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Optimized tissue culture protocol for high-frequency multiplication and genetic fidelity evaluation in ginger (*Zingiber officinale* Roscoe)

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Abstract
Ginger (*Zingiber officinale* Roscoe) is a widely valued spice and medicinal plant, primarily propagated vegetatively through rhizomes. However, conventional propagation methods are inefficient and prone to soil-borne pathogens. The present study reports an optimized *in vitro* micropropagation protocol for large scale and disease-free production of ginger using rhizome bud as explants. Axenic cultures were successfully established using a surface sterilization protocol comprising 70% ethanol (30 sec), 1000 ppm Cefotaxime (15 min), 1000 ppm Streptomycin (15 min), Kasugamycin (5 mL L^{-1}) for 15 min, 2500 ppm Bavistin (20 min) and 0.1% $HgCl_2$ (6 min), resulting in 60% contamination free cultures. For sprout induction, MS basal medium supplemented with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA led to 100% bud break, with 3.00 sprouts and 2.00 leaves per explant. During shoot multiplication, the average highest number of shoots (5.81) was observed on MS medium containing 5.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, while the average best multiplication rate (3.15), leaf number (13.55), and mean shoot length (2.98 cm) were recorded on medium with 3.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA. *in vitro* rooting was most effective in ½ MS medium with 2.0 mg L^{-1} IAA, with 100% rooting in most treatments, and maximum root number (7.80) recorded in these media, whereas highest root length was observed in MS medium containing 2.0 mg L^{-1} IBA. *Ex vitro* hardening with a mixture of Cocopeat: Vermiculite (1:1) resulted in 90% survival rate. Genetic fidelity of 55 *in vitro* regenerated ginger plants was assessed using eight ISSR primers, of which six produced 100% monomorphic banding patterns, indicating complete genetic uniformity among all samples. This optimized protocol offers a reproducible, scalable system for commercial propagation and conservation of genetically stable ginger plants.

Keywords: *Zingiber officinale*, micropropagation, rhizome bud explant, shoot multiplication, *in vitro* rooting, genetic fidelity, ISSR markers

1. Introduction
India has long been known as the “land of spices” due to its rich history of spice cultivation, trade and medicinal usage. Among these, ginger (*Zingiber officinale* Roscoe), a tropical perennial herb belonging to the family Zingiberaceae, stands out as one of the most economically and medicinally significant. The genus name Zingiber originates from the Sanskrit word *Srngaverram*, meaning “shaped like a horn” aptly describing the morphology of its rhizome. Ginger is a modified underground stem (rhizome) with nodes, internodes and scale leaves and it is propagated vegetatively. It has sterile diploid chromosomes (2n = 2x = 22), with a genome size of ~3.1 Gb (Cheng *et al.*, 2021). As of 2022, global ginger production was 4.87 million tons, with India contributing 2.22 million tons, making it the world’s leading producer (FAOSTAT, 2024).

Ginger has been widely used in traditional medicine for centuries. Its therapeutic potential is attributed to bioactive compounds like gingerols, shogaols, zingiberene and bisabolene (Al-Achi, 2008) [1], which possess anti-inflammatory, analgesic, antioxidant, antifungal, antiemetic and anti-platelet properties (Mowrey and Clayson, 1982) [18]. These properties make ginger valuable in treating ailments ranging from sore throat and asthma to diabetes and muscle pain.

Despite its importance, ginger suffers from major limitations in propagation. It does not produce viable seeds and hence relies solely on vegetative reproduction through rhizomes

(Nair, 2019) [20]. This method is inherently inefficient, producing only 5-6 plants per rhizome annually (Solanki *et al.*, 2014) [31]. Furthermore, vegetative propagation facilitates the spread of soil borne pathogens such as *Pseudomonas solanacearum* (bacterial wilt), *Pythium aphanidermatum* (soft rot), and *Meloidogyne* spp. (root-knot nematodes), significantly impacting plant health and productivity (Estouka *et al.*, 2021) [8].

In this context, plant tissue culture provides a viable solution for large scale, rapid and disease-free propagation of ginger. *in vitro* techniques allow for the production of genetically uniform, pathogen free plantlets with enhanced multiplication rates (Shaaban *et al.*, 2023) [27]. Micropropagation, in particular, offers significant advantages including high turnover, genetic stability and year-round production of quality planting material (Da Silva *et al.*, 2014) [6]. However, rhizome bud explants though responsive are often contaminated due to exposure to soil-borne microbes, making surface sterilization and media optimization critical (Meenu and Kaushal, 2017; Manisha *et al.*, 2018) [16, 15]. Several factors such as explant type, growth regulator combinations and culture conditions influence successful regeneration and must be carefully standardized for commercial scale applications.

With this background, the present study was undertaken to develop an optimized and reproducible micropropagation protocol for *Z. officinale*. The objectives were to establish axenic cultures from rhizome buds, optimize media compositions for shoot induction, multiplication, rooting, identify suitable hardening substrates and evaluate the clonal fidelity of regenerated plants using ISSR markers.

2. Materials and Methods

2.1 Plant Material and Explants

Healthy rhizomes of *Z. officinale* '*Piplod Local*' were obtained from the Agricultural Research Station (ARS), Anand Agricultural University (AAU), Dahod, Gujarat, India. Daughter rhizomes were dry stored for 1-2 weeks to promote sprouting, after which 0.5 cm bud segments containing active meristems were excised. Explants were thoroughly washed under running tap water to remove soil and debris prior to surface sterilization. All micropropagation and hardening work was conducted at the Centre for Advanced Research in Plant Tissue Culture, AAU, Anand, Gujarat, India.

