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ORIGINAL ARTICLE

# Induction of in vitro micro rhizomes and assessment of yield, quality, and clonal fidelity in *ex vitro* established plants of ginger (*Zingiber officinale* Rosc.)

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## Abstract

In vitro micro rhizome technology is a highly effective approach in combating seed-borne diseases and ensuring the production of healthy and high-quality planting material in ginger (*Zingiber officinale* Rosc.). To gauge the efficiency of micro rhizome production and their viability ex vitro, an experiment was conducted on several ginger varieties viz., IISR Varada, IISR Mahima, IISR Rejatha, and Karthika, at ICAR- Indian Institute of Spices Research, Kozhikode, Kerala, India. This experiment adhered to established protocols and standardized procedures. All four varieties exhibited varying rates of micro rhizome production after 180 days of culture. Among these, IISR Varada demonstrated the highest mean weight of cultured plant mass (96.0 ± 4.41 g), followed by Karthika (91.4 ± 5.72 g), IISR Rejatha (78.45 ± 5.59 g), and IISR Mahima (72.4 ± 3.56 g). IISR Rejatha exhibited the maximum number of micro rhizomes per bottle (11.35 ± 0.81) compared to IISR Mahima (10.8 ± 0.54), Karthika (9.8 ± 0.58), and IISR Varada (9.0 ± 0.63). The highest total weight of micro rhizome and mean weight of a single micro rhizome per bottle were recorded in IISR Varada (32.6 ± 1.92 g and 3.9 ± 0.29 g, respectively), followed by Karthika (27.1 ± 1.19 g and 2.9 ± 0.16 g, respectively), IISR Rejatha (27.0 ± 1.79 g and 2.5 ± 0.18 g, respectively) and IISR Mahima (24.5 ± 1.10 g and 2.4 ± 0.16 g, respectively). Besides, IISR Varada, followed by Karthika, emerged as the most promising varieties for micro rhizome production in terms of their multiplication rate. The evaluation extended to the first and second-generation progenies of micro rhizomes from IISR Varada. Results indicated the successful establishment of first-generation micro rhizomes in grow bags and second-generation micro rhizomes in the field, employing both direct planting and transplanting methods. Assessment of quality parameters revealed that the second-generation (V2) transplanted plants of micro rhizomes of IISR Varada exhibited the highest essential oil content 0.78%. The total phenolic content was highest in second-generation (V2) rhizomes directly planted in soil (23 mg GAE/g), whereas the first-generation micro rhizomes raised in grow bags registered the highest total flavonoid content (TFC) of 1.39 mg QE/g. Moreover, the genetic fidelity test conducted on the first and second generations (V1 and V2, respectively) of micro rhizome-derived plants, using molecular markers, exhibited a monomorphic banding pattern similar to that of the mother plant, confirming their genetic stability.

## Key message

In vitro micro rhizomes of ginger have the potential to combat pathogens transmitted through seed materials, while simultaneously preserving clonal fidelity and ensuring high quality standards.

**Keywords** Disease-free planting material · Essential oil · Genetic fidelity · Ginger varieties · Total phenol

## Introduction

Ginger (*Zingiber officinale* Rosc.), renowned globally for its medicinal, flavor, and culinary attributes, holds a prominent place in the Ayurvedic system of medicine and is valued for its therapeutic properties (Wardani et al. 2023; Dusabumuremyi

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et al. 2022; Sharifi-Rad et al. 2017). Despite its significance, ginger cultivation faces formidable challenges, notably from seed and soil borne diseases such as rhizome rot (*Pythium aphanidermatum*), bacterial wilt (*Ralstonia solanacearum*), and nematodes (*Meloidogyne* spp.), leading to substantial crop and yield losses ranging from 50 to 90% (Archana et al. 2023; Yan et al. 2022; Guji et al. 2019).

Since it is vegetatively propagated, these diseases persist through successive generations, emphasizing the urgent requirement for disease-free planting materials particularly in the absence of tolerant or resistant varieties. Plant tissue culture emerges as a promising solution for the rapid multiplication of disease-free ginger plantlets (Shaaban et al. 2023; Zahid et al. 2021a, b; Estouka et al. 2021; Tewelde et al. 2020; Miri 2020; Higgins et al. 2020). However, commercial adoption remains limited due to prolonged incubation periods required for rhizome development compared to conventional seed materials (rhizomes) (Zhao et al. 2023).

Micro rhizome technology offers a potential breakthrough, enabling the production of pathogen-free seed rhizomes in vitro, albeit smaller in size yet retaining essential anatomical and aromatic properties of conventional rhizomes (Zahid et al. 2021a, b; Abbas et al. 2014). Micro rhizomes, comprising one to six buds and two to four nodes (Kandiannan et al. 2020), exhibit promise for germplasm conservation (Gezahegn et al. 2024) and synthetic seed production, boasting extended storage capabilities and non-seasonal availability (Shylaja et al. 2018; Lo-Apirukkul et al. 2012). Noteworthy studies by Mehaboob et al. (2019), Babu et al. (2016), Abbas et al. (2014) and Zheng et al. (2008) have outlined effective protocols for in vitro micro rhizome production, ensuring disease-free outcomes (Archana et al. 2013c). Factors such as sucrose concentration, photoperiod, and medium nature have significantly influenced the aseptic development of ginger micro rhizomes (Gezahegn et al. 2024; Swarnathilaka et al. 2016; Abbas et al. 2014;).The major objective of the study was to assess the suitability of various ginger varieties for micro rhizome production under in vitro conditions and evaluate their performance under field conditions. The aim was to ensure availability of disease-free planting materials of elite varieties of ginger for enhanced production.

## Materials and methods

### Preparation of explants and surface sterilization

Healthy, disease-free rhizomes from four ginger varieties viz., IISR Varada (a high-yielding, high-quality type), IISR Mahima (nematode-tolerant type), IISR Rejatha (another high-quality variant), and Karthika (dual purpose type) were sourced from the ginger germplasm of ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India. These rhizomes were first treated with 0.2% Bavistin (Crystal Crop Protection Pvt. Limited, Delhi, India) followed by shade-drying and incubation under sand for rhizome bud development in a shade net house. This process served as the source of explants for the study conducted from 2019–2022.

