

Efficient *in vitro* propagation and comparative phytochemical profiling of *Kaempferia galanga* L. - An important medicinal plant of Bangladesh

Joyantee Biswas, Sweety Majumder, Tapash Kumar Bhowmik*, Md Mahbubur Rahman

Plant Tissue Culture and Biotechnology Laboratory, Department of Botany, Faculty of Biological Sciences, University of Chittagong, Chattogram, Bangladesh

Corresponding Author: Tapash Kumar Bhowmik

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Abstract

Kaempferia galanga L. (Zingiberaceae) is a commercially and therapeutically vital endangered medicinal plant whose natural populations are rapidly depleting due to over harvesting and sluggish conventional propagation methods. This study aimed to establish a highly efficient, reproducible *in vitro* propagation protocol using rhizome bud explants, alongside a comparative qualitative phytochemical profiling of *in vitro* regenerated versus naturally grown plants. Explants were cultured on Murashige and Skoog (MS) basal medium supplemented with varying concentrations and combinations of 6-benzyl aminopurine (BAP), Kinetin (Kn), α -Naphthalene acetic acid (NAA), Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA). The quantitative evaluation of plant growth regulator combinations revealed that the maximum multiple shoot buds (MSBs) induction frequency (96%) and the highest number of shoots per explant (4.53 ± 0.25) were recorded on MS medium supplemented with 1.5 mg/l BAP and 1.0 mg/l NAA within 15-23 days of culture. For shoot elongation, MS medium fortified with 2.0 mg/l BAP and 0.5 mg/l Kn exhibited the optimum response, expanding initial 2.16 ± 0.07 cm buds to a maximum length of 4.54 ± 0.04 cm. Superior rhizogenesis (90% rooting frequency; 4.25 ± 0.10 roots/shoot; 3.65 ± 0.07 cm root length) was achieved on full strength MS medium supplemented synergistically with 0.5 mg/l IBA within 14-17 days. Hardened seedlings demonstrated an 80% survival rate upon successful acclimatization to *ex vitro* conditions. Comparative qualitative phytochemical screening for secondary metabolites revealed that *in vitro* developed plantlets possessed elevated levels of specific alkaloids (responding intensely to Hager's, Mayer's and Tannic acid reagents) and coumarin (++++) compared to naturally grown plants (+), whereas saponins were absent in the tissue cultured clones. These findings substantiate that the optimized micropropagation protocol not only facilitates rapid mass scale conservation but also enhances the elicitation of highly valued pharmaceutical bioactive compounds, offering a sustainable alternative for commercial therapeutic exploitation.

Keywords: Acclimatization, *In vitro* propagation, *Kaempferia galanga*, phytochemical profiling

Introduction

Kaempferia galanga L., commonly known as aromatic ginger, is a high value, rhizomatous perennial herb belonging to the family Zingiberaceae (Wang *et al.* 2021) [1]. Native to India and widely cultivated across Southeast Asia, including Bangladesh, the plant possesses immense ethno-pharmacological and industrial significance. The rhizomes are deep reservoirs of potent bioactive secondary metabolites, fundamentally recognized for their antimicrobial, anti-inflammatory, antioxidant and substantial antineoplastic properties against various carcinoma lines (Kumar 2020, Ali *et al.* 2018) [2, 3]. The therapeutic efficacy of *K. galanga* is primarily attributed to its diverse phytochemical profile, specifically volatile oils containing ethyl p-methoxy cinnamate and ethyl cinnamate, which renders it an indispensable ingredient in traditional Ayurvedic formulations (such as *Dasamularishta*) and modern pharmaceutical applications (Muzzazinah *et al.* 2024, Sahoo *et al.* 2014) [4, 5].

Despite its tremendous pharmacological value, the natural germplasm of *K. galanga* in Bangladesh and globally faces severe threats. Conventional propagation strictly depends on the vegetative division of underground rhizomes, as natural

seed set is exceedingly rare (Kala *et al.* 2006, Rout *et al.* 2000) [6, 7]. This traditional method is constrained by seasonal dependency, low multiplication rates and a high susceptibility to soil borne pathogens causing rhizome rot and wilt diseases (Fowler *et al.* 1993) [8]. The continuous, unmanaged wild harvesting of these slow growing rhizomes to satisfy the exponential global demand for herbal supplements and cosmetics has propelled *K. galanga* to the brink of endangerment, necessitating immediate conservation strategies. Consequently, establishing a robust, pathogen free and rapid micropropagation system is an urgent imperative for the sustainable commercial cultivation and *ex situ* conservation of this species (Das and Handique 2023, Rahman *et al.* 2004) [9, 10].