2.2 Surface Sterilization and Establishment of Axenic Cultures

Surface sterilization involved sequential antibacterial, antifungal and chemical treatments comprising Ethanol (70%), Cefotaxime, Streptomycin, Kasugamycin, Bavistin and $HgCl_2$ at specified concentrations and durations, followed by thorough rinsing with sterile distilled water (Fig 1). Four treatment were used: ST_1-70% Ethanol (30s), Cefotaxime (1000 mg L^{-1}, 15 min), Streptomycin (1000 mg L^{-1}, 15 min), Kasugamycin (5 mL L^{-1}, 15 min), Bavistin (2500 mg L^{-1}, 20 min), and $HgCl_2$ (0.1%, 10 min); ST_2-Ethanol exposure increased to 45s and Bavistin to 22 min, with other steps as in ST_1; ST_3 same as ST_1 but with $HgCl_2$ reduced to 6 min; ST_4 Bavistin reduced to 15 min and $HgCl_2$ to 5 min. Explants were then inoculated on culture medium and incubated under controlled conditions. Contamination was assessed at 7, 14 and 21 days recording bacterial, fungal and total contamination rates along with exudation (medium browning, necrosis, or death).

2.3 Culture Media Preparation and Conditions

Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) [19] was prepared with 3% sucrose, 0.8% agar and plant growth regulators (PGRs) as per the experimental design. The pH was adjusted to 5.8 before autoclaving at 121 °C (15 psi) for 15 min. Forty millilitres of medium were dispensed into culture vessels, which were incubated at 25±1 °C under a 16 h light/8 h dark photoperiod and 2500 lux intensity.

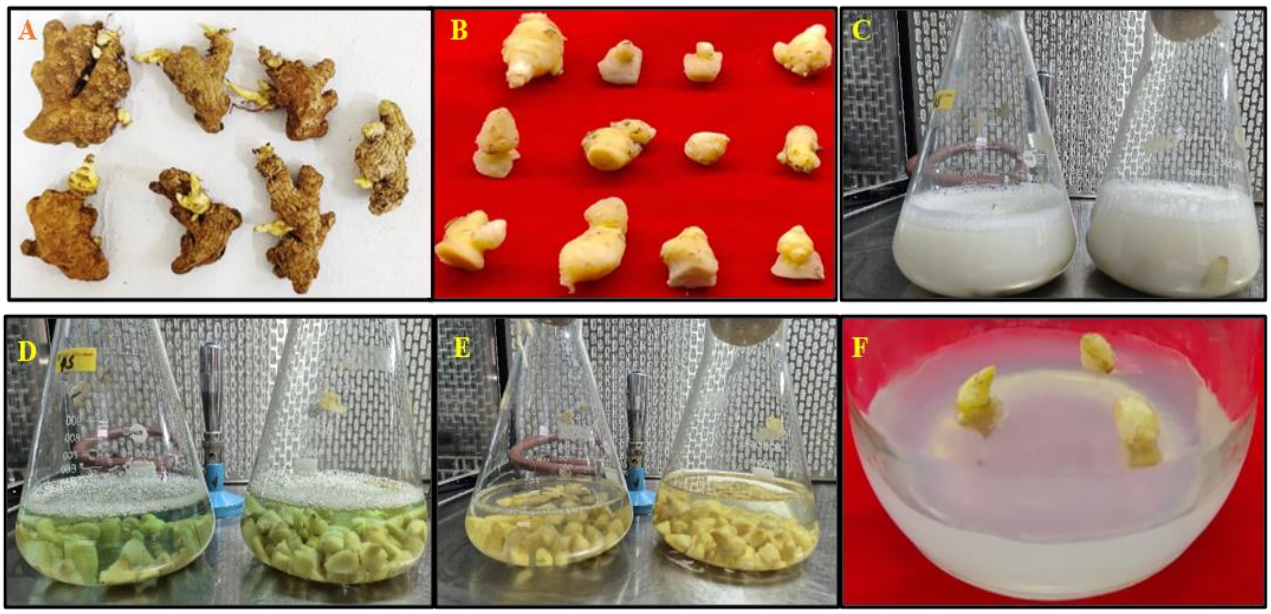


Fig 1: Establishment of axenic cultures of *Z. officinale* from rhizome bud explants.
(A) Fresh rhizome sections with visible buds selected for culture initiation. (B) Excised rhizome bud explants prior to surface sterilization. (C) Explants undergoing antibiotic and antifungal treatments during surface sterilization. (D) Final chemical sterilization step using $HgCl_2$. (E) Thoroughly rinsed explants after sterilization, ready for inoculation. (F) Initiated in culture medias after successful establishment of axenic cultures.

2.4 Culture Initiation and Shoot Multiplication

Axenic rhizome buds (~0.5 cm) were cultured on MS medium supplemented with BAP (1.0-4.0 mg L^{-1}) and NAA (0.5-1.5 mg L^{-1}), or without PGRs (control), for sprout induction. Data on sprouting percentage, days to sprout, number of sprouts, and leaves per explant were recorded after 21 days. Sprouts from the initiation stage were subcultured for multiplication. The initial screening tested BAP (5, 10 mg L^{-1}) with NAA (0.5, 1.0 mg L^{-1}), IAA (1.0 mg L^{-1}), or kinetin (1.0 mg L^{-1}), compared with a PGR-free control. Based on results, refined treatments used BAP (1.0, 3.0, 5.0 mg L^{-1}) with NAA (0.1, 0.2 mg L^{-1}) or IAA (0.1, 0.5 mg L^{-1}). Subcultures were maintained for three cycles recording shoot number, leaf number, shoot length and multiplication rate to determine the optimal PGR combination for high frequency propagation.

2.5 *in vitro* Rooting

Root induction was evaluated using half-strength and full-strength MS media containing 3% (w/v) sucrose and 0.8% (w/v) agar. Auxins tested included IBA, IAA and NAA at concentrations of (0.5, 1.0 and 2.0 mg L^{-1}) along with hormone-free controls. Microshoots (3-4 cm) from the multiplication stage were aseptically transferred to the respective rooting media. Observations included days to root initiation, rooting percentage, number of roots per shoot, and root length.

