Research Article

A Study on Callus Induction and the Successful Regeneration of Plantlets from Rhizome Explants of *Alpinia galanga* (L.) Willd., a Valuable Medicinal Plant

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ABSTRACT

Aim: The primary focus of our study is to establish a reliable approach for plant regeneration via indirect organogenesis of *Alpinia galanga*, utilizing rhizome buds. **Materials and Methods:** The rhizome explants were placed onto MS media supplemented with 0.5 to 2.5 mg/L 2,4-D to induce callus formation. The more effective callus induction was observed on MS media containing 1.5 mg/L 2,4-D with a light green, compact nature of callus. The callus transferred to MS media supplemented with various cytokinin's such as BAP, Kinetin and TDZ in different concentrations for shoot induction. **Results:** The highest shoot induction frequency (91.0±3.21%), the maximum number of shoots per callus (9.66±0.88) and the highest plantlet length (91.13±1.44 mm) were observed in 1.0 mg/L BAP. The best-regenerated shoot buds were then transferred to auxins such as NAA and IBA with various concentrations (0.25 mg/L, 0.5 mg/L, 0.75 mg/L and 1.0 mg/L) for root initiation. High rooting frequency (93.00±1.73%), maximum number of roots (21.66±1.76) and maximum root length (93.33±1.76 mm) were induced in MS medium containing 0.5 mg/L IBA. **Conclusion:** We have developed a reliable and easily replicable protocol for callus induction and subsequent plant regeneration of *A. galanga* in controlled laboratory settings. Following this, the *in vitro* plantlets were successfully acclimatized to field conditions.

Keywords: *Alpinia galanga,* Callus, Rhizome, Auxin.

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INTRODUCTION

Alpinia galanga (L.) Willd. is an important valuable medicinal plant belonging to the family Zingiberaceae. The major supplier of *A. galanga* is India along with Indonesia and Thailand.[1] It shows abundant growth along the eastern Himalayas and it is cultivated in the Western Ghats. Because of its significant medicinal value, its volatile oil is drawing greater international attention.

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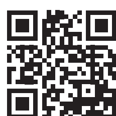

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Flowers and young shoots are used as a vegetable or as a spice.[2] The rhizomes have a strong aromatic smell with conspicuously nodes and internodes.[3] Rhizomes of *A. galanga* are richest in carbohydrates and lowest in fat.[4] *A. galanga* can be used as a good source of anti-bacterial, antioxidant,[5] anti-cancer,[6] anti-diabetic,[7] anti-microbial and anti-fungal[8] properties. It is used to cure diseases such as fever, ulcers, throat infections, bad breath, whooping cough, rheumatism and bronchial catarrh.[9] Rhizomes serve as the primary vegetative propagules for commercial cultivation, but their regeneration is notably sluggish and they are extremely vulnerable to a range of environmental stressors, including temperature fluctuations during storage.[10] The conventional breeding method is not sufficient to

meet its commercial demand. So large-scale propagation methods are needed to meet the market demand for *A. galanga*. In this aspect, callus induction techniques could be used to produce plantlets.

MATERIALS AND METHODS

Plant materials

The plants were procured from the garden situated at Unani Medical College Campus in Kozhikode, Kerala. Following this, they were carefully tended and raised in the garden maintained by the Department of Botany, Jamal Mohamed College (Autonomous), Tiruchirappalli, Tamil Nadu. Healthy rhizome buds were used as the explants and cut into pieces (1.5-2.0 cm). They were thoroughly cleaned with tap water for 20 min and dipped in detergent (teepol) for 5 min. All explants were surface sterilized by using 70% (v/v) ethanol for 1 min, followed by washes using sterile distilled water. Lastly, the explants were treated with a 0.1% (w/v) aqueous solution of mercuric chloride for 2 min, after which they underwent repeated washing with sterile distilled water

Culture medium and condition

The explants were transferred to MS Medium[11] with 0.8% (w/v) agar and 3% (w/v) sucrose. Prior to autoclaving, the medium's pH was adjusted to 5.8. Autoclaving was conducted at 121ºC and 104 kPa pressure for 20 min. Subsequently, the culture was incubated under a photoperiod of 16 hr of light and 8 hr of darkness, at a temperature of 22±2ºC, with a light intensity of 40 µmol m s.