Plant tissue culture provides a biotechnological platform capable of overcoming conventional reproductive barriers, offering mass clonal multiplication independent of seasonal constraints. Specifically, manipulating *in vitro* environments using varied concentrations and combinations of cytokinins and auxins can precisely direct the morphogenetic pathways of specific explants, such as rhizome buds, toward accelerated direct organogenesis without an intervening somaclonal-variant callus phase (Vidya *et al.* 2021, Preetha *et al.* 2016) [11, 12]. Furthermore, *in vitro* cellular

environments inherently exert controlled physical and chemical stresses, which frequently act as potent elicitors modifying the biosynthetic pathways of plant secondary metabolites (Oksman-Caldentey and Inze 2004, Wink *et al.* 2005) ^[13, 14]. Profiling these phytochemical constituents is crucial for pharmaceutical validation, ensuring that tissue-cultured clones retain or exceed the therapeutic efficacy of their wild counterparts (Parida *et al.* 2022) ^[15].

Identifying the comparative qualitative phytochemical disparity between natural and *in vitro* propagated tissues addresses a critical gap in confirming the viability of propagated *K. galanga* as an industrial raw material. Therefore, the core objectives of this study were to (a) optimize a highly reproducible and rapid *in vitro* multiple shoot regeneration and rooting protocol from rhizome bud explants of *K. galanga* using specific Plant Growth Regulators (PGRs) combinations and (b) critically compare the qualitative phytochemical profiles of the *in vitro* raised plantlets against naturally grown plants to validate their potential as an enhanced source of medicinal secondary metabolites.

Materials and Methods

1. Plant material and explant preparation

Healthy, field grown *Kaempferia galanga* L. plants were source to procure mature rhizome buds, which served as the primary explants. The rhizomes were washed meticulously under running tap water to remove soil debris. The excised buds were subjected to surface sterilization using a 1% Savlon and liquid soap solution for 5-10 minutes under constant agitation. Following 3-4 rinses with distilled water, the explants were transferred to a laminar airflow cabinet. Subsequent sterilization involved a quick rinse with 70% ethanol for less than 60 seconds, followed by submersion in 0.1% mercuric chloride (HgCl₂) for designated durations to eliminate deep-seated pathogens. Finally, the explants were washed 4-5 times with sterile distilled water to eliminate any residual sterilant before being trimmed to 0.5-1.0 cm pieces using a sterile surgical blade.

2. Media preparation and culture conditions

Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) ^[16] was utilized for all *in vitro* experimental phases. The medium was supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) bacteriological grade agar. Stock solutions of PGRs were prepared and supplemented to the basal medium at varying concentrations. The pH of media formulations was strictly adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl prior to autoclaving at 121°C and 1.15 kg/cm² pressure for 20 minutes. All inoculated culture vessels were incubated in a highly controlled culture room maintained at 25 ± 2°C with a 14-hour light and 10-hour dark photoperiod under cool white fluorescent tubes providing a 2000-3000 lux illumination intensity.

3. Multiple shoot regeneration and elongation

To induce multiple shoot buds (MSBs), the surface sterilized rhizome buds were cultured on full strength MS medium supplemented with singular and combined treatments of cytokinins, specifically BAP (0.5-3.0 mg/l) and Kn (0.5-3.0 mg/l) and the auxin NAA (0.5-1.0 mg/l). Developing microshoots were aseptically excised and

transferred to designated elongation media, which consisted of MS basal medium fortified with various synergistic combinations of BAP (1.0-2.0 mg/l), Kn (0.5-1.0 mg/l) and NAA (0.5-1.0 mg/l).