Sprouting buds measuring 2 cm in length from all four varieties were carefully excised for culture initiation. The buds were rigorously cleaned under running tap water for 10–15 min, followed by soaking in Tween 20 (Himedia Laboratories, Mumbai, India) for 20 min and rinsing with tap water five times to ensure removal of residues. Subsequently, the buds were surface sterilized with Bavistin (0.3%) for 30 min, followed by treatment with ethyl alcohol (70%) for 30 s and mercuric chloride (Himedia Laboratories) (0.1%) for 10 min under aseptic conditions. The explants were then rinsed 3–4 times with sterile double-distilled water to eliminate any remaining surface sterilants and air-dried.

The aseptically prepared buds were then inoculated into MS medium, made up of macro- and micronutrients, and vitamins (Murashige and Skoog 1962) (Annexure 1) supplemented with 3% (w/v) sucrose (Sisco Research Laboratories, Mumbai, India), without specific growth regulators for culture initiation. The nutrient media was prepared by mixing stock solutions of various chemical ingredients, adjusting the pH to 5.75 using dilute NaOH or HCl (Himedia Laboratories), and incorporating a gelling agent, agar (Himedia Laboratories) (0.6% w/v), for solidification. Tubes (25 × 150 mm) containing the medium (10 mL) were autoclaved for 15 min at 121 °C under a pressure of 1.06 kg $cm^{-2}$. Following inoculation, the cultures were incubated at 25 ± 2 °C with a 14 h photoperiod at 3000 lx, with regular monitoring for shoot induction.

### Establishment of cultures and micro rhizome formation

After 40 days, the established cultures devoid of contamination were transferred to in vitro shoot multiplication medium (90 mL) in tissue culture bottles (250 mL capacity), comprising MS medium supplemented with 3 mg $L^{-1}$ of 6-benzylaminopurine (BAP) (Himedia Laboratories) and 0.5 mg $L^{-1}$ of 1-Naphthaleneacetic acid (NAA) (Himedia Laboratories), as per our prior unpublished study. These cultures were subcultured thrice at 40-day intervals. The resultant multiple shoots served as the basis for explant preparation for micro rhizome production in vitro.

Regenerated shoots from all four ginger varieties, measuring 3–4 cm in length, were excised, with their top shoots and roots trimmed off. These shoots were then transferred to phytajars (75 × 74x138 mm) (Himedia Laboratories) containing micro rhizome induction media (120 mL), consisting of basal MS with increased sucrose concentration (9%), agar (7 g $L^{-1}$), and BAP (0.5 mg $L^{-1}$) (Babu et al. 2005). Cultures

were maintained at a constant temperature of 25 ± 2 °C with a 12-h light period for 150–180 days.

## Ex vitro establishment of micro rhizomes

The harvested micro rhizomes were thoroughly washed to eliminate any adhering media, shoots and roots trimmed off and planted in pro trays filled with a mixture of coir pith compost and vermicompost (1:1 ratio) to initiate budding. To ensure adequate soil moisture, the plants were irrigated once daily through misting. For the study, twenty micro rhizomes of each variety were replicated four times. The survival rate and morphological parameters of the plantlets derived from the micro rhizomes of different varieties were determined after 40 days of planting.

## Comparative evaluation of the performance of first-generation and second-generation micro rhizomes

Following the hardening phase, the plantlets originating from the micro rhizomes of the ginger variety IISR Varada were transplanted into grow bags (40 × 24x24cm) containing a potting mixture of soil, sand, and FYM (2:1:1 ratio) in August 2020 under shade net. These ginger plants represented first-generation plants (V1). The recommended package of practices were followed during cultivation.

Morphological characterization of these plantlets was conducted 150 days post-transplantation. Thirty plantlets were randomly selected and tagged for observation. Parameters including plant height (measured from soil level to the tip of the top leaf of the main shoot), number of tillers (cm), height of the tallest tiller (measured from soil level to the tip of the main shoot) (cm), diameter of the tiller (cm), number of leaves on the tiller, length and width of leaves (cm), and length of petiole (measured from the tip of the leaf sheath to the base of the blade) (cm) were recorded. Subsequently, in March 2021, the ginger plants were harvested, and observations on rhizome parameters such as thickness (cm) and weight (g) were recorded. The harvested ginger were then analysed for various quality parameters like essential oil content, total phenolic content, and total flavonoid content.

The rhizomes harvested from the grow bags were stored in a cold room and utilized as seed material for the subsequent generation (V2). In June 2021, half of the stored rhizomes was directly planted in the field, while the remaining half was used to cultivate pro tray plants as single bud rhizomes. Forty-day-old pro tray plants were transplanted in the field in June 2021 following recommended package of practices. Morphological parameters were observed and recorded 150 days post-transplantation. Thirty plantlets were randomly selected (from both direct planting and transplantation) and tagged for observation. The second-generation ginger plants, cultivated through both direct seed planting and transplantation were harvested in January 2022. Rhizome parameters such as thickness (cm) and weight (g) were recorded including quality parameters like essential oil content, total phenolic content, and total flavonoid content.

## Quality analysis

The harvested rhizomes from both the V1 generation ginger grown in grow bags and the V2 generation ginger cultivated in the field (both directly planted and transplanted) were assessed for quality parameters like essential oil content, total phenolic content, and total flavonoid content.

a. Estimation of Essential oil (EO) content

The essential oil was extracted from fresh rhizomes using a hydro distillation procedure following the ASTA method (American Spice Trade Association 1997). Approximately 150 g of fresh ginger was crushed and transferred into a 1 L round bottom flask, which was then filled with water. The distillation process began by placing the flask over a heating mantle, with the flask connected to both the Clevenger trap and condenser. Distillation proceeded for 4–5 h until consecutive readings showed no further variation. The percentage of essential oil was calculated using the formula:

$$\text{Essential oil (V/W)(\%)} = (\text{Amount of oil collected (mL)}/\text{Weight of sample (g)}) \times 100$$

b. Preparation of ginger extract

Briefly, 1 g of fresh rhizome was ground and extracted with 80% methanol (Himedia Laboratories) through centrifugation and the supernatant collected. This process was repeated twice, and the resulting extract was adjusted to a final volume of 25 mL. From this, separate aliquots were collected for the subsequent two assays.

c. Determination of total phenolic content (TPC)

The total phenolic content (TPC) of ginger rhizome was assessed using the Folin–Ciocalteu reagent (FCR) (SRL chemicals) based method (Singleton and Rossi 1965). Briefly, 0.2 mL of extract was thoroughly mixed with 2.8 mL double distilled water, followed by the addition of 0.5 mL of 1N FCR. The mixture was then incubated for 3 min at room

temperature. Subsequently, 2 mL of 10% sodium carbonate ($Na_2CO_3$) (SRL chemicals) was added, vortexed well, and incubated for 60 min at room temperature. The absorbance of the resulting blue color was measured at 650 nm using gallic acid (Himedia Laboratories) as the standard. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram fresh weight.

d. Determination of total flavonoid content (TFC)

The total flavonoid content was determined using the aluminum chloride reagent (Chang et al. 2002). About 0.2 mL of extract, 1.8 mL of double distilled water, 0.1 mL of 10% aluminum chloride (SRL chemicals), 0.1 mL of 1 M potassium acetate (SRL chemicals) were added to a test tube and mixed well using a vortexer. The tubes were then incubated at room temperature for 30 min, and the absorbance of the resulting yellow color complex was measured at 415 nm using Quercetin (Himedia Laboratories) as the standard. The results were expressed as milligrams of quercetin equivalents (QE) per gram of fresh weight.