2.6 Hardening of *in vitro* Propagated Plants

Rooted plantlets were gently removed from culture vessels, rinsed with distilled water and treated with 50% WP copper oxychloride (2 min) followed by 0.2% carbendazim (2 min) to prevent fungal contamination. They were transplanted into sterilized substrates cocopeat alone or mixed with perlite (4:1), vermiculite (3:1), perlite + vermiculite (2:1:1) or Pindstrup® (1:1) in plastic cups, irrigated with fine mist and covered with polythene bags to maintain humidity. After primary hardening, plantlets were transferred to larger pots and acclimatized in the greenhouse. Survival rate, mortality rate and shoot and root lengths were recorded after 45 days.

2.7 Assessment of Genetic Fidelity

Genomic DNA was extracted from young leaves of *in vitro* regenerated plants using the CTAB method described by Doyle and Doyle (1987) [7]. DNA concentration and quality were evaluated spectrophotometrically and by agarose gel electrophoresis. PCR amplification was performed with eight ISSR primers in 10 µl reactions containing 50 ng template DNA, 10 pmol primer, and 5 µl PCR master mix. The thermal profile comprised an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer specific annealing at 38-69 °C for 90 s and extension at 72 °C for 90 s with a final extension at 72 °C for 10 min. Amplicons were separated on 1.8% agarose gels, stained with ethidium bromide and visualized under UV light. Banding patterns were scored as present or absent to assess clonal fidelity.

2.8 Statistical Analysis

Data on culture initiation, shoot multiplication, rooting, and hardening were analyzed using a Completely Randomized Design (CRD) with four replications per treatment (Panse and Sukhatme, 1954) [23]. Observations were recorded after 3 weeks and analysis of variance (ANOVA) was performed to test treatment effects. Significance of differences among treatment means was assessed using the critical difference (CD) at the 5% probability level. The coefficient of variation (CV%) was also computed to assess experimental precision.

3. Results

3.1 Establishment of Axenic Culture

Since rhizome explants of *Z. officinale* were collected from the underground portion of the plant, contamination risk is high due to the presence of both exogenous and endogenous pathogens. Different surface sterilization treatments combining ethanol, antibiotics, fungicides and mercuric chloride were evaluated for their effect on bacterial and fungal contamination, total contamination, axenic culture establishment and exudation. Among the treatments, ST_3 comprising 70% Ethanol for 30 sec, Cefotaxime 1000 ppm for 15 min, Streptomycin 1000 ppm for 15 min, Kasugamycin 5 mL L^{-1} for 15 min, Bavistin 2500 ppm for 20 min and 0.1% $HgCl_2$ for 6 min recorded the lowest total contamination (40%) and the highest axenic culture establishment (60%), with bacterial and fungal contamination at 30% and 10% respectively. ST_2 had the lowest bacterial contamination (10%) but was less effective overall due to high fungal contamination (70%). ST_4 completely eliminated fungal contamination (0%) but resulted in 100% bacterial contamination and 0% axenic culture survival. No exudation was observed across treatments, as regular subculturing to fresh medium prevented accumulation of phenolic exudates. While none of the treatments completely eliminated contamination likely due to endophytic microbes. Optimized protocols minimized contamination while maintaining explant viability. This optimized $HgCl_2$ exposure time effectively suppressed both bacterial and fungal contaminants without causing the reduced bud break or explant death reported at longer exposures (≥12-15 min) (Khatun *et al.*, 2016; Sinchana *et al.*, 2020) [13, 29]. Previous reports using prolonged ethanol (Nongalleima *et al.*, 2014) [22], sodium hypochlorite (Kone *et al.*, 2022) [14], or Clorox (Zahid *et al.*, 2021; Al-Taha *et al.*, 2020) [33, 2] achieved lower contamination levels but often at the expense of tissue viability or chlorophyll integrity. Our findings demonstrate that precise adjustment of sterilant type and exposure time is essential to balance microbial control with explant survival.

3.2 Initiation

Axenic rhizome buds obtained after sterilization were cultured on MS basal medium supplemented with varying concentrations of BAP (0-4.0 mg L^{-1}) in combination with NAA (0-2.0 mg L^{-1}) to evaluate sprout induction (Table 2). ANOVA revealed significant differences among media combinations for all traits measured ($p < 0.05$) including days to sprout induction, number of sprouts and number of leaves. The highest sprouting percentage (100%) was recorded in GI8 (3.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA) and GI2 (1.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA), whereas the lowest (33.33%) occurred in GI9 and GI13. The shortest time to sprouting (10 days) was observed in GI8, while GI1 (MS basal media without PGRs), GI5, and GI13 required 17 days. The maximum number of sprouts (3.00) was produced in GI2, followed by GI8 (2.25), whereas most other treatments yielded ≤ 2.0 sprouts. An average of two leaves per explant was recorded in GI2, GI6, GI8 and GI11; other treatments produced only one leaf.

Table 2: Effect of different BAP and NAA combinations on sprout induction in ginger.

Treatment Code	BAP (mg L^{-1})	NAA(mg L^{-1})	Sprouting (%)	Days to Sprout Induction	No. of Sprouts	No. of Leaves
GI1	0.0	0.0	58.34	17.00	1.00	1.00
GI2	1.0	0.5	100.00	11.25	3.00	2.00
GI3	2.0	0.5	66.67	12.50	1.00	1.00
GI4	3.0	0.5	50.00	16.25	1.00	1.00
GI5	4.0	0.5	58.34	17.00	2.00	1.00
GI6	1.0	1.0	66.67	13.25	2.00	2.00
GI7	2.0	1.0	75.00	11.50	2.00	1.00
GI8	3.0	1.0	100.00	10.00	2.25	2.00
GI9	4.0	1.0	33.33	12.00	1.00	1.25
GI10	1.0	2.0	66.67	13.50	1.00	1.00
GI11	2.0	2.0	50.00	15.00	2.00	2.00
GI12	3.0	2.0	50.00	12.00	1.00	1.00
GI13	4.0	2.0	33.33	17.00	1.00	1.00
S. Em±				0.46	0.07	0.07
CD (5%)				1.32	0.20	0.20
CV (%)				6.73	8.90	10.45