4. Root induction and acclimatization

For rhizogenesis, elongated individual micro shoots measuring 3.5-4.0 cm were carefully excised and inoculated onto rooting media. These media comprised half- and full-strength MS basal formulations, augmented with auxins including IBA (0.5-2.0 mg/l), IAA (0.5-1.0 mg/l) and NAA (0.5-1.0 mg/l). Following profuse root development, the culture vessels were exposed to ambient room temperatures for up to 5 days to initiate hardening. Plantlets were subsequently extracted, their roots washed gently to remove the agar matrix and transplanted into small plastic pots containing a 1:1 mixture of sterile garden soil and compost. After 3-5 days of primary room temperature acclimatization, the established plantlets were transferred to an *ex vitro* natural environment.

5. Phytochemical screening protocol

For secondary metabolite extraction, 25g of dried, pulverized plant material (from both *in vitro* regenerated and naturally grown *K. galanga*) was mixed with 50 ml of methanol, shaken for 30 minutes, stored overnight and sonicated for 10 minutes. The extract was filtered using Whatman filter paper; this procedure was triplicated. The methanolic extracts were concentrated using a rotary evaporator below 51°C. Qualitative screening for alkaloids utilized five standard reagents: Dragendorff's (D), Hager's (H), Mayer's (M), Wagner's (W) and Tannic acid (T) (Harborne 1973, Sofowara 1993) ^[17, 18]. The relative presence of secondary metabolites (steroids, flavonoids, saponins, terpenoids, glycosides, tannins, phlobatannins, quinines, anthraquinones, coumarins and phenols) was recorded colorimetrically and designated incrementally from '+' (minimum) to '++++' (highest quantity), while '-' denoted complete absence.

6. Statistical analysis

Experiments were executed systematically with a minimum of five replicates. Data regarding percentage responses, days required and morphometric measurements were collected accurately and expressed as Mean ± Standard Error (SE).

Results

Rhizome buds explant cultured on MS medium responded directly by producing MSBs across various PGRs combinations and concentrations without an intervening callus phase. The quantitative evaluation (Table 1) demonstrated a broad variance in explant proliferation frequency, ranging from a minimum of 62% to a maximum of 96% depending on the precise PGRs combinations. While BAP alone at 2.0 mg/l induced a favorable 85% response with 3.45 ± 0.32 shoots (Fig. 1), the synergistic application of a cytokinin (BAP) and an auxin (NAA) proved markedly superior to singular treatments or dual-cytokinin combinations. The absolute highest frequency of MSB induction (96%) was achieved on MS medium supplemented with 1.5 mg/l BAP and 1.0 mg/l NAA (Fig. 2). This optimal formulation not only generated the highest average number of MSBs per explant (4.53 ± 0.25) but also

did so in an accelerated timeframe of 15-23 days. Conversely, media fortified with dual cytokinins lacking auxin supplementation exhibited depressed morphogenetic responses. For instance, the minimum proliferation response (62%) combined with the lowest average number of shoots (1.32 ± 0.32) occurred in the medium fortified with 1.0 mg/l BAP and 1.0 mg/l Kn, which also delayed induction to 22-26 days.

Following isolation, the directly produced MSBs were culture on specific elongation media. As detailed in Table 2, the empirical data dictates that for shoot internode elongation, combinations of two cytokinins drastically outperformed cytokinin-auxin pairings over a 30-day period. Maximum elongation was distinctly recorded on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l Kn, where initial shoot buds measuring 2.16 ± 0.07 cm rapidly expanded to 4.54 ± 0.04 cm, demonstrating an absolute net elongation of 2.38 cm (Fig. 3-6). In sharp contrast, the minimum elongation capacity was noted on MS medium containing 1.0 mg/l BAP and 1.0 mg/l NAA, which stunted growth, allowing shoots to grow from an initial length of 1.12 ± 0.02 cm to only 2.63 ± 0.03 cm, representing a net increase of only 1.51 cm.

