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Full Length Article

Effort on *In Vitro* Culture for Obtaining True-to-Type and Healthy Red Ginger (*Zingiber officinale* Rosc. var. rubrum) Jahira 2 Variety

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Abstract

Ginger production requires healthy planting material. Bacterial wilt caused by *Ralstonia solanacearum* and leaf spot by *Pyricularia zingiberi* frequently resulted in production loss in red ginger. *In vitro* culture provides a means for producing healthy, true-to-type planting materials. This study aims to learn about the effects of subculture frequency on growth, yield, and somaclonal variation in Jahira 2, the most popular red ginger variety, using flow cytometry and chloroplast counts on stomata guard cells. This is the first report about the *in vitro* production of the Jahira 2 variety and the detection of somaclonal variation. Plantlets derived from the third and fourth subcultures of the Jahira 2 variety grown in an MS medium supplemented with BAP 2.0 mg/L were transferred to a hormone-free MS medium for four weeks before acclimatization. The plantlets could be acclimatized effectively in the greenhouse, with a 90% survival rate. However, first-generation red ginger grown in the greenhouse yielded less rhizome weight (79-98 g) than conventional red ginger (318 g). All ginger plants remained disease-free and healthy. When *in vitro* cultured red ginger plants were compared to conventional plants, there were no significant variations in the number of stomata guard cell chloroplasts or genome size. Somaclonal variation in the Jahira 2 variety may be hampered by reducing subculture frequency to four times. In crux, stable and disease-free Jahira 2 variety of red ginger can be recommended for large-scale *in vitro* production and germplasm conservation as well. © 2024 Friends Science Publishers

Keywords: Chloroplast count; Flow cytometry; *In vitro* culture; Rhizome yield; *Z. officinale*

Introduction

Red ginger (*Zingiber officinale* var. rubrum) is an herbaceous perennial that grows annual pseudostems. It is primarily grown in Indonesia and Peninsular Malaya (Purseglove 1972) but is now spreading to the West and other tropical regions (Africa, India). The red ginger looks similar to the ordinary ginger (white ginger) but has smaller rhizomes and a stronger flavor. Furthermore, the base of its leaf shoot has a red hue (Sivashothy *et al.* 2011) or a scarlet color on the rhizome's scaly leaves (Suciyati and Adnyana 2017; Gnasekaran *et al.* 2021). Red ginger, unlike common ginger, contains higher levels of essential oil than white ginger types (Supu *et al.* 2018), making it primarily used for medicinal purposes and as a source of ginger oil rather than spices and culinary uses (Gnasekaran *et al.* 2021).

Red ginger has been used in herbal medicines to treat various diseases, including inflammation, vomiting, rubella, tuberculosis, growth disorder, atherosclerosis, and cancer (Zhang *et al.* 2022). Several studies have verified that red ginger has anti-inflammatory, antiemetic, antitumor, analgesic, anti-hemorrhagic, neuronal-cell protective, anti-rheumatic, antifungal, and anti-bacterial properties (Supu *et al.* 2018).

The efficacy and safety of herbal products depend on intrinsic and extrinsic factors. Besides environmental conditions, cultivation practices, processing and genetic factors also play an important role in the quality of herbal products. The most challenging aspect of cultivating ginger is bacterial wilt disease caused by *Ralstonia solanacearum* (Horita *et al.* 2023) and leaf spot disease caused by *Pyricularia zingiberi* (Wahyuno *et al.* 2020). Both pathogens infected all varieties of ginger, including red ginger. Bacterial wilt disease is commonly transmitted through rhizomes used as planting materials and the pathogen's attack reduces ginger productivity and causes significant crop loss.

Similarly, the leaf spot adds to the complexities faced by ginger farmers. This fungal infection manifests as small, dark spots on the leaves, which can coalesce and spread, ultimately affecting the plant's health. Although leaf spot disease might not directly lead to the death of the plant as

bacterial wilt does, it can weaken the ginger and make it more susceptible to other stresses and diseases, further impacting its productivity. According to Rostiana and Syahid (2008) the pathogen causing wilt disease remains challenging to control, and as of now, no resistant varieties have been identified. Similarly, leaf spot disease has no resistant variety (Wahyuno *et al.* 2020).