### Genetic fidelity assessment using SSR markers

A total of 20 samples were included in the study, comprising leaf samples from the mother plant of IISR Varada, four stages of in vitro subcultures (5, 6, 7, and 8), five in vitro micro rhizome leaf samples, five pro tray plants of the V1 generation, and five pro tray plants of the V2 generation. The samples cultivated in MS medium and leaves from the mother plant of IISR Varada were collected from ICAR-IISR, Kozhikode, Kerala, India. The leaves were thoroughly cleaned, and 100 mg was utilized for DNA isolation following the method described by Doyle (1991). The quantity and quality of the extracted DNA were assessed using a Denovix Nanodrop (DeNovix Wilmington, DE). Additionally, confirmation was performed via agarose gel (0.8%) electrophoresis, with visualization under UV light using a gel documentation system (Syngene Gel Doc; Syngene Synoptics Ltd, Cambridge, UK).The genetic fidelity of the micro rhizome-derived plantlets, as well as their V1 and V2 generations, mother plants, and various stages of in vitro subcultures, was evaluated using SSR markers (Akshitha et al. 2022).

The PCR reaction was conducted with a total reaction volume of 20 µL, comprising 1 µL of DNA, 8 µL of nuclease-free water (Thermo Fisher Scientific, Waltham, MA), 10 µL of Takara Emerald mastermix (Takara Bio Inc., Shiga, Japan), and 1.0 µL of primer. PCR amplification proceeded according to the following protocol: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at the appropriate temperature for 45 s depending on the primer used, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min, carried out using Invitrogen Pro Flex PCR system, 19,105 (Applied Biosystems, Waltham, MA). The PCR products were then loaded onto an agarose gel stained with ethidium bromide (Sigma-Aldrich), along with a 100 bp ladder. Gel electrophoresis was conducted at 100 V, and the results were documented using a Gel Documentation System (Syngene Gel Doc; Syngene Synoptics Ltd, Cambridge, UK).

### Statistical analysis

Under in vitro conditions, each ginger variety was considered as a treatment, with 20 bottles per variety replicated four times. Micro rhizome formation was indicated by swelling of the pseudo stem at the bottom part of the bottle. After 180 days of incubation in micro rhizome media, data on mean weight of cultures, number of micro rhizomes per bottle, total weight of micro rhizomes per bottle, and weight of single micro rhizome were recorded. All collected data were subjected to statistical analysis using single-factor ANOVA. Mean values for each treatment were compared using Tukey's HSD test at $p < 0.05$. Correlations between variables were determined using scatter plot. A t-test was performed to compare the performance of first and second-generation micro rhizomes. All statistical analyses were conducted using R.

## Results

### Variability among ginger varieties for in vitro micro rhizome production

The methodology employed for the micro rhizome production in ginger under in vitro conditions are depicted in Fig. 1. All four varieties of ginger viz., IISR Varada, IISR Mahima, IISR Rejatha, and Karthika, exhibited robust response to in vitro multiplication and micro rhizome development (Fig. 2). Micro rhizome induction was observed after 90–100 days of culture, characterized by swelling at the shoot base and reached maturity within 150–180 days. Marked variation between ginger varieties were observed with respect to mean weight of cultures, total weight of micro rhizomes, and weight of single micro rhizomes per bottle (Table 1). In fact, the mean weight of culture for IISR Varada (96.0 ± 4.41 g) was significantly higher than that of IISR Mahima (72.4 ± 3.56 g), IISR Rejatha (78.4 ± 5.59 g), and Karthika (91.4 ± 5.72 g) ($p < 0.05$). Also, the mean weight of culture of Karthika was significantly higher than that of IISR Mahima and IISR Rejatha ($p < 0.05$).

The number of micro rhizomes per bottle did not exhibit significant variation among the four ginger varieties. However, IISR Rejatha produced higher number of micro rhizomes (11.2 ± 0.81) compared to IISR Mahima (10.8 ± 0.54), Karthika


**Fig. 1** In vitro culture initiation, multiplication and micro rhizome induction in ginger. **a** Healthy disease free rhizomes; Explant **b** Culture initiation **c** Multiple shoot formation **d** Induction in micro rhizome media **e** Multiple shoot formation in micro rhizome media **f** 150 day old micro rhizome culture **g** In vitro plants with micro rhizomes **h** Micro rhizomes for planting out **i** Plantlets developed from micro rhizomes in pro tray **j** Established plants of V1 generation of micro rhizomes grown in grow bags. Scale Bars: a = 1 cm; b, c, d, e, f, g, h, i, j = 2 cm