These results demonstrate that 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and 3.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA was the most effective combination, ensuring maximum sprouting percentage, early bud break and satisfactory shoot development. These results align with earlier findings in ginger and related Zingiberaceae where moderate BAP concentrations promoted optimal cytokinin-auxin ratio (Bejoy *et al.*, 2012; Sathyagowri & Seran, 2011; Kambaska & Santilata, 2009) [3, 26, 12]. Higher BAP levels (≥4 mg L^{-1}) reduced sprouting efficiency, likely due to cytokinin induced apical dominance inhibition and excessive callus formation, underscoring the importance of maintaining a balanced cytokinin-to-auxin ratio during initiation.

3.3 Multiplication

3.3.1 Initial Screening for Shoot Multiplication (Three Cycle Evaluation)

Multiplication is the determining step for obtaining a higher number of plantlets during commercial micropropagation. In the present study, the effect of phytohormone BAP in combination with NAA, IAA and Kinetin was evaluated for its influence on shoot proliferation parameters, namely number of shoots, number of leaves, shoot length and multiplication rate over three successive multiplication cycles (21, 42, and 63 days). ANOVA revealed significant differences ($p < 0.05$) among media combinations for all recorded parameters across all cycles, indicating that the growth regulator composition played a critical role in determining multiplication efficiency.

Across the three cycles, treatment GM2 (5.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA) consistently produced the highest number of shoots (5.88, 5.75 and 5.80 in Cycles 1-3, respectively). Treatment GM3 (5.0 mg L^{-1} BAP + 1.0 mg L^{-1} IAA) (Fig 2C) excelled in leaf production (10.08, 10.00, and 9.93 leaves per shoot) and shoot length (2.65, 2.58 and 2.80 cm) and maintained a high multiplication rate (2.35, 2.53 and 2.45) across all 3 cycles. Higher BAP concentrations (10 mg L^{-1}) in GM4 and GM5 tended to reduce shoot length and overall multiplication efficiency, particularly in GM5 (10 mg L^{-1} BAP + 1.0 mg L^{-1} Kinetin), which recorded the lowest multiplication rates (1.08, 1.25). The combined analysis highlights that moderate BAP concentrations (5 mg L^{-1}) supplemented with either low NAA or IAA provided the optimal balance for both shoot proliferation and growth. The detailed performance of each treatment across the three multiplication cycles is presented in (Table 3). Excessive cytokinin (GM5: 10 mg L^{-1} BAP + 1.0 mg L^{-1} kinetin) reduced multiplication rates, a trend also reported by Faridah *et al.* (2011) [10] and Miri (2020) [17].

3.3.2 Optimized Media Screening for Shoot Multiplication (Three Cycle Evaluation)

Following the initial screening of media compositions, three consecutive multiplication cycles were conducted (Cycle 1-21 days, Cycle 2-42 days and Cycle 3-63 days) using refined concentrations of BAP in combination with NAA and IAA to determine the most effective hormonal combination for shoot proliferation in ginger. ANOVA revealed significant differences among treatments for all recorded parameters number of shoots, number of leaves, shoot length, and multiplication rate in each cycle ($p < 0.05$). Mean values from all three cycles are summarized in (Table 4). Across all cycles, the treatment GM7 (3 mg L^{-1} BAP + 0.1 mg L^{-1} NAA) (Fig 2A) consistently produced the highest number of shoots (4.98, 4.85 and 5.15) in cycles 1, 2 and 3, respectively; the number of leaves per shoot was (13.45, 13.65, and 13.55) and length of shoot measured (3.02 cm, 2.98 cm and 2.95 cm), and the multiplication rate was (3.15, 3.05, and 3.08) respectively. The treatment GM8 (5 mg L^{-1} BAP + 0.2 mg L^{-1} NAA) (Fig 2B.) also performed well in terms of shoot number, particularly in Cycle 2 (5.08 shoots) and Cycle 3 (5.03 shoots), though leaf numbers and multiplication rates remained lower than GM7. Treatments incorporating IAA (GM9 and GM11) generally produced fewer shoots and lower multiplication rates across cycles, with GM11 (1 mg L^{-1} BAP + 0.2 mg L^{-1} IAA) showing the poorest performance (multiplication rate 1.25-1.45 across all the cycles). These results are consistent with Taghavi *et al.* (2021) [32] and Shaik & Kanth (2018) [28], who found that a moderate BAP-NAA ratio sustained axillary shoot proliferation without inducing physiological stress. Physiologically, moderate BAP levels in combination with low NAA enhances meristematic cell division without diverting resources to root primordia, ensuring vigorous shoot development over multiple cycles.