The tissue culture approach is a possible method for obtaining healthy red ginger planting materials. This technique is an effective and promising protocol for propagating *Zingiber officinale in vitro*, employing sprouting buds (Gnasekaran *et al.* 2021; Karyanti *et al.* 2021), but no report concerning *in vitro* propagation using the Jahira 2 variety. Jahira 2 is the most popular red ginger superior variety in Indonesia with a strong spicy flavor. This variety has been widely cultivated and is frequently used as a raw material for medicines in herbal industries and as a source of essential oil, ginger drinks and extracts.

Preliminary research of micropropagation of the Jahira 2 variety produced healthy plantlets in the greenhouse, but the first-generation observation of their growth and yield has yet to be reported. The field performance of *in vitro*-regenerated plants is a critical factor that needs to be overcome before commercial production is permitted. *In vitro* propagation is expected to yield true-to-type and pathogen-free planting materials. However, this technique can also lead to somaclonal variations, encompassing genetic and epigenetic alterations, as has been reported in ginger during tissue culture (Saleh *et al*. 2019; Zhao *et al*. 2023). However, in some cases, somaclonal variation could also provide a valuable source of genetic variation for crop productivity and quality improvement. Somaclonal variation during *in vitro* cultures has been attributed to frequent sub-cultures and high hormone concentrations. In this study, low hormone concentrations are used, and sub-cultures are minimized to mitigate genetic changes. Various methods, including morphological, cytological, biochemical and molecular approaches, have identified somaclonal variation resulting from tissue cultures Zhao *et al.* (2023). This study assessed ginger plants derived from tissue cultures using chloroplast count on the stomata guard cells and flow cytometry.

The number of chloroplasts in stomata guard cells is correlated with the ploidy levels of the plants. Counting chloroplast in guard cells of epidermal peels using a light microscope allows for accurate assessment of chloroplasts within a single cell in one focal plane. This technique has been successfully applied to various plant species, including pepper (*Capsicum annum* L.) and eggplant (*Solanum melongena*) (Bat *et al.* 2021). In addition, flow cytometry (FCM) is another technique used commonly or detecting ploidy levels (Zhang *et al*. 2019; Eng *et al.* 2021). Ploidy determination is important to numerous plants breeding applications, including detecting somaclonal variations in tissue cultures. This method has successfully assessed DNA content in numerous plant species, such as ginger (Zhao *et al.* 2023) and agave (Zonneveld 2021). Moreover, flow cytometry is an excellent method for detailed analysis of proliferating cell populations as it provides the distributions of nuclei based on their DNA content (Mickelson-Young *et al*. 2016).

Therefore, besides chloroplast counting, FCM analysis is employed to detect ploidy levels and DNA content status of ginger plants derived from *in vitro* culture, and morphological characters also validate the results. *In vitro* multiplication utilizing shoot tip explants with a low Benzyl adenine (BA) concentration and less frequent subcultures has shown promise in minimizing somaclonal variations and yielding healthy planting materials. These novel findings, especially in the Jahira 2 red ginger variety context, have yet to be explored. Therefore, this study was conducted observe morphological characteristics, yield, chloroplast number, and ploidy levels of the red ginger Jahira 2 variety derived from tissue culture.

Materials and Methods

Experimental material

The experiment was performed at the Indonesian Spice and Medicinal Crops Research Institute, Bogor, Indonesia, from January 2021 to February 2023. The red ginger (*Z. officinale* var. rubrum) Jahira 2 variety was used in this study. The tissue culture methods used in this study to produce healthy and stable planting materials by applying a low concentration of Benzyl adenine (BA) and a low frequency of subcultures. The tissue-cultured red ginger Jahira 2 variety plants were obtained by shoots tip cultured in an MS medium with 2.0 mg/L Benzyl adenine. The plantlets were proven to be disease–free by visual observation (Data not published). Plantlets of red ginger plants derived from the third and fourth subcultures were employed as planting materials. Before acclimatization in the greenhouse, red ginger plantlets were moved from Murashige and Skoog (MS) + BA 2.0 mg/L into a BA-free MS medium and maintained for four weeks.

