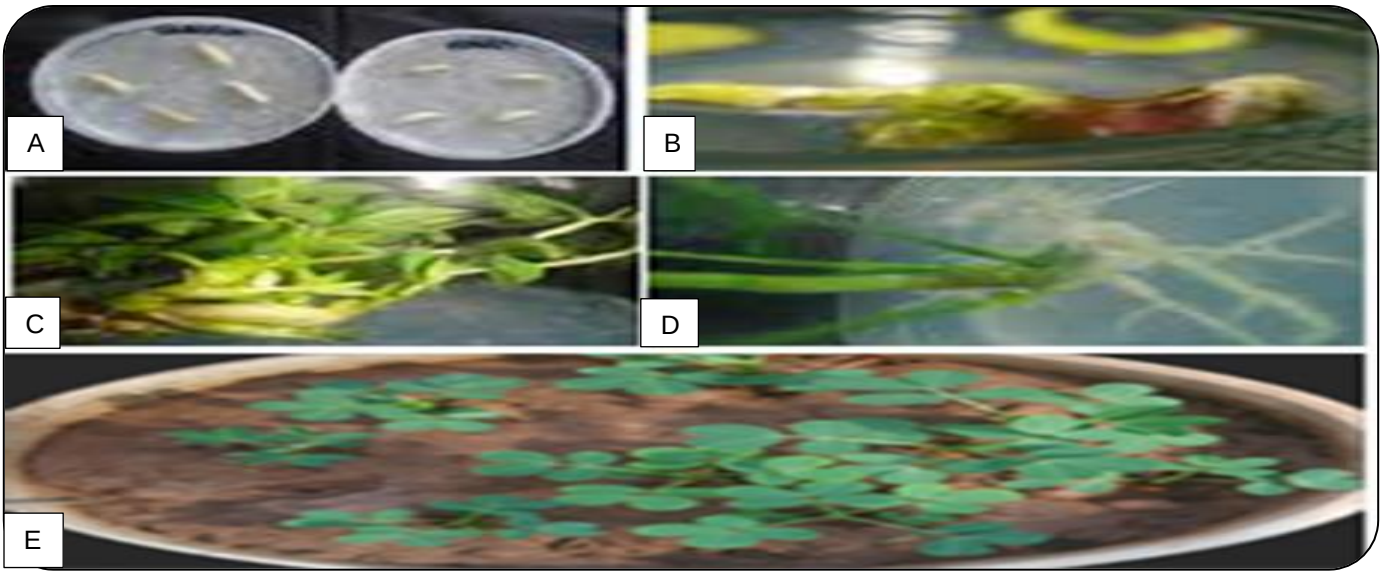

Figure 3. (A) Hardening, (B) acclimatization of rooted plants (C-D) shifted to the pods and (E) finally shifted to the field.

Table 5. Embryogenesis for shoot induction media of BARD 479.

Replication	400 mg/ml BAP & 100 mg/ ml NAA		400 ml/ml BAP & 200 mg/ml NAA		400 mg/ml BAP & 500 mg/ml NAA		200 mg/ml BAP &100 mg/ ml NAA		200 mg/l bap & NAA 200 mg/ml		200 mg/l bap & NAA 500 mg/ml	
	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq
1	22	88	21	84	6	24	2	8	0	0	0	0
2	21	84	20	80	8	32	2	8	0	0	0	0
3	20	80	18	72	6	24	4	16	2	8	1	4
4	23	92	18	72	9	36	4	16	3	12	2	8
Total	86		77		29		12		5		3	

Table 6: Embryogenesis for shoot induction media of Potohar.