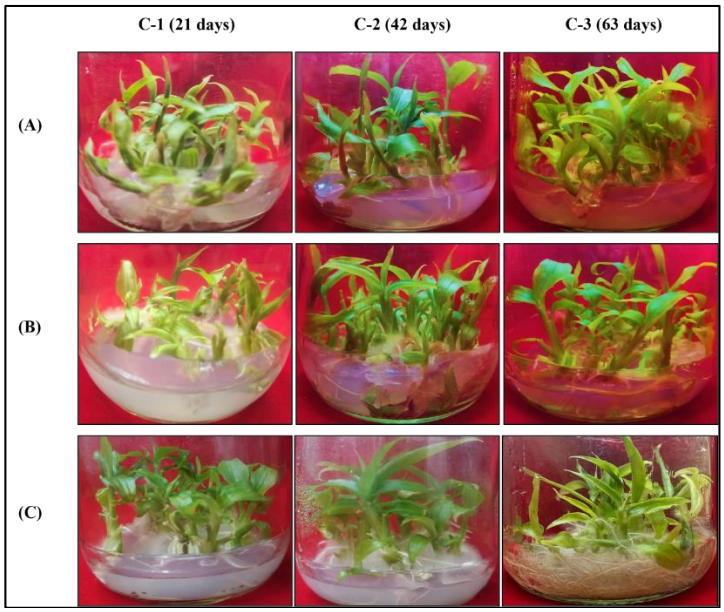


Fig 2: Shoot proliferation of *Z. officinale* after three multiplication cycle (21, 42 and 63 days)
C-1 = cycle 1, C-2 = cycle 2, C-3 = cycle 3, A = GM7 (3 mg L^{-1} BAP + 0.1 mg L^{-1} NAA), B = GM8 (5 mg L^{-1} BAP + 0.2 mg L^{-1} NAA), C = GM3 (5 mgl^{-1} BAP + 1.0 mgl^{-1} IAA)

Table 3: Effect of Different Media Compositions on Shoot Multiplication of Ginger (*Z. officinale* Roscoe) Across Three Successive Multiplication Cycles (Initial Screening Stage).

Treatment Code	BAP (mg L^{-1})	NAA (mg L^{-1})	IAA (mg L^{-1})	Kinetin (mg L^{-1})	Shoots C1	Leaves C1	Length (cm) C1	Rate C1	Shoots C2	Leaves C2	Length (cm) C2	Rate C2	Shoots C3	Leaves C3	Length (cm) C3	Rate C3
GM1	-	-	-	-	2.63	3.23	2.28	1.15	2.75	3.40	2.38	1.35	2.88	3.70	2.35	1.38
GM2	5.0	0.5	-	-	5.88	7.90	1.68	2.35	5.75	7.70	1.64	2.25	5.80	8.00	1.95	2.30
GM3	5.0	-	1	-	4.80	10.08	2.65	2.35	4.33	10.00	2.58	2.53	4.58	9.93	2.80	2.45
GM4	10	1	-	-	4.45	5.58	1.38	1.53	4.13	5.68	1.26	1.55	4.05	5.38	1.53	1.63
GM5	10	-	-	1	3.38	8.38	1.00	1.08	3.03	8.48	1.15	1.15	3.05	8.33	1.08	1.25
S. Em±					0.11	0.20	0.09	0.06	0.22	0.17	0.07	0.08	0.20	0.20	0.08	0.07
CD (5%)					0.34	0.59	0.27	0.19	0.66	0.52	0.20	0.24	0.59	0.59	0.25	0.21
CV%					5.30	5.61	10.09	7.25	11.01	4.85	7.54	8.87	9.57	5.57	8.57	7.52

Notes: GM = Ginger Multiplication medium; BAP = 6-benzylaminopurine; NAA = α-naphthaleneacetic acid; IAA = indole-3-acetic acid; Kn = kinetin; MS = Murashige and Skoog basal medium; C1 = Cycle 1 (21 days); C2 = Cycle 2 (42 days); C3 = Cycle 3 (63 days).

Table 4: Effect of Optimized Concentrations of BAP in Combination with NAA and IAA on Shoot Multiplication of Ginger (*Z. officinale* Roscoe) Across Three Successive Multiplication Cycles.

Treatment Code	BAP (mg L^{-1})	NAA (mg L^{-1})	IAA (mg L^{-1})	Shoots C1	Leaves C1	Length (cm) C1	Rate C1	Shoots C2	Leaves C2	Length (cm) C2	Rate C2	Shoots C3	Leaves C3	Length (cm) C3	Rate C3
GM6	3	-	0.1	4.35	5.85	2.42	2.30	4.40	6.00	2.28	2.25	4.48	6.03	2.40	2.38
GM7	3	0.1	-	4.98	13.45	3.02	3.15	4.85	13.65	2.98	3.05	5.15	13.55	2.95	3.08
GM8	5	0.2	-	4.90	11.65	2.60	2.18	5.08	11.80	2.55	2.23	5.03	11.98	2.70	2.18
GM9	5	-	0.5	3.58	11.45	2.65	2.25	3.53	11.20	2.50	2.20	3.78	11.35	2.60	2.13
GM10	1	0.1	-	3.63	10.30	2.43	1.63	3.43	10.38	2.43	1.58	3.40	10.40	2.45	1.40
GM11	1	-	0.2	3.33	10.00	2.38	1.45	3.25	10.25	2.30	1.35	3.90	10.10	2.28	1.25
S. Em±				0.13	0.23	0.06	0.10	0.11	0.27	0.06	0.07	0.14	0.30	0.05	0.08
CD (5%)				0.38	0.67	0.17	0.29	0.33	0.79	0.18	0.19	0.42	0.88	0.15	0.24
CV%				6.21	4.33	4.47	9.04	5.36	5.07	4.87	6.17	6.63	5.61	3.98	7.90

Notes: GM = Ginger Multiplication medium; BAP = 6-Benzylaminopurine; NAA = α-Naphthaleneacetic acid; IAA = Indole-3-acetic acid; C1 = First cycle (21 days); C2 = Second cycle (42 days); C3 = Third cycle (63 days)