Rhizogenesis initiation occurred within 14-22 days of transferring the elongated microshoots to rooting media (Table 3). Rooting efficiency was fundamentally dependent on exogenous auxin supplementation, as half strength MS media with PGRs or supplemented exclusively with NAA (0.5 and 1.0 mg/l) entirely minimum to induce roots. The most prolific rhizogenesis occurred on full strength MS medium supplemented synergistically with 0.5 mg/l IBA (Fig. 7). This optimal treatment achieved a 90% rooting response within a rapid 14-17 days, yielding the highest average number of roots per micro shoot (4.25 ± 0.10) and an extended average root length of 3.65 ± 0.07 cm after 30 days (Fig. 8). Altering this ratio to 1.0 mg/l IAA severely

depressed the rooting parameters, dropping the successful response rate to 68% and producing only 2.03 ± 0.03 roots measuring a minimal 1.29 ± 0.16 cm. Following successful *in vitro* development, the hardened seedlings were transitioned to natural soil. An excellent 80% of the transferred *in vitro* seedlings successfully established normal, healthy growth *ex vitro* (Fig. 9).

The comparative qualitative screening unveiled distinct biochemical variations between the methanolic extracts of *in vitro* developed plantlets and naturally grown *K. galanga* (Table 4). Both plant sources expressed maximum baseline alkaloid concentrations when tested with Dragendorff's (D) and Wagner's (W) reagents, scoring identically (+++). Crucially, however, the *in vitro* plantlets displayed moderately higher alkaloid concentrations when reacted with Hager's (H) and Mayer's (M) reagents (++ vs. + in natural plants), as well as Tannic acid (T) (+++ vs. ++ in natural plants). This explicitly signifies an enhancement of specific alkaloidal complexity under *in vitro* propagation.

Further assessment of ten secondary metabolites (Table 5) affirmed the retention of core bioactive compounds, indicating equivalently high concentrations (+++) of steroids, flavonoids, glycosides, quinines, tannins, phlobatannins and terpenoids across both *in vitro* and natural samples. A prominent and biologically valuable distinction emerged concerning coumarin: *in vitro* developed plants exhibited maximum concentrations (+++), whereas naturally grown plants showed only minimal trace presence (+). Conversely, saponin was completely absent (-) in the *in vitro* plants but detected at a minimum level (+) in the wild plants. Neither anthraquinone nor phenol was detected in either sample group.

Table 1: Effects of different concentrations and combinations of PGRs for induction and development of MSBs from rhizome budsexplant of *K. galanga*.

PGRs	Conc. (mg/l)	% of explants response	Time (d) require for induction of MSBs	Average no. of MSBs/explant ($\bar{x} \pm SE$)
BAP	0.5	65	22 - 26	1.37 ± 0.32
	1.0	75	15 - 27	2.22 ± 0.26
	2.0	85	15 - 18	3.45 ± 0.32
	3.0	72	15 - 20	2.18 ± 0.18
Kn	0.5	72	18 - 21	2.16 ± 0.15
	1.0	70	20 - 30	2.02 ± 0.02
	2.0	86	22 - 24	3.52 ± 0.17
	3.0	73	24 - 28	2.24 ± 0.33
BAP + Kn	1.0 + 0.5	75	16 - 20	2.43 ± 0.37
	1.0 + 1.0	62	22 - 26	1.32 ± 0.32
	1.5 + 0.5	76	17 - 23	2.27 ± 0.16
	1.5 + 1.0	65	18 - 30	1.39 ± 0.32
	2.0 + 0.5	87	21 - 24	4.05 ± 0.04
BAP + NAA	2.0 + 1.0	88	19 - 30	4.13 ± 0.08
	1.0 + 0.5	76	15 - 25	2.12 ± 0.16
	1.0 + 1.0	75	17 - 22	2.16 ± 0.10
	1.5 + 0.5	78	15 - 20	2.54 ± 0.14
	1.5 + 1.0	96	15 - 23	4.53 ± 0.25
	2.0 + 0.5	78	16 - 30	2.53 ± 0.15
	2.0 + 1.0	75	19 - 27	2.22 ± 0.19

d = days; Values are the means of 5 replicates.

Table 2: Effects of different concentrations and combinations of PGRs fortified on MS medium on elongation of directly produced multiple shoot buds of *K. galanga*.