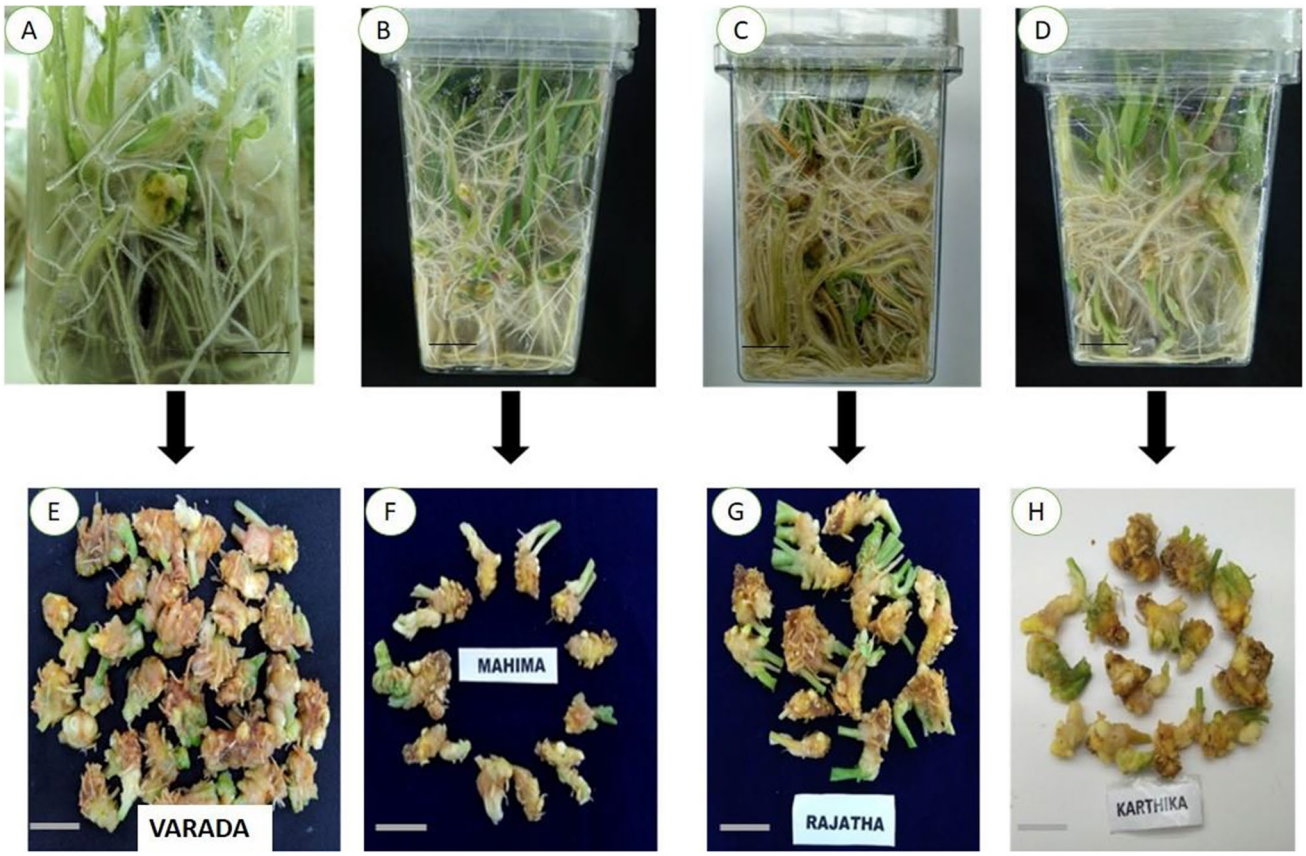


**Fig. 2** In vitro micro rhizome formation of ginger varieties. **A** IISR Varada, **B** IISR Mahima **C** IISR Rejatha **D** Karthika **E** Extracted micro rhizomes of IISR Varada **F** Extracted micro rhizomes of IISR Mahima **G** Extracted micro rhizomes of IISR Rejatha **H** Extracted micro rhizomes of Karthika. Scale Bars: A, B, C, D = 1 cm; E, F, G, H = 2 cm

(9.8 ± 0.58), and IISR Varada (9.0 ± 0.63) (Table 1). Significantly higher total weight of micro rhizomes per bottle and mean weight of single micro rhizomes per bottle were observed for IISR Varada compared to the other three varieties ($p < 0.05$). However, there were no significant differences in these parameters among IISR Mahima, IISR Rejatha, and Karthika. The highest mean total weight of micro rhizomes and mean weight of a single micro rhizome per bottle were recorded in IISR

**Table 1** Genotypic response of ginger varieties to in vitro micro rhizome production

| Variety | Mean wt. of culture (g) | No. of micro rhizomes/bottle | Total wt. of micro rhizomes/bottle (g) | Mean wt. of single micro rhizome/bottle (g) |
|---|---|---|---|---|
| IISR Varada | 96.0 ± 4.41a | 9.0 ± 0.63b | 32.6 ± 1.92a | 3.9 ± 0.29a |
| IISR Mahima | 72.4 ± 3.56c | 10.8 ± 0.54ab | 24.5 ± 1.10b | 2.4 ± 0.16b |
| IISR Rejatha | 78.4 ± 5.59bc | 11.2 ± 0.81a | 27.0 ± 1.79b | 2.5 ± 0.18b |
| Karthika | 91.4 ± 5.72ab | 9.8 ± 0.58ab | 27.1 ± 1.19b | 2.9 ± 0.16b |
| *F*-Value | 7.567*** | 3.553** | 7.379*** | 10.578*** |
| *p-value* | 0.000 | 0.017 | 0.000 | 0.000 |
| CV (%) | 25.92 | 28.30 | 24.80 | 29.87 |
| MSD | 14.75 | 1.94 | 4.64 | 0.61 |

Values are means ± SE ($n = 20$). Means followed by the same letters in each column are not significantly different at * $p < 0.05$ (Tukey's HSD test). F value represents ** = $p < 0.01$, *** = $p < 0.001$, and ns = non significant. *CV* Coefficient of variation; *MSD* Minimum Significant Difference

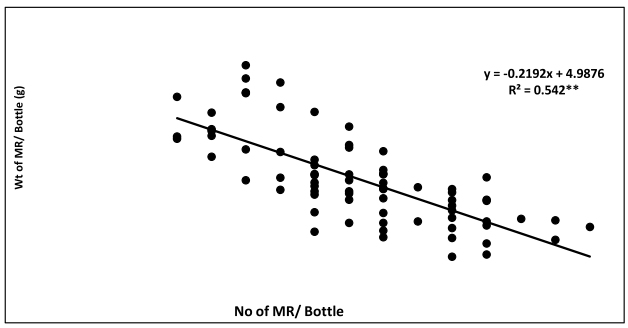


**Fig. 3** Scattered plot diagram of correlation of variables of micro rhizomes

Varada (32.6 ± 1.92 g and 3.9 ± 0.29 g, respectively), followed by Karthika (27.1 ± 1.19 g and 2.9 ± 0.16 g, respectively), IISR Rejatha (27.0 ± 1.79 g and 2.5 ± 0.18 g, respectively), and IISR Mahima (24.5 ± 1.10 g and 2.4 ± 0.16 g, respectively) (Table 1).

There was no significant correlation between mean weight of cultures, number of micro rhizomes per bottle, total weight of micro rhizomes per bottle, and weight of single micro rhizomes. However, the scatter plot diagram (Fig. 3) depicted a negative relationship between the weight of micro rhizomes and the number of micro rhizomes developed per bottle.