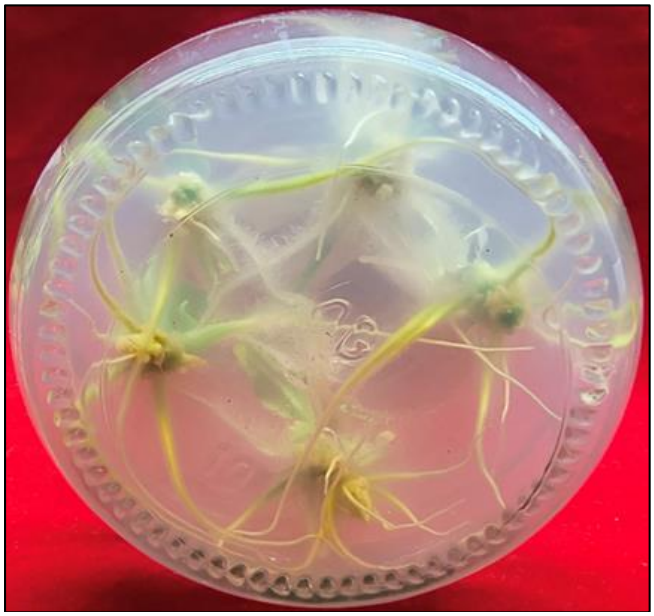
3.4 *In vitro* rooting

In vitro rooting is a crucial step in the micropropagation process, as it determines the successful acclimatization of regenerated plantlets under *ex vitro* conditions. Poorly developed root systems can severely reduce survival rates during hardening. In the present study, micro shoots obtained from the proliferation stage were transferred to rooting media for root induction. Two separate rooting experiments were conducted: (i) using half-strength MS medium and (ii) using full-strength MS medium. Both media were supplemented with different concentrations of IBA, IAA and NAA along with 3% sucrose and 0.8% agar.

3.4.1 Effect of IBA, IAA and NAA on root induction in half-strength MS medium

Minimal days to root induction (5 days) were recorded in GR3 (1.0 mg L^{-1} IBA), GR4 (2.0 mg L^{-1} IBA), and GR6 (1.0 mg L^{-1} IAA), whereas the maximum time (10 days) was

taken by GR10 (2.0 mg L^{-1} NAA). Rooting percentage reached 100% in most treatments (GR1, GR2, GR3, GR4, GR5, GR6, GR7, GR10), while GR8 (0.5 mg L^{-1} NAA) and GR9 (1.0 mg L^{-1} NAA) recorded 95% rooting. The highest number of roots (7.80) was observed in GR7 (2.0 mg L^{-1} IAA) (Fig 3A), which was statistically at par with GR8 (0.5 mg L^{-1} NAA) and GR9 (1.0 mg L^{-1}NAA). The lowest number of roots (4.90) occurred in GR3 (1.0 mg L^{-1} IBA). The longest roots (6.83 cm) were obtained in GR4 (2.0 mg L^{-1} IBA) (Fig 3B), which was at par with GR6 (1.0 mg L^{-1} IAA) and GR2 (0.5 mg L^{-1} IBA). The lowest root length (3.30 cm) occurred in the hormone free control (GR1) (Table 5a). ANOVA results revealed significant differences among treatments for all measured parameters, including number of roots and root length ($p < 0.05$). Days to root induction and rooting percentage also varied considerably among treatments.



GR7 (½ MS + 2.0 mg L^{-1}

B. GR9 (½ MS +2.0 mg L^{-1} IBA)

Fig 3. Effect of IBA, IAA and NAA on root induction in ½ MS media

3.4.2 Effect of IBA, IAA and NAA on root induction in full-strength MS medium

The fastest root induction (5 days) recorded in GR14 (2.0 mg L^{-1} IBA) and GR16 (1.0 mg L^{-1} IAA), whereas GR11 (control), GR15 (0.5 mg L^{-1} IAA), and GR20 (2.0 mg L^{-1} NAA) took 8 days. Rooting percentage reached 100% in most treatments except GR13 (1.0 mg l^{-1} IBA) and GR19 (1.0 mg L^{-1} NAA), which recorded 95%. The highest number of roots (7.58) was obtained in GR17 (2.0 mg L^{-1} IAA) (Fig 4A), while the lowest (5.08) was in GR13 (1.0 mg L^{-1} IBA). Root length was highest (7.73 cm) in GR14 (2.0 mg L^{-1} IBA) (Fig 4B), followed by GR17 (2.0 mg L^{-1} IAA), and lowest (3.43 cm) in GR11 (control) (Table 5b). ANOVA results indicated significant treatment effects ($p < 0.05$) on number of roots and root length.

A. GR17 (MS + 2.0 mg L^{-1}IAA

B. GR14 (MS + 2.0 mg L^{-1}IBA)

Fig 4: Effect of IBA, IAA and NAA on root induction in full strength MS media

3.4.3 Comparison between half-strength and full-strength MS media

Overall, both media strengths supported satisfactory rooting in ginger micro shoots, but some differences were evident. Half-strength MS medium supplemented with 2.0 mg L^{-1} IAA and MS medium supplemented with 2.0 mg L^{-1} IBA induced the highest root numbers (7.80) and longest roots (7.73 cm). Reduced salt strength favoured root elongation, as also reported in ginger by Rahman *et al.* (2005) [24] and Sinchana *et al.* (2020) [29], possibly due to lowered osmotic stress facilitating cell expansion. IBA promoted longer, thicker roots, while IAA yielded higher root counts differences attributable to auxin stability and tissue transport properties. NAA was less effective for elongation, in agreement with Sarker *et al.* (2023) [25] and Miri (2020) [17]. These findings confirm that auxin type and medium strength should be optimized to enhance root biomass or to promote root elongation.

Table 5: Effect of Different Concentrations of IBA, IAA and NAA on Root Induction in Half-Strength and Full-Strength MS Media in *Z. officinale*