Replication	400 ml/ml BAP & 100 mg/ml NAA		400 mg/ml BAP & 200 mg/ ml NAA		400 mg/ml BAP & 500 mg/ml NAA		200 mg/ml BAP &100 mg/ ml NAA		200 mg/l bap & NAA 200 mg/ml		200 mg/l bap & NAA 500 mg/ml	
	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq
1	19	76	17	68	6	24	2	8	3	12	1	4
2	18	72	16	64	4	16	5	20	3	12	0	0
3	19	76	14	56	5	20	3	12	0	0	1	4
4	19	76	12	48	3	12	3	12	1	4	1	4
Total	75		59		18		13		7		3	

Table 7: Embryogenesis for Root induction media of BARD 479.

Replication	200 mg/ml IBA &300 mg/ ml NAA		300 mg/ml IAB & 200 mg/ml NAA		300 mg/ml IBA & 100 mg/ml NAA		100 mg/ml IBA & 500 mg/ ml NAA		300 mg/l IBA & NAA 300 mg/ml		200 mg/l IBA & NAA 100 mg/ml	
	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq
1	23	92	19	76	5	20	3	12	3	12	2	8
2	24	96	20	80	5	20	4	16	2	8	0	0
3	24	96	20	80	2	8	4	16	2	8	1	4
4	24	96	21	84	6	24	3	12	0	0	1	4
Total	95		80		18		14		7		4	

Table 8: Embryogenesis for Root induction media of Potohar.

Replication	200 mg/ml IBA &300 mg/ ml NAA		300 mg/ml IBA& 200 mg/ml NAA		300 mg/ml IBA & 100 mg/ml NAA		100 mg/ml IBA & 500 mg/ ml NAA		300 mg/l IBA & NAA 300 mg/ml		200 mg/l IBA & NAA 100 mg/ml	
	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq	No. of plants	Freq
1	21	84	14	56	3	12	2	8	2	8	2	8
2	22	88	14	56	4	16	1	4	0	0	0	0
3	22	88	17	68	4	16	0	0	1	4	0	0
4	20	80	19	76	5	20	3	12	0	0	0	0
Total	85		64		16		6		3		2	

DISCUSSION

Agriculture plays a crucial role in ensuring global food security, economic stability, and sustainable development (Khattak et al., 2024; Shaheen et al., 2023; Tahir et al., 2023; Khattak[b] et al., 2022). Among various crop types, oilseed crops are particularly significant due to their contribution to edible oil production, protein-rich byproducts, and industrial applications

(Arif et al., 2024; Alam et al., 2023). These crops, including soybean, sunflower, canola, and groundnut, provide essential nutrients and enhance soil fertility through crop rotation. Groundnut (*Arachis hypogaea L*.), a major oilseed crop, is widely cultivated for its high oil content, nutritional value, and adaptability to diverse agro-climatic conditions. Recent studies have emphasized the importance of improving groundnut genotypes through biotechnological approaches to enhance yield, stress tolerance, and nutritional quality (Kumar et al., 2023; Patel et al., 2023).

In this study, two peanut genotypes, BARD-479 and POTOHAR, were used to develop a protocol for high regeneration frequency from directly developed somatic embryoids and callus. Somatic embryoid induction has been successfully carried out in more than one hundred species (Aslam et al., 2017; Tautorus et al., 1991). Recent advancements in somatic embryogenesis have highlighted the role of plant growth regulators (PGRs) in optimizing callus and embryo induction (Zhang et al., 2023; Singh et al., 2022). Most of the 2,4-D explants were turned into the callus phase and appeared brown in indirect somatic embryogenesis compared to picloram. Previous studies also observed low percentage induction in 2,4-D and high picloram efficacy in embryoids (Ahmed et al., 2014; Bhanumathi et al., 2005; Eapen & George, 1990). The ANOVA (analysis of variance), as mentioned in Table 1-8, showed that callus and embryo induction was highly significant. For Bard-479, the highest rate of callus and embryo induction was 73% and 86% with 2,4-D (300 mg/l), BAP (400 mg/l), and the addition of NAA at 100 mg/l. Potohar genotype gave a maximum callus and embryo induction rate of 65% and 75%, respectively, with 2,4-D (300 mg/l), BAP (400 mg/l), along with NAA (200 mg/l) and 400 mg/l BAP, 100 mg/l NAA. A combination of 2,4-D, BAP, and NAA proved to be the best in callus induction compared to NAA and BAP used individually. Similarly, increasing or decreasing the concentration of PGRs significantly affected the results. The optimum concentrations of the hormones NAA, BAP, and 2,4-D induced the highest callus induction rate of 75% for Bard-479 and 65% for Potohar, with embryo induction rates of 87% and 75%, respectively.