PGRs	Conc. (mg/l)	Initial length (cm) of individual shoot bud ($\bar{x} \pm SE$)	Length (cm) of individual shoot bud after 30d of culture ($\bar{x} \pm SE$)
BAP + NAA	1.0 + 0.5	2.12 $\pm$ 0.02	3.14 $\pm$ 0.08
	1.0 + 1.0	1.12 $\pm$ 0.02	2.63 $\pm$ 0.03
	1.5 + 0.5	2.13 $\pm$ 0.05	3.16 $\pm$ 0.06
	1.5 + 1.0	2.31 $\pm$ 0.64	4.16 $\pm$ 0.05
	2.0 + 0.5	2.15 $\pm$ 0.04	3.16 $\pm$ 0.04
	2.0 + 1.0	2.13 $\pm$ 0.05	3.16 $\pm$ 0.05
BAP + Kn	1.0 + 0.5	2.62 $\pm$ 0.03	3.15 $\pm$ 0.05
	1.0 + 1.0	1.56 $\pm$ 0.15	3.23 $\pm$ 0.03
	1.5 + 0.5	1.25 $\pm$ 0.03	3.35 $\pm$ 0.03
	1.5 + 1.0	2.15 $\pm$ 0.04	3.24 $\pm$ 0.05
	2.0 + 0.5	2.16 $\pm$ 0.07	4.54 $\pm$ 0.04
	2.0 + 1.0	2.16 $\pm$ 0.07	4.46 $\pm$ 0.07

d = days; Values are the means of 5 replicates.

Table 3: Effects of different concentrations and combinations of PGRs on the development of roots in elongated MSBs of *K. galanga* on half and full-strength MS medium.

PGRs	Conc. (mg/l)	Days to root induction	% of micro shoot produced roots	Average* number of roots/micro shoot ($\bar{x} \pm SE$)	Average* length (cm) roots after 30d of culture ($\bar{x} \pm SE$)
½ MS0	----	18 - 20	70	2.01 $\pm$ 0.14	3.01 $\pm$ 0.13
½ MS + IBA	0.5	14 - 18	75	2.13 $\pm$ 0.03	3.17 $\pm$ 0.06
	1.0	14 - 19	74	2.10 $\pm$ 0.07	3.14 $\pm$ 0.04
	1.5	16 - 18	72	2.02 $\pm$ 0.52	3.06 $\pm$ 0.03
	2.0	16 - 22	76	2.14 $\pm$ 0.05	3.22 $\pm$ 0.02
½ MS + NAA	0.5	18 - 22	74	2.24 $\pm$ 0.27	3.06 $\pm$ 0.04
	1.0	16 - 18	72	2.01 $\pm$ 0.35	3.06 $\pm$ 0.03
MS + IBA	0.5	14 - 17	90	4.25 $\pm$ 0.10	3.65 $\pm$ 0.07
	1.0	16 - 20	78	2.53 $\pm$ 0.09	3.16 $\pm$ 0.04
MS + IAA	0.5	17 - 22	75	2.24 $\pm$ 0.27	3.06 $\pm$ 0.04
	1.0	20 - 22	68	2.03 $\pm$ 0.03	1.29 $\pm$ 0.16

d = days; --- = No response; Values are the mean of 5 replicates.