## Ex vitro establishment

Micro rhizomes from all varieties were able to successfully germinated and produced plantlets in pro trays. There was no significant variation among varieties regarding the survival rate of micro rhizomes in pro trays. Notably, micro rhizomes of the variety IISR Varada exhibited the highest survival rate (91%) in pro trays, followed by Karthika (85%), IISR Rejatha (81%) and IISR Mahima (79%) (Fig. 4). This suggested that the survival rate of micro rhizomes ex vitro was independent of genotype. Apparently, all the varieties demonstrated equal proficiency in responding to micro rhizome production technique. Furthermore, morphological evaluation of the pro tray plants revealed that the ginger variety IISR Varada displayed superiority over other varieties (Table 2), after 40 days of planting in pro trays.

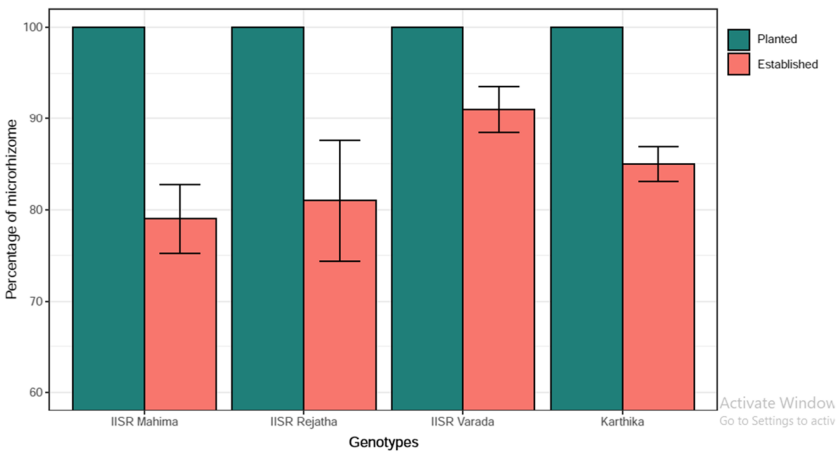


**Fig. 4** Genotypic response of ginger varieties to survival rate of *ex vitro* cultured micro rhizomes

**Table 2** Morphological evaluation of micro rhizome induced plants planted on pro trays after 40 days of planting

| Variety | Plant height (cm) | No. of tillers | Leaf length (cm) | Leaf breadth (cm) | Stem diameter (cm) | No. of leaves |
|---|---|---|---|---|---|---|
| IISR Varada | 11.6 ± 0.38a | 0.223 ± 0.03 | 10.6 ± 0.22a | 1.95 ± 0.26a | 1.95 ± 0.26a | 6.0 ± 0.20a |
| IISR Mahima | 10.7 ± 0.2ab | 0.202 ± 0.02 | 9.4 ± 0.15b | 1.4 ± 0.01ab | 1.4 ± 0.01ab | 5.3 ± 0.15b |
| IISR Rejatha | 10.8 ± 0.16b | 0.181 ± 0.02 | 9.3 ± 0.18b | 1.48 ± 0.02b | 1.48 ± 0.02bc | 4.5 ± 0.05c |
| Karthika | 9.7 ± 0.09c | 0.239 ± 0.05 | 9 ± 0.15b | 1.4 ± 0.01b | 1.4 ± 0.01c | 4.2 ± 0.04c |
| *F*-Value | 10.967*** | 0.654 ns | 15.995*** | 4.221** | 8.551*** | 40.000*** |
| *p-value* | 0.000 | 0.582 | 0.000 | 0.007 | 0.000 | 0.000 |
| CV (%) | 12.04 | 31.23 | 10.07 | 25.25 | 4.06 | 14.07 |
| MSD | 0.87 | NA | 0.65 | 0.47 | 0.03 | 0.47 |

Values are means ± standard error ($n = 20$). Means followed by the same letters in each column are not significantly different at * $p < 0.05$ threshold (Tukey's HSD test). *F* value represented ** = $p < 0.01$, *** = $p < 0.001$, and ns = non-significant. *CV* Coefficient of variation; *MSD* Minimum Significant Difference

The results demonstrated significant differences between the varieties for all morphological parameters, except for number of tillers. To be specific, IISR Varada produced the tallest plants (11.6 ± 0.38 cm), longest leaves (10.6 ± 0.22 cm), widest leaves (1.95 ± 0.26 cm), thickest stems (1.95 ± 0.26 cm), and highest number of leaves (6.0 ± 0.20). On the other hand, IISR Mahima had shorter plants (10.7 ± 0.2 cm) with smaller leaves compared to IISR Varada, although they were still significantly taller and had more prominent leaves than IISR Rejatha and Karthika. IISR Rejatha and Karthika exhibited similar plant height and leaf length, but IISR Rejatha had broader leaves and thicker stems compared to Karthika.

## Comparative evaluation of the performance of first-generation and second-generation micro rhizomes

Though there were no significant differences between the morphological parameters (Table 3), the first-generation plants (V1) grown in grow bags exhibited greater plant height (87.43 ± 2.31 cm) and height of the tallest tiller (69.32 ± 1.57 cm) compared to second-generation plants (V2). However, second-generation plants directly sown displayed a higher number of tillers (26.26 ± 1.50), leaves (22.90 ± 0.93), and longer (24.56 ± 0.69 cm) and wider (2.67 ± 0.07 cm) leaves compared to transplanted

**Table 3** Comparison of first generation grow bag planted micro rhizomes (V1) with second generation (V2) direct field planted seed rhizome and transplanted seedlings from seed produced with micro rhizome plants

| Observations | First generation micro -rhizome grow bag planted (V1) | Second generation (V2) | | SE(d) | CD | CV% |
|---|---|---|---|---|---|---|
| | | Direct seed rhizome field planted | Transplanted in the field | | | |
| Plant height (cm) | 87.43a | 74.85b | 70.74b | 2.311 | 4.623 | 15.83 |
| Number of tillers | 24.90a | 26.26a | 24.06a | 1.504 | 3.009 | 24.77 |
| Height of the tallest tiller (cm) | 69.32a | 55.55b | 55.97b | 1.577 | 3.154 | 15.58 |
| Shoot diameter | 3.04b | 3.44a | 3.01b | 0.109 | 0.218 | 14.76 |
| Number of leaves on main shoot | 21.13a | 22.90a | 19.26b | 0.931 | 1.861 | 18.8 |
| Leaf length (cm) | 22.97b | 24.56a | 22.53b | 0.696 | 1.392 | 11.8 |
| Leaf width (cm) | 1.97c | 2.67a | 2.40b | 0.079 | 0.158 | 17.67 |
| Leaf petiole length (cm) | 0.46b | 0.53a | 0.55a | 0.023 | 0.047 | 19.85 |
| Rhizome thickness (cm) | 1.70c | 2.13a | 2.02b | 0.047 | 0.095 | 13.44 |
| Rhizome weight (g) | 377.70b | 495.45a | 378.06b | 40.409 | 80.818 | 32.75 |
| Essential oil content (%) | 0.53a | 0.53a | 0.78b | 0.02 | 0.06 | 4.60 |
| Total phenolic content (mg GAE/g) | 23.41b | 18.04a | 21.49b | 0.73 | 2.29 | 8.48 |
| Total flavonoid content (mg QE/g) | 1.23b | 1.39b | 0.97a | 0.10 | 0.32 | 21.04 |