Callus induction

Rhizome explants (3 mm thick circular discs) were utilized to attempt callus initiation on MS media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) ranging from 0.5 to 2.5 mg/L. The cultures were exposed to light conditions for 16 weeks. The callus from the explants frequently sub-cultured within two to three weeks.

Indirect organogenesis

The undifferentiated mass of cells thus formed was transferred to MS media supplemented with various cytokinins concentrations (BAP, Kinetin and TDZ with 0.5, 1.0, 1.5 mg/L) for shoot induction and later to auxins such as NAA and IBA containing different concentrations (0.25 mg/L,0.5 mg/L, 0.75 mg/L and 1.0 mg/L) for root initiation.

Acclimatization

The *in vitro* regenerated plantlets were taken from the culture medium and agar was removed from the plantlets by washing them under running tap water and individually transferred to 200 mL paper cups filled with a mixture of vermiculite and sand (3:1) under controlled conditions for primary hardening. The potted plants were irrigated every day continuously for 4 weeks. The percentage of survival rate was calculated after one month. Subsequently, they were relocated to earthen pots filled with a mixture of cattle manure, soil, and sand (in a ratio of 1:2:1) to undergo the secondary hardening phase.

Statistical analysis

All experiments were carried out 3 times with 6 explants. Data were subjected to the homogenous subsets and descriptive analysis (Mean, SD). All data on various parameters were evaluated by the Ducan *post hoc* hypothesis and ANOVA. The results were compared using SPSS (Version 20, IBM, NY).

RESULTS

Effect of 2,4-D on callus induction

The effect of different concentrations of 2,4-D on callus induction of *A. galanga* was studied. Various concentrations such as 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L of 2,4-D were utilized for the *A. galanga* callus formation and were statistically analyzed and tabulated as mean±SD (Table 1). No callus formation was observed in 0.5mg/L 2,4-D concentration whereas increased concentrations of 2,4-D significantly increased the percentage of callus induction with different morphological features. Among the various 2,4-D that were analyzed, 86.66±1.20% of callus induction was observed in 1.5 mg/L culture with light green and compact callus.

Table 1: Effect of 2,4-D on the callus formation of *A. galanga*.

2,4-D (mg/L)	Percentage of callus induction	Nature of callus
0.5	0.00[d]	No callus formed
1.0	51.66±2.18[b]	Light brown, fragile
1.5	86.66±1.20[a]	Light green, compact
2	48.33±3.48[b]	Light yellow, fragile
2.5	24.66±6.11[c]	Dark brown, compact

Table 2: Effect of cytokinins on shoot regeneration from *in vitro* grown callus of *A. galanga*.

Plant growth regulators (mg/L)			Shoot induction (%)	Mean number of shoots/calluses	Mean plantlet length(mm)
TDZ	BAP	Kinetin			
0.5			32.0±2.08[def]	4.0±1.15[def]	62.0±1.89[d]
1.0			39.66±1.45[d]	6.33±0.88[bcd]	71.36±2.49[c]
1.5			33.66±3.52[de]	4.66±0.33[cde]	69.26±2.5[c]
	0.5		54.33±2.33[c]	7.33±0.88[ab]	79.16±2.54[b]
	1.0		91.0±3.21[a]	9.66±0.88[a]	91.13±1.44[a]
	1.5		71.66±2.18[b]	7.0±0.57[bc]	89.26±0.66[a]
		0.5	23.33±4.25[fg]	3.66±0.88[ef]	45.1±2.91[f]
		1.0	25.0±3.21[efg]	3.0±0.57[ef]	55.0±2.22[e]
		1.5	18.33±2.18[g]	1.66±0.66[f]	43.0±1.21[f]

Effect of cytokinins on shoot organogenesis

Following a six-week culture period, shoot induction was noted to emerge from the callus. The callus was subsequently transferred to MS media, where separate supplementation with varying concentrations of TDZ, BAP, and Kinetin was performed. The process of shoot induction, along with the number of shoots per callus and the length of the resulting plantlets, exhibited variability across different cytokinin concentrations. The highest number of shoot induction was observed in 1.0 mg/L BAP (91.00±3.21%). The maximum number of shoots per callus was developed in 1.0 mg/L BAP (9.66±0.88). The plantlet length about 91.13±1.44 mm was maximum in 1.0 mg/L BAP. Among various concentrations (0.5 mg/L, 1.0 mg/L, 1.5 mg/L) of TDZ and kinetin, the highest shoot induction and maximum number of shoots per callus was shown on MS medium with 1.0 mg/L TDZ or kinetin (Table 2).