Table 4: Qualitative test for alkaloids of *K. galangal*

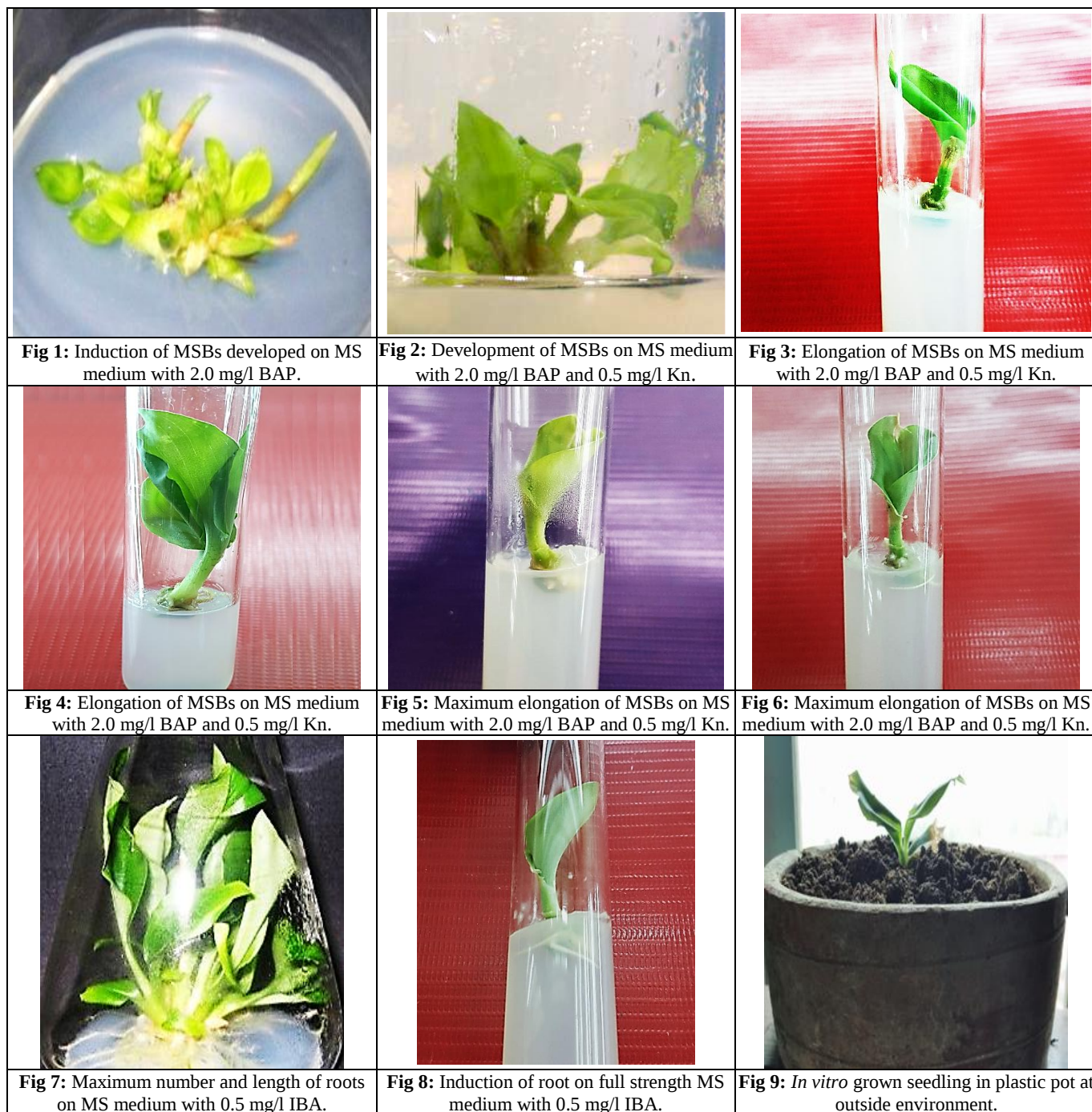
Plant sample used	Qualitative estimation of alkaloids by different reagents				
	D	H	M	W	T
<i>In vitro</i> developed plant	+++	++	++	+++	+++
Naturally grown plant	+++	+	+	+++	++

Notes: Name of the reagents, D-Dragendroff's reagent, H-Hager's reagent, M-Mayer's reagent, W-Wagner's reagent and T- Tannic acid reagent.

Table 5: Qualitative test for ten secondary metabolites of *K. galangal*

Plant sample Used	Secondary metabolites (% of coloration)										
	Phl.	Flv.	Ter.	Str.	Gly.	Qui.	Cou.	Anthro.	Sap.	Tann.	Phe.
<i>In vitro</i> developed plant	+++	+++	-	+++	+++	+++	+++	+++	-	+++	-
Naturally grown plant	+++	+++	-	+++	+++	+++	+++	+++	-	+	-

Notes: Phl= Phlobatannins, Flv = Flavonoids, Ter.= Terpenoids, Str.= Steroids, Gly = Glycosides, Qui = Quinine, Cou = Coumarin, Anthro =Anthroquinone, Sap =Saponins, Tann = Tannin, Phe = Phenol.