Values are means for $n = 30$. Means followed by the same letters in each column are not significantly different at * $p < 0.05$ threshold using paired t test. *SE (d)* Standard error of a difference; *CD* Critical Difference; *CV* Coefficient of variation

second-generation plants (V2) and first-generation plants (V1) grown in grow bags. Analysis of yield parameters revealed that seed-propagated second-generation plants exhibited superior per plant rhizome yield (495.45 ± 40.41 g) and thickness (2.13 ± 0.04 cm) compared to transplanted second-generation plants (378.06 ± 40.41 g fresh rhizome yield with 2.02 ± 0.04 cm thickness) and first-generation grow bag plants (377.70 ± 40.41 g fresh rhizome yield with 1.70 ± 0.04 cm thickness) (Fig. 5).

## Quality parameters

The essential oil (EO) content of fresh ginger rhizomes ranged from 0.53% to 0.78% (Table 3). Notably, second-generation (V2) transplanted plants of micro rhizomes of IISR Varada exhibited the highest EO content of 0.78% among treatments ($p < 0.05$). Conversely, the rhizomes of directly planted second-generation plants (V2) and first-generation plants (V1) grown in grow bags displayed almost similar EO content.

Regarding total phenolic content (TPC), a significant variation was observed among the rhizomes of different generations. Specifically, the direct planted second-generation (V2) rhizomes of ginger variety IISR Varada registered the highest TPC of 23 mg GAE/g ($p < 0.05$), followed by the rhizomes of second-generation transplanted ginger (21 mg GAE/g). Conversely, the first-generation micro rhizomes raised in grow bags exhibited the highest total flavonoid content (TFC) of 1.39 mg QE/g, significantly higher than ($p < 0.05$) the rhizomes of V2 generations. The lowest flavonoid content was recorded in the second-generation (V2) transplanted plants (0.97 mg QE/g) (Table 3).

## Genetic fidelity

The primers ZOC 11, ZOC 28, ZOC 92, ZOC 98, and ZOC 100 generated monomorphic bands ranging from 100 to 300 bp (Table 4). These monomorphic bands were consistent across V1 and V2 plants, as well as subcultured in vitro plants and micro rhizome plants, resembling those of the mother plant. Only ZOC 100 produced a heterozygotic band. A representative gel image illustrating the amplification of ZOC 28 SSR primers for genetic fidelity testing of different micro rhizome-derived plants compared to the mother plant is provided in Fig. 6.

# Discussion

Ginger propagation traditionally relies on seed rhizomes, which can carry pests and pathogens, impacting crop growth and yield. Therefore, the use of disease-free seed rhizomes

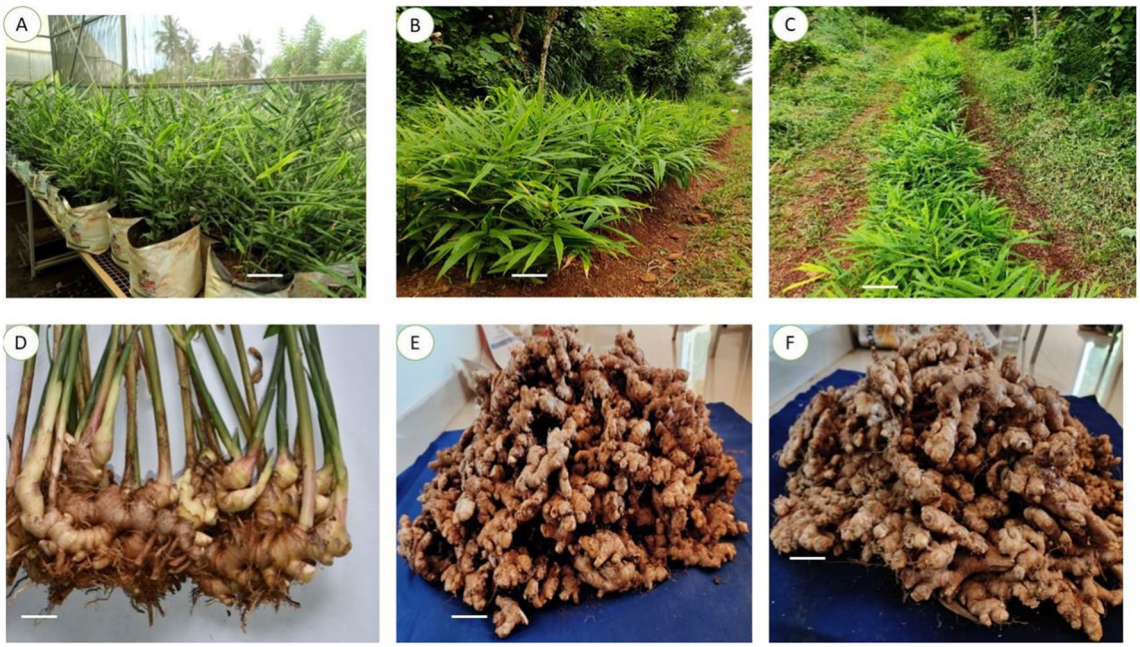


**Fig. 5** View of the first and second generation of micro rhizome derived plants of ginger variety IISR Varada and their harvested rhizomes. **A** V1 generation grown in grow bags **B** V2 generation direct seed planting **C** V2 generation transplanting **D** Yield obtained from one grow bag of V1 generation **E** Harvested rhizomes of V2 generation direct rhizome planting **F** Harvested rhizomes of V2 generation transplanting. Scale Bars: A, B, C, D, E, F = 1 cm