Effect of NAA and IBA on root induction

The shoots with 5 cm height were excised and transferred to MS medium containing various concentrations of auxins like IBA and NAA (0.25, 0.5, 0.75 and 1.0 mg/L) for rooting. After 3 weeks, root initiation, number of roots and root length were recorded. High rooting frequency (93.00±1.73%) with maximum number of roots (21.66±1.76) was induced in MS medium supplemented with 0.5 mg/L IBA. The maximum root length (93.33±1.76 mm) was observed in MS medium containing 0.5 mg/L IBA (Table 3).

Acclimatization

The micro propagated plantlets are treated to acclimatization process to increase growth and reduce mortality. Regenerated plantlets were transferred to paper cups containing a mixture of vermiculite and sand (3:1) (Figure 1 H and I). They were watered twice every

Table 3: Effect of auxins (IBA and NAA) on root induction from *in vitro* regenerated shoots of *A. galanga*.

Plant growth regulator (mg/L)	Frequency of root initiation (%)	Mean number of roots	Mean root length (mm)
IBA			
0.25	77.66±1.45[c]	13.66±1.45[cd]	61.33±1.2[d]
0.5	93.00±1.73[a]	21.66±1.76[a]	93.33±1.76[a]
0.75	86.33±0.88[b]	15.66±0.66[bcd]	64.66±1.45[d]
1.0	71.33±1.45[d]	17.33±1.20[bc]	77.33±0.33[b]
NAA			
0.25	76.66±1.45[c]	15.0±1.15[bcd]	61.33±1.85[d]
0.5	87.33±0.33[b]	18.0±2.08[ab]	72.33±1.85[c]
0.75	73.66±1.20[cd]	15.33±0.33[bcd]	52.66±1.25[e]
1.0	61.33±3.17[e]	12.33±0.66[d]	47.66±0.33[g]