Discussion

The successful establishment of a highly reproducible *in vitro* regeneration protocol for *Kaempferia galanga* structurally validates previous findings detailing the superior regenerative totipotency of meristematic tissues in Zingiberaceae species under precisely controlled environments (Rakkimuthu *et al.* 2011, Joshi and Dhar 2003) ^[19, 20]. In the present quantitative study, the combination of 1.5 mg/l BAP and 1.0 mg/l NAA achieved a remarkable 96% direct shoot multiplication rate. Physiologically, BAP acts as an aggressive cytokinin to break axillary bud dormancy and suppress apical dominance, forcing localized mitotic division (Kannan and Anbazhakan 2016) ^[21]. The supplementation of NAA, a synthetic auxin, acts synergistically with BAP to maintain proper cellular polarity and vascular tissue differentiation in the emerging shoot primordia. This strong auxin-cytokinin synergy bypassed the need for an unstable callus phase, an

absolute requisite for maintaining true-to-type clonal fidelity in commercial pharmacological crops.

During the subsequent shoot elongation phase, an exclusive dual cytokinin interaction proved vastly superior to auxin-cytokinin combinations. Maximum shoot elongation (net growth of 2.38 cm) was driven by 2.0 mg/l BAP combined with 0.5 mg/l Kn. This synergistic interaction likely modulates intracellular gibberellic acid synthesis and enhances cellular turgor, precipitating rapid internodal cellular expansion (Rani *et al.* 2014) ^[22]. Following elongation, robust adventitious root formation is structurally critical for the survival of *in vitro* raised clones during *ex vitro* acclimatization (Panigrahi and Patel 2014) ^[23]. The application of auxin, IBA (0.5 mg/l) on full strength MS medium, catalyzed maximum rhizogenesis (90%). IBA fundamentally initiates adventitious root primordia at the basal wound by altering local endogenous auxin gradients, while the supplemental IAA acts to sustain the downstream

elongation of these primordial root cells (Shekhawat *et al.* 2015) ^[24].

The qualitative phytochemical screening unveiled compelling biochemical distinctions. The *in vitro* plantlets demonstrated a markedly enhanced accumulation of alkaloids (detected universally across Hager's, Mayer's and Tannic acid reagents) and coumarin compared to their wild counterparts. *In vitro* conditions represent a unique microenvironment characterized by controlled osmotic, light and nutrient stresses; furthermore, exogenous PGRs often behave as chemical elicitors that upregulate secondary metabolic cascade pathways (Kharde *et al.* 2018) ^[25]. The highly elevated coumarin content in tissue cultured *K. galanga* is of tremendous pharmacological significance, implying that the *in vitro* environment efficiently forces the transcriptional activation of the phenyl propanoid pathway. Recent studies similarly confirmed that metabolic profiling of Zingiberaceae species *in vitro* cultures yields elevated structurally complex metabolites compared to field grown counterparts due to controlled stress induced elicitation (Parida *et al.* 2022) ^[15].

Conversely, the complete absence of saponins in the *in vitro* raised clones provides insight into the ecological triggers of specific secondary metabolites. Saponin biosynthesis frequently requires symbiotic associations with specific soil microbiota, fungal pathogens or distinct abiotic edaphic stressors that are inherently absent in a sterile, heterotrophic agar matrix (Simes *et al.* 1959) ^[26]. Overall, this robust phytochemical retention and the significant elicitation of coumarin and specific alkaloids establish *in vitro* propagated *K. galanga* as an uncompromised, structurally superior bio-factory for continuous therapeutic exploitation.

Conclusion

The present investigation successfully established a highly efficient propagation protocol for the endangered medicinal plant *Kaempferia galanga* L. utilizing dormant rhizome buds. The optimal synergistic treatment of 1.5 mg/l BAP and 1.0 mg/l NAA secured an exceptional 96% multiple shoot regeneration, while robust rhizogenesis was optimally driven by an IBA and IAA combination. The hardened seedlings demonstrated an excellent 80% *ex vitro* survival rate. Crucially, comparative phytochemical profiling validated that the *in vitro* cultivated clones not only retained their primary therapeutic secondary metabolites but also exhibited augmented levels of valuable alkaloids and coumarin, likely driven by *in vitro* elicitation pathways. This optimized protocol simultaneously serves vital conservation objectives by mitigating destructive wild-harvesting pressures and establishes a commercially viable, rapid pathway for the pharmaceutical industry to access high quality, biochemically superior medicinal raw materials.

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