**Table 4** Details of SSR markers used for testing genetic fidelity of micro rhizomes, V1, V2 generations, in vitro subcultures and mother plant of ginger variety IISR Varada

| Primers | Sequence (5'-3') | Annealing Temperature | Total allele | Products size (bp) |
|---|---|---|---|---|
| ZOC 11 | GCTGCTGGTACTTGGCTTTC CTCCTTCCTTTGCCTATCAAGA | 60.0 | 1 | 225 |
| ZOC 28 | GCCTTCTTCGGAGTGTCCTT AACAAAGCCTAATCCAAAACC | 60.0 | 1 | 280 |
| ZOC 92 | GTAGTCCCCAAACAGAAACTCG AGATCGAGGTGGTCAGCAAT | 60.0 | 1 | 200 |
| ZOC 98 | TGCCACTCAATAACATGAACC CGATACATAACAAGCAAGCAAC | 58.0 | 1 | 280 |
| ZOC100 | CATCCCACTGGAAGCGTACAAAC AGGTCGGAGGTGAAGTCTCTG | 61.0 | 2 | 150 |

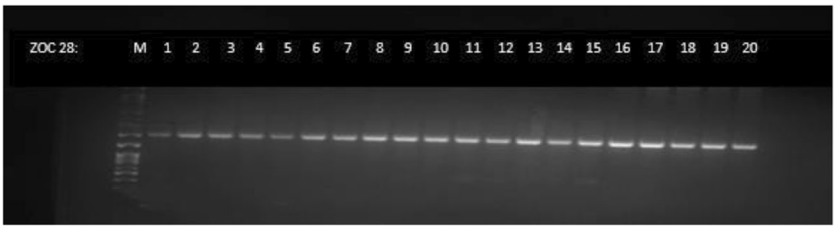


**Fig. 6** SSR banding profile of ginger variety IISR Varada using primer ZOC 28**.** M- 100 bp DNA ladder, 1- Mother plant, 2-fifth in vitro subculture, 3- sixth in vitro subculture, 4- seventh in vitro subculture, 5- eighth in vitro subcultures, 6 to 10- V1 generation, 11 to 15- V2 generation, 16 to 20- Micro rhizome leaf sample

is crucial for successful cultivation. Various studies have explored rapid multiplication techniques such as in vitro propagation and micro rhizome production for ginger (Kandiannan et al. 2020). These techniques involve inducing micro rhizomes in shoots cultured in a medium supplemented with growth regulators like 6-benzyl adenine (BA), sucrose, and reduced photoperiod (Swarnathilaka et al. 2016).

In vitro conditions facilitate the proliferation of shoots through the development of axillary buds, a process influenced by the specific varieties. Notably, each variety exhibits distinct tendencies during the generation of multiple shoots and during initial swelling and greening of vegetative buds. In this study, micro rhizomes were successfully induced in four ginger varieties and this variability can be attributed to genetic differences and their response to sucrose concentrations. Higher sucrose levels in the medium signal the formation of micro rhizomes (Gezahegn et al. 2024; Rout et al. 2001) and the concentration of sucrose and hormones in the medium influenced the development of storage organs like rhizomes (Askari et al. 2018).

Genetic similarities among plants yielded comparable regeneration frequencies (Kavyashree 2009) and IISR Rejatha demonstrated a pronounced propensity towards generating significant quantity of micro rhizomes. This is consistent with the findings of Archana et al. (2013b), in their study on the effects of various sucrose levels on micro rhizome production. Similarly, Shylaja et al. (2017) reported approximately 12 micro rhizomes per bottle using the variety Karthika. Therefore, it is apparent that distinct varieties exhibit varying abilities to induce micro rhizomes under aseptic conditions (Archana et al. 2013a). Further, the quantity of micro rhizomes produced per bottle demonstrated an inverse relationship with the total weight of micro rhizomes, likely attributed to reduced sucrose availability and limited space for induction. This is identical to observations in *Curcuma longa*, where both high (11%) and low (0–5%) sucrose concentrations inhibited micro rhizome formation (Islam et al. 2004). Nevertheless, the mean weight of in vitro micro rhizomes across the four ginger varieties were markedly higher than the previous reports (Zahid et al. 2021a, b; Shylaja et al. 2017).

Though the induction and maturation of micro rhizomes in our study required approximately 150–180 days, the results were remarkable. A micro rhizome weighing between 2–4 g, once extracted from in vitro and hardened, can yield an impressive 300 to 400 g of disease-free ginger rhizomes in the first year of planting in grow bags. This method surpasses conventional approaches, where 25 g of seed rhizome typically yield 600–800 g (or even less as noted by Dev and Sharma 2022; Ravi et al. 2022) but lack assurance regarding disease resistance. Notably, prior studies have not explored the yield performance of micro rhizomes under ex vitro conditions. Furthermore, the production of in vitro micro rhizomes operates as a continuous, year-round process, unaffected by seasonal limitations. This characteristic minimizes time delays after the initial phase, ensuring consistent efficiency of in vitro micro rhizome production over time.

The successful establishment of micro rhizomes in pro trays indicated their potential for field transplantation. Although the survival rate varied among ginger varieties, overall, the technology holds great promise for producing disease-free planting materials, though the survival rate of micro rhizomes ranged from 79 – 91%. Morphological characterization revealed variations among plantlets that can be attributed to genetic diversity. While this in vitro micro rhizome technology can be extended to other ginger varieties for ensuring the production of healthy planting materials, careful management of subculture cycles is necessary to prevent somaclonal variation. Also, regular initiation of cultures from fresh rhizomes is recommended to maintain genetic fidelity.

The evaluation of first and second-generation progenies of micro rhizomes from IISR Varada demonstrated successful establishment in both grow bags and field conditions through direct planting and transplanting methods. Interestingly, the fresh rhizome yield obtained from these progenies was comparable to conventionally propagated plants, contrary to the usual reduction observed in first-generation micro-propagated plants (Hemanthakumar and Preetha 2023). The variation in tiller production in direct-planted second-generation micro rhizomes may be attributed to increased rhizome bud production, resulting in higher yields compared to transplanted plants. Notably, no incidence of diseases was observed in the micro rhizome-derived plantlets across generations, highlighting the potential for disease-free cultivation. This study marks the first comprehensive report on the performance of various generations of micro rhizome-derived seed materials of ginger.