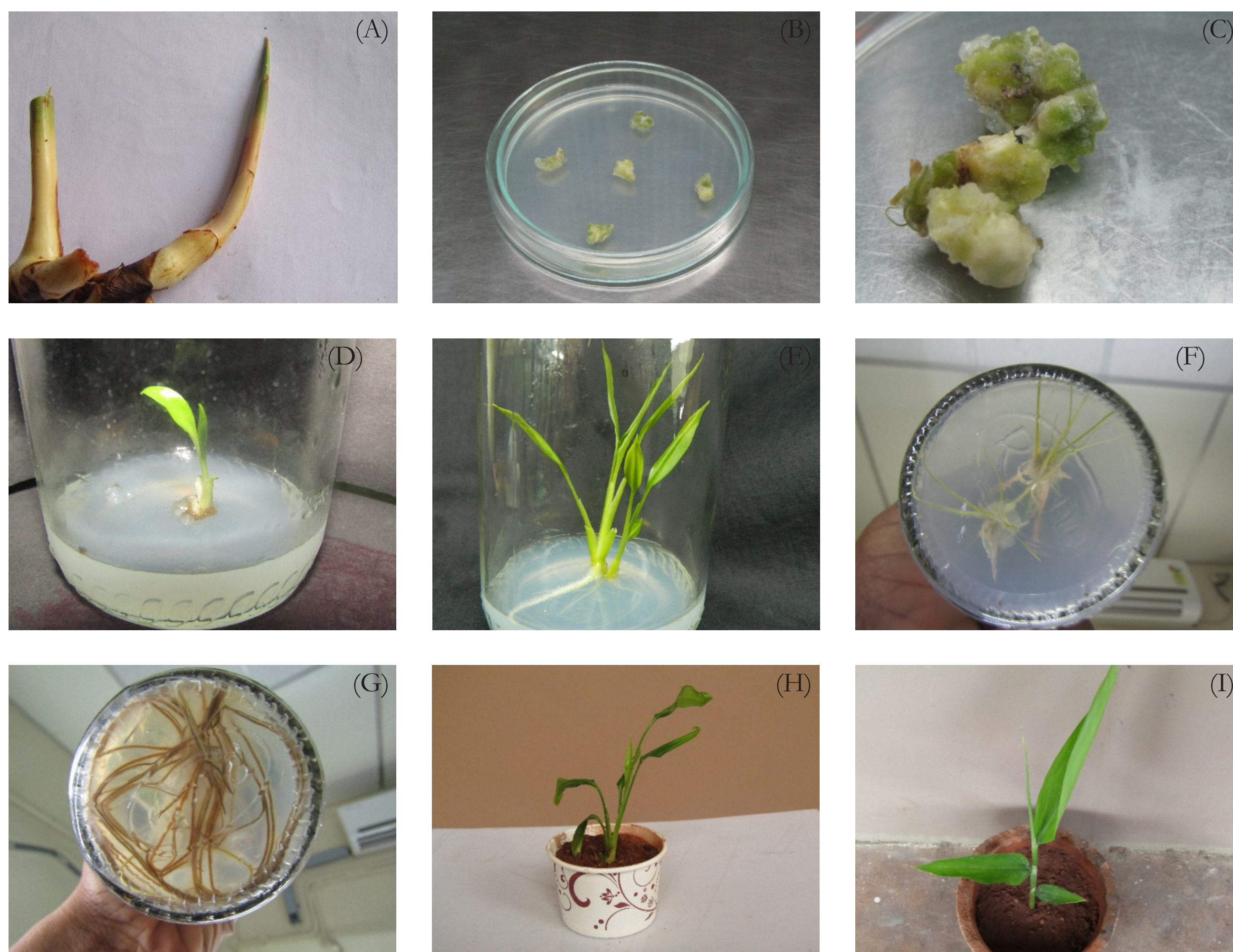

Figure 1: Callus induction and plantlet regeneration in *A. galanga* (A) Rhizome bud explant (B&C) White to light green callus on callus induction medium (D&E) Indirect organogenesis on shoot induction medium (F) Root development in rooting medium (G) Mature root after three month of culture (H) Primary hardened plant on paper cup (I) Secondary hardened plant on earthen pot.

day. 84% survival was observed after one month of primary hardening. Following the secondary hardening phase, the plantlets were transplanted into earthen pots. Under shaded conditions, a survival rate of 72% was observed and recorded.

DISCUSSION

Our results were in accordance with the effect of 2,4-D was also recorded in indirect organogenesis of *Zingiber officinale*.[12] The best result of callus production from young and primordial leaves of ginger was recorded in MS medium with 3 mg/L 2,4-D.[13] The highest frequency of callus induction was shown at 1 mg/L 2,4-D containing MS medium in *Zea mays*.[14] Maximum induction of callus obtained from the leaves of *Achyranthes aspera* was from the combination of 2.0 mg/L 2,4-D and 0.5 mg/L NAA.[15] Callus yield and regeneration frequencies of wheat (*Triticum aestivum*) were higher with 1.0 mg/L 2,4-D.[16]

The most significant callus induction percentage documented in *Barringtonia racemosa* during endosperm culture was observed, with a complete callusing rate of 100% achieved in MS medium supplemented with 1.5 mg/L of 2,4-D and 0.5 mg/L of kinetin.[17] *Curcuma selenosis* exhibited a callus formation rate of 91.1% when rhizome buds were cultured on MS medium supplemented with 0.55 mg/L TDZ, 0.45 mg/L BA, and 0.26 mg/L 2,4-D.[18] Significant improvement in friable callus production was observed in *Curcuma caesia* when cultivated on MS medium containing 5 mg/L BAP and 2 mg/L 2,4-D. [19] The embryogenic callus of *Kaempferia rotunda* were induced on MS medium containing 2.5 mg/L 2,4-D and 0.5 mg/L BAP.[20] The best result for callus initiation in *Alpinia purpurata* was in MS media supplemented with 2,4-D (2 ppm) and kinetin (2ppm).[21] Suitable MS medium for callus induction in *A. zerumbet* was 1.5 mg/L BA and 0.3 mg/L 2,4-D, and for callus differentiation was 2 mg/L TDZ and 0.1 mg/L 2,4-D.[22]

Stfaan *et al.*[23] reported that BAP is the most useful cytokinin for obtaining the shoot multiplication. High-frequency multiple shoot induction from rhizome explants of *Kaempferia galangal* was noted on MS medium with 0.5 mg/L of IAA and 2.5 mg/L of BAP.[24] The shoot induction frequency (49.8%) of *Boesenbergia rotunda* was maximum on MS medium containing 2 mg/L BAP.[25] The maximum number of shoots (6.2) from rhizomatous bud explants of *A. calcarata* were produced on MS medium containing 1.125 mg/L BAP, 2.15 mg/L kinetin, and 0.465 mg/L NAA.[26] Different concentrations of IAA and BAP were used for culturing shoot bud explants of *Etlingera elatior*. The best mean numbers of shoots (3.67) were obtained in MS medium with 5 mg/L BAP. The highest shoot length (4.2 cm) was produced from MS medium containing 6 mg/L of BAP.[27]