(a) Effect on Root Induction in Half-Strength MS Medium							
Treatment Code	**IBA (mg L^{-1})**	**IAA (mg L^{-1})**	**NAA (mg L^{-1})**	**Days to root induction**	**Rooting (%)**	**Number of roots**	**Length of roots (cm)**
GR1	-	-	-	8	100	6.85	3.30
GR2	0.5	-	-	6	100	5.55	6.75
GR3	1	-	-	5	100	4.90	4.84
GR4	2	-	-	5	100	5.35	6.83
GR5	-	0.5	-	7	100	6.35	5.55
GR6	-	1	-	5	100	6.18	6.78
GR7	-	2	-	7	100	7.80	5.08
GR8	-	-	0.5	6	95	7.20	5.65
GR9	-	-	1	7	95	7.25	6.33
GR10	-	-	2	10	100	7.00	6.18
S. Em.±	-	-	-	-	-	0.24	0.14
CD at 5%	-	-	-	-	-	0.70	0.41
CV%	-	-	-	-	-	7.50	4.90
(b) Effect on Root Induction in Full-Strength MS Medium							
Treatment Code	**IBA (mg L^{-1})**	**IAA (mg L^{-1})**	**NAA (mg L^{-1})**	**Days to root induction**	**Rooting (%)**	**Number of roots**	**Length of roots (cm)**
GR11	-	-	-	8	100	6.00	3.43
GR12	0.5	-	-	6	100	5.43	5.73
GR13	1	-	-	6	95	5.08	5.35
GR14	2	-	-	5	100	6.28	7.73
GR15	-	0.5	-	8	100	6.03	5.25
GR16	-	1	-	5	100	7.08	5.31
GR17	-	2	-	6	100	7.58	6.23
GR18	-	-	0.5	7	100	6.40	5.00
GR19	-	-	1	7	95	6.65	4.58
GR20	-	-	2	8	100	6.63	5.50
S. Em.±	-	-	-	-	-	0.07	0.08
CD at 5%	-	-	-	-	-	0.21	0.23
CV%	-	-	-	-	-	2.28	2.89

MS: Murashige and Skoog medium, IBA: Indole-3-butyric acid, IAA: Indole-3-acetic acid, NAA: α-Naphthaleneacetic acid, S. Em.±: Standard Error of Mean, CD: Critical Difference at 5% level of significance, CV%: Coefficient of Variation

3.5 Hardening of *in vitro* Regenerated Plants

In vitro rooted plantlets of *Z. officinale* were transferred to *ex vitro* conditions for acclimatization using five different potting substrate combinations Cocopeat, Cocopeat + Perlite (4:1), Cocopeat + Pindstrup® (3:1), Cocopeat + Vermiculite (1:1), and Cocopeat + Perlite + Vermiculite (2:1:1). After 45 days of hardening, survival percentage, mortality percentage, shoot length and root length were recorded for all replications to assess the effectiveness of each substrate in supporting successful acclimatization.

3.5.1 Primary and Secondary Hardening

Primary hardening was conducted in a fan-pad greenhouse with daily irrigation and fertigation every three days. Among the five substrates, Cocopeat + Vermiculite (1:1) recorded the highest survival percentage (90%), lowest mortality (10%), longest shoots (10.90 cm) and longest roots (9.90 cm) (Fig 5). This was followed by Cocopeat + Pindstrup® (3:1) with 80% survival, 20% mortality, shoot length of 10.35 cm, and root length of 9.25 cm. The lowest survival (50%) and highest mortality (50%) were recorded in Cocopeat alone, with shorter shoots (8.18 cm) and roots (7.28 cm). Vermiculite likely improved root aeration and moisture retention, reducing transplant shock. Similar benefits of mixed substrates have been observed by Zahid *et al.* (2021) [33] in ginger and Singh (2016) [30] in teak and Zhou *et al.* (2022) [34] in other species. Substrate structure and water-holding capacity thus play a pivotal role in transitioning *in vitro* grown plantlets to ambient conditions.

Fig 5: Primary and Secondary hardening of *in vitro* regenerated plantlets of ginger
a = *in vitro* rooted plants, b = primary hardened plants, c = secondary hardened plants

3.6 Assessment of Genetic Fidelity of Micropropagated Plants

Genetic uniformity of 55 *in vitro* regenerated *Z. officinale* plants was assessed using Inter Simple Sequence Repeat (ISSR) markers. Genomic DNA was extracted from all plantlets using the CTAB method (Doyle and Doyle, 1987) [7], and its quality was evaluated by electrophoresis on a 0.8% agarose gel. DNA quantity was determined using a Nanodrop spectrophotometer, and working solutions were prepared from the stock DNA samples. The A_{260}/A_{280} ratios ranged from 1.70 to 2.09, indicating acceptable purity and suitability for downstream molecular analyses.

PCR amplification was performed in a thermal cycler, and amplicons were resolved on 1.8% agarose gels. Across all primers, a total of 39 scorable bands ranging from 200 to 1310 bp were generated, of which 35 were monomorphic and 4 polymorphic, corresponding to 90.83% monomorphism and 9.16% polymorphism. The number of bands amplified per primer ranged from 3 (UBC-888) to 6 (ISSR 01, ISSR 06, ISSR 20), with an average of 4.9 bands per primer. Six primers ISSR 01, ISSR 2, ISSR 10, ISSR 20, UBC-888, and ISSR 79 (Fig 6) produced 100% monomorphic bands, while ISSR 06 and ISSR 26 showed limited polymorphism (33.33% and 40%, respectively) (Table 6). Primer wise banding patterns revealed consistent, sharp and well resolved bands across all samples, confirming high genetic stability. Comparable high-fidelity levels have been reported in banana (96.3%; Nandhakumar *et al.* 2017) [21] and ginger (George *et al.* 2022) [11], while Chirumamilla *et al.* (2021) [5] observed complete monomorphism in *Kaempferia parviflora*. The minimal polymorphism detected here may reflect rare somaclonal variation during prolonged culture, but overall, the data confirm that this protocol reliably produces true to type planting material suitable for commercial scale production.