Phytochemical analysis of first and second-generation micro rhizomes of IISR Varada revealed variation in essential oil content, total phenolic content, and total flavonoid content. Essential oil content holds particular significance in evaluating ginger quality according to ISO and Codex Alimentarius standards. Sakamura and Suga (1989) noted marked variations in the composition of volatile oils in micro-propagated ginger rhizomes and plantlets grown in different media. However, upon transfer to soil medium, the essential oil composition reverted to that of traditionally cultivated rhizomes. While our study did not involve compositional analysis, it is noteworthy that one of our treatments showed an increase in essential oil content. Interestingly, we observed a negative correlation between essential oil and total flavonoid content, with second-generation (V2) transplanted plants exhibiting higher essential oil and lower total flavonoid content. This finding contrasted with the results of Zahid et al. (2021a), who reported no significant differences in total phenolic and flavonoid content values between micro-propagated and conventionally cultivated plants of the 'Bentong' ginger variety. This study, therefore, holds great significance in the context of cultivating crops in tissue culture mediums primarily to produce secondary metabolites. Macalalad et al. (2016) found that ginger shoot cultures in MS medium supplemented with benzyl amino purine (BAP) yielded the highest level of gingerol. Our findings suggested that higher production of secondary metabolites, such as phenolics and flavonoids, can be achieved in successive generations of in vitro-induced micro rhizomes of ginger.

The variation in the quality components among the first and second-generation progenies of micro rhizomes from the ginger variety IISR Varada may be attributed to environmental factors and the quantity of seed materials employed. In the V1 and V2 generations (directly planted), rhizomes serve as the seed material, whereas in V2 (transplanted), plants originating from single-bud rhizomes were utilized, potentially accounting for the observed variation in quality parameters. Similarly, Prasath et al. (2018) have documented an increased concentration of oleoresin in single sprout transplanting compared to direct-seeded rhizomes, corroborating our findings. Furthermore, the synthesis and accumulation of phenolics, flavonoids, and terpenoids (components of EO) are influenced by various factors such as the nutrient content of the media, tissue culture plant generation, environmental conditions, and the health of the mother rhizomes (Hussain et al. 2022; Moghaddam and Mehdizadeh 2017).

Genetic fidelity was tested using SSR markers, a widely accepted method for testing genetic purity and diversity (George et al. 2022; Akshitha et al. 2022; Akshitha 2018; Cyriac et al. 2016). The presence of monomorphic bands across various stages of in vitro subcultures, micro rhizomes, and subsequent generations maintained ex vitro, indicated the success of micro rhizome culture in maintaining true-to-type characteristics akin to the mother plant (Fig. 6). This confirmed the genetic stability of subsequent generations of micro rhizome-derived seed materials, a critical aspect for advocating this technology without concerns of genetic impurities or somatic mutations. Our findings are consistent with genetic stability analyses conducted in other crops like red ginger (George et al. 2022), turmeric (Nayak et al. 2011; Babu et al. 2005), and banana (Uma et al. 2023) in banana. Moreover, the clonal fidelity of subsequent generations of micro rhizome-derived seed materials was confirmed for the first time through this study.

Although, the study confirmed the genetic stability of subsequent generations of micro rhizome-derived seed materials, the variation observed in quality parameters might stem from the absence of trait-linked SSR markers associated with genes involved in secondary metabolite biosynthesis. Consequently, our study was unable to establish a direct association between maintaining genetic fidelity and the accumulation of secondary metabolites. Similarly, Nazir et al. (2021) reported variations in the chemical profile among the mother plant, acclimatized plant, and in vitro regenerants of *Dioscorea deltoidea*, despite maintaining genetic fidelity among them.

## Conclusion

Significant variations were observed among ginger varieties in terms of in vitro micro rhizome production, primarily attributed to genotypic variability and their responses to increased sucrose concentration. In vitro micro rhizome formation methodology holds great promise for producing healthy planting materials for elite ginger varieties on a large scale. Besides, high-tech intensive cultivation of micro rhizomes in polyhouses under controlled conditions is feasible using this technology, enabling consistent production.

The yield obtained from the first and second generations of in vitro micro rhizome-produced ginger plants was comparable to that of traditionally propagated plants. Moreover, molecular marker-based genetic fidelity tests conducted on first and second-generation micro rhizome-derived plantlets (V1 and V2) revealed a monomorphic banding pattern similar to the mother plant, indicating genetic stability. Remarkably, no diseases were observed throughout the study across the generations of micro rhizomes. The percentage disease index was measured and found to be 0%.This underscores the potential of in vitro micro rhizomes in mitigating diseases transmitted through seed materials, thereby enhancing ginger production nationwide.

## Annexure 1

**Table 5** MS media composition

| Stock | Components | Quantity (1X) (mg/l) | Volume to be pipette for 1L |
|---|---|---|---|
| Stock A | Ammonium Nitrate | 1650 | 25 ml |
| | Potassium Nitrate | 1900 | |
| | Magnesium sulphate | 370 | |
| | Potassium dihydrogen phosphate | 170 | |
| Stock B | Calcium Chloride | 440 | 5 ml |
| | Potassium iodide | 0.83 | |
| Stock C | Boric acid | 6.20 | 1 ml |
| | Manganese sulphate | 22.30 | |
| | Zinc sulphate | 8.6 | |
| | Sodium molybdate | 0.25 | |
| Stock D | Sodium EDTA | 37.3 | 5 ml |
| | Ferrous sulphate | 27.8 | |
| Stock E | Copper sulphate | 0.025 | 0.2 ml |
| | Cobalt chloride | 0.025 | |
| Stock F | Glycine | 2.0 | 1 ml |
| | Thiamine HCl | 0.1 | |
| | Pyridoxine HCl | 0.5 | |
| | Nicotinic acid | 0.5 | |
| Others | Inositol | 100 mg | |
| | Sucrose | 30 g | |
| | Agar | 6 g | |

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**Author contribution** Sharon Aravind conceptualized the project, conducted investigation, developed the methodology, wrote the original draft, and reviewed the work. Nisthar E prepared cultures, induced micro rhizomes, and collected data. Chaithanya K.C analyzed genetic fidelity. R Sivaranjani studied the biochemical aspects of micro rhizomes and analyzed the data. K. Kandiannan performed formal analysis, curated data, and reviewed and revised the manuscript. V. Srinivasan administered the project, analyzed data, and reviewed and revised the manuscript. Mukesh Sankar conducted formal data analysis. K. Nirmal Babu contributed to the methodology, provided resources, and supervised the project.

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**Data availability** All data supporting the findings of this study are available within the paper.

## Declarations

**Ethical approval** This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

**Ethical responsibility** The submitted work is original and has not been published elsewhere in any form or language.

**Competing interests** The authors have no relevant financial or non-financial interests to disclose.

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