Recent studies have also demonstrated the importance of optimizing PGR concentrations for efficient somatic embryogenesis and plant regeneration in various crops. For instance, Zhang et al. (2023) reported that a balanced combination of auxins and cytokinins is critical for maximizing callus induction and embryogenesis in legumes. Similarly, Singh et al. (2022) emphasized the role of NAA and BAP in enhancing shoot and root regeneration in groundnut genotypes under in vitro conditions. These findings align with our results, where the combination of 2,4-D, BAP, and NAA yielded the best outcomes for callus and embryo induction.

Similarly, some media could produce root-like structures with less than 10% frequency. For Bard-479, 300 mg/l IBA and 200 mg/l NAA favored the best rate in root formation (95%), while Potohar root formation was recorded at 85%. Similarly, Bard-479 results for shoot induction were also good (86%) compared to Potohar (75%). Both genotypes showed promising results, but Bard-479 outperformed Potohar in terms of callus, embryo, and shoot induction. Callus and embryogenesis malfunction was observed at higher concentrations of NAA, which is consistent with previous findings that excessive auxin levels can inhibit embryogenesis and lead to callus browning (Kumar et al., 2023; Patel et al., 2023). Roots were induced within 15-20 days, and the developed plants were shifted to polythene bags for hardening.

The successful regeneration of groundnut genotypes through somatic embryogenesis holds significant potential for crop improvement programs. Recent studies have highlighted the application of somatic embryogenesis in developing stress-tolerant and high-yielding groundnut varieties (Kumar et al., 2023; Patel et al., 2023). Furthermore, the integration of advanced biotechnological tools, such as CRISPR-Cas9 genome editing, with somatic embryogenesis can accelerate the development of improved groundnut genotypes with enhanced nutritional and agronomic traits (Zhang et al., 2023; Singh et al., 2022).

CONCLUSION AND RECOMMENDATIONS

The protocol developed here has provided efficient approaches for the micro-propagation of peanuts through mature embryogenesis and callus. The results obtained with cotyledonary nodes confirmed that embryos are suitable explants for somatic embryogenesis induction and callus induction, and this methodology could be extended to other species. But for regeneration into shoots and roots, somatic embryogenesis protocols are the best way to get the desired plant quickly.

It is recommended to go for direct embryogenesis to save time and reduce the chances of contamination. Moreover, if hardening of plant or root or shoot induction is not required, one can go with callusing as it has more regeneration rate. Still, if hardening of the plant is needed, somatic embryogenesis is the ultimate method. Moreover, root and shoot frequency in embryogenesis is more than other methods. The protocol's effectiveness also depends on the type of explant and species as in the present study, and variety Bard 479 performed better. So our results can vary slightly if other varieties or accessions are used.

AUTHOR CONTRIBUTIONS

Conceptualization, Methodology, Validation and writing, S. Begum; S.H. Khattak, Data curation & Funding acquisition; Analysis, S. Begum; M.A. Aslam; G. Zahid; M. Murtaza, Methodology, Investigation & reviewing and

editing; Software & Validation, S.H. Khattak; F. Jabeen; F. Amin; S. Komal, Resources, Supervision, Project administration, Funding acquisition and Resources, S. Begum; S.H. Khattak; G.M. Ali; Methodology, Reviewing and editing, S.H. Khattak; M.A. Aslam; S. Begum.

COMPETING OF INTEREST

All the authors declare no conflict/competing interest and confirm that no industry or private sector was involved in the study.

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