Table 6: PCR amplification profile and marker efficiency parameters for ISSR primers used in genetic fidelity analysis of *Z. officinale*

Primer Code	Primer Sequence (5′-3′)	Tm (°C)	% GC	Total Bands	Monomorphic Bands (%)	Polymorphic Bands (%)	Band Size Range (bp)
ISSR 10	ACACACACACACACACG	52.77	53	4	100	0	200-1000
ISSR 06	GAGAGAGAGAGAGAGAC	52.77	53	6	66.67	33.33	360-1190
ISSR 01	CACACACACACACAAG	49.18	50	6	100	0	380-1010
ISSR 20	GGAGGAGGAGGA	42.47	67	4	100	0	650-1200
UBC-888	GCTAGTGCTCACACACACACACA	62.43	52	3	100	0	220-910
ISSR 79	GATCTGGTGGTGGTGGTGGTGGTGG	71.04	63	5	100	0	220-1270
ISSR 26	ACACACACACACACACC	52.77	53	5	60	40	480-1230
ISSR 2	AGAGAGAGAGAGAGAGC	52.77	53	6	100	0	350-1310
Total / Mean				39	90.83	9.16	-

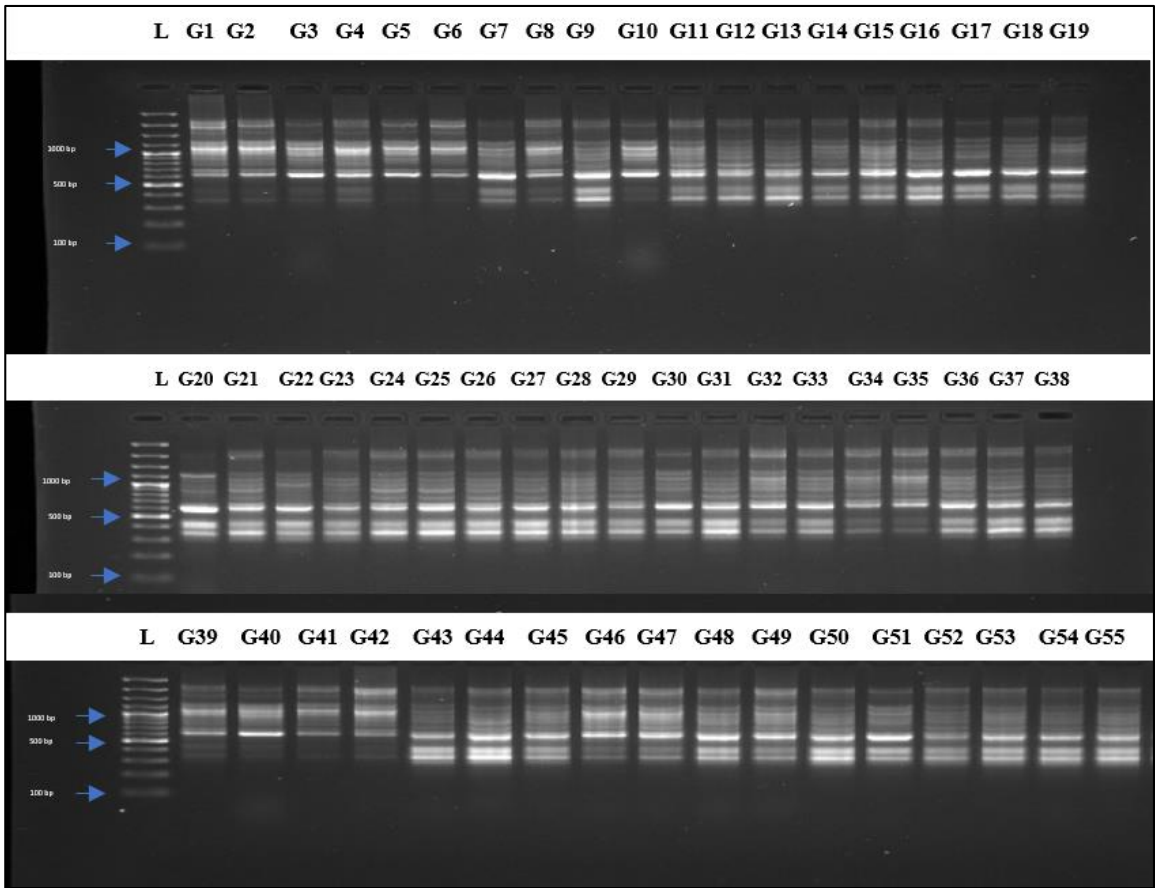


Fig 6: PCR amplification pattern of *Z. officinale* genomic DNA using ISSR primer 2.
L: 100 bp DNA ladder; G1-G55: *in vitro* regenerated plants of ginger. All amplified bands are monomorphic, confirming genetic uniformity across the regenerants.

4. Conclusion

This study established an optimized *in vitro* tissue culture protocol for *Z. officinale* capable of high frequency multiplication and genetic fidelity maintenance. Systematic optimization of each stage from axenic culture establishment, sprout induction and shoot proliferation to efficient rooting and *ex vitro* hardening resulted in a reproducible system for large scale plantlet production. The best multiplication rate (3.08) was achieved with 3.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA, while half-strength MS medium with 2.0 mg L^{-1} IAA produced superior rooting. Genetic stability was verified using eight ISSR primers across 55 micropropagated genotypes, with six primers producing 100% monomorphic and well-resolved banding patterns, confirming complete genetic homogeneity. These markers proved highly reliable for distinguishing true-to-type plants from potential variants and are well suited for routine fidelity monitoring in ginger micropropagation systems. Their sensitivity to minor genetic changes also makes them valuable for detecting somaclonal variation in tissue culture derived plants. This molecular validation confirms that the optimized protocol maintains genetic integrity while supporting reliable, large-scale production of pathogen free, true-to-type plants for both commercial cultivation and germplasm conservation.

5. Author Contributions

S.C. conducted the tissue culture experiments and data collection. G.B.P. conceptualized the study, supervised the experimental design, and contributed to data interpretation. A.M.P. and A.S.P. assisted in protocol optimization, statistical analysis, molecular analysis, figure preparation, and drafting of the manuscript. All authors reviewed and approved the final manuscript.

6. Data Availability Statement

All data generated or analysed during this study are included in this published article. Further inquiries can be directed to the corresponding author.

7. Conflicts of Interest

The authors declare that there are no conflicts of interest related to this study.

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