Rhizomatous bud explants of *Z. cassumunar* were inoculated on MS medium with various concentrations of NAA, BAP and Kinetin. 1 mg/L BAP observed 100% response giving both single shoots (30%) and multiple shoots (70%).[28]

The *in vitro* propagated shoots of *C. zedoaria* were cultivated within an MS medium containing varying levels of NAA and BAP. The most notable occurrence of rhizomes, both in terms of frequency and quantity, was documented at 4.0 mg/L BAP coupled with 6% sucrose.[29] Concerning *Z. zerumbet,* the highest average count of shoots (5.6) per explant was observed when utilizing an MS medium supplemented with a combination of 2.0 mg/L IAA and 5.0 mg/L BAP. The optimal shoot length (9.44 cm) was achieved using a medium enriched with 2.0 mg/L IAA and 1.0 mg/L BAP.[30] BAP is also very efficient to high frequent shoot induction from callus in different species of Zingiberaceae like *C. longa*,[31] *C. amada*[32] and *K. angustifolia*.[33]

Isolated shoots of *Z. officinale,* the highest root development reported on MS media containing 1.0 mg/L IBA or IAA and 2% (w/v) sucrose.[34] The best rooting in shoot cluster of *Etlingera coccinea* was obtained on MS medium fortified with 0.203 mg/L IBA (3.5±0.08 cm root length with 90±2.24% response).[35] The maximum adventitious root production from friable callus of *C. amada* was noted in MS medium supplemented with 3% of sucrose along 0.3 mg/L IBA.[36] In *C. longa*, best rooting percentage (83.3%) was showed in MS medium with moderate quantity of IBA.[37] In *C. caesia*, the high frequency of rooting (89.76%) was observed in half strength MS medium containing 0.609 mg/L IBA.[38] Healthy rooting observed in the shoot buds of *A. calcarata* was on MS medium containing 0.5 mg/L IBA.[39] In *C. amada,* 9 mg/L BAP with MS medium was the best for shoot multiplication while 1 mg/L NAA with MS medium gave the highest root number.[40]

CONCLUSION

Our study was dedicated to establishing a robust methodology for the indirect organogenesis and plant regeneration of *A. galanga* via rhizome buds. The initial phase involved rhizome explants placed on MS media, with callus formation induced using a range of 2,4-D concentrations. Notably, the most effective callus induction was achieved with 1.5 mg/L 2,4-D, resulting in light green and compact callus. Subsequent steps included the transfer of callus to MS media supplemented with various cytokinins (BAP, Kinetin, TDZ) to stimulate shoot formation. Remarkable outcomes were observed, with the highest shoot induction frequency (91.0±3.21%), a maximum of 9.66±0.88 shoots per callus, and a peak plantlet length of 91.13±1.44 mm recorded at 1.0 mg/L BAP concentration.

The protocol also successfully guided the transition of regenerated shoot buds to auxin treatments, where NAA and IBA at different concentrations (0.25 mg/L, 0.5 mg/L, 0.75 mg/L, and 1.0 mg/L) facilitated root initiation. Remarkable rooting results were attained, showcasing a high rooting frequency (93.00±1.73%), a maximum of 21.66±1.76 roots per plantlet, and an impressive maximal root length of 93.33±1.76 mm, particularly noteworthy in MS medium containing 0.5 mg/L IBA.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

2,4-D: 2,4-Dichlorophenoxyacetic acid; **NAA:** Naphthaleneacetic acid; **IBA:** Indole-3-butyric acid; **BAP:** 6- Benzylaminopurine; **Kn:** Kinetin; **TDZ:** Thidiazuron; **MS:** Murashige and Skoog medium.

SUMMARY

This study culminates in the successful development of a reliable and reproducible protocol for callus induction and subsequent plantlet regeneration of *A. galanga*

under controlled laboratory conditions. Importantly, the *in vitro* plantlets were effectively acclimatized to field conditions, underscoring the practical potential of our approach. This research not only contributes to the advancement of plant tissue culture techniques but also holds promise for the conservation and propagation of *A. galanga*, an important medicinal plant.

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