Treatments

The treatment consisted of Jahira 2 plantlets derived from the third and fourth subcultures. Before acclimatization, the ginger plantlets were thoroughly washed with tap water to remove the agar from the roots. The plantlets were then transplanted into pots filled with media according to Sharma and Singh (1997), modified in media composition with roasted husk and soil. The cultured pots were covered with polyethylene bags to maintain high humidity around plantlets. After two weeks, the top corner of the bags was clipped and after eight weeks, they were removed and the plants were transplanted into the 15 x 20 cm polybag containing soil, manure and husk (2:1:1) and kept for eight

weeks (Fig. 1). After the plant's performance appeared optimal, they were transferred into a 40 × 40 cm polybag with soil, manure, and husk (2:1:1) and kept at the greenhouse for nine months.

Observations recorded

The growth parameters were recorded using four months' plants after being moved to the pots (eight months after acclimatization). It included the number of tillers, plant height, leaf length, leaf width, stem diameter, rhizome weight, rhizome length, width, thickness, and plant health. Leaf length and width were observed by measuring each plant's longest and widest leaf. Plant height was measured from the soil surface to the highest leaves on each plant. The stem diameter was measured on the tallest tillers of each plant, five centimeters from the soil surface, using a digital caliper.

The rhizome was harvested eight months after being moved to the pots. The parameters observed were rhizome weight, length, width and thickness. The observations were conducted on all plants of each treatment.

Cytological characteristics were evaluated by counting the number of stomata guard cell chloroplasts, according to Zhang *et al.* (2019). The number of chloroplasts was observed on leaves seven months after acclimatization. One leaf sample was obtained from each treatment. The leaves utilized have grown to a typical size, allowing the leaves to be easily sliced and the cell density to be low enough to count the chloroplast guard cells of the stomata. A tiny incision is made from the underside of the leaf's epidermis. The thin incision is placed on a glass object, covered with glass, and examined under a microscope at 400 x magnification. The number of chloroplasts was counted from 3 to 7 stomata guard cells. Photographs are taken instantly by connecting the microscope and camera to the computer. The camera magnification is double that of the microscope magnification.

Observation on ploidy levels

The number of DNA concentration among treatments were observed on leaves seven months after acclimatization. A flow cytometer assessed the quantity of nuclear DNA in young leaf samples of acclimatized ginger grown in the greenhouse. The nuclei were isolated following the method developed by (Galbraith *et al*. 1983). Approximately 1 cm^2 of young leaf tissue from each variant was chopped with a sharp scalpel blade in a glass petri dish containing 0.5 mL isolation buffer LBOI (Dolezel *et al.* 1989). The samples were stained with 50 *u*g/mL Propidium-iodide (PI) and 50 *u*g/mL RNAse, respectively. FCM used the *Glycine max* young leaf sample as an internal reference standard to determine the nuclear DNA concentration. The homogenate-stained nuclei were filtered through 30 m nylon mesh into a 1 mL Eppendorf tube, and their fluorescence was measured using a Millipore Guava simple Cyte-5 Flow Cytometer. A sample's absolute nuclear DNA quantity is estimated using the G1 peak means. It was determined as follows:

Sample 2C nuclear DNA value (pg or Mbp) = [sample G1 peak mean / standard G1 peak mean] x standard 2C DNA content Converting DNA mass in picograms (pg) to the numbers of base pairs (bp) as follows: 1 pg DNA = 0.978 x 10^9 bp (Dolezel *et al*. 2007). The *Glycine max* nuclear DNA sample (2.5 pg) was used as a standard (Tiersch *et al.* 1989).

Statistical analysis

The experiment was set up with three replications and a completely randomized design. The data of various parameters were statistically evaluated using one-way ANOVA in SPSS statistical software (version 23.0) and significant differences between means (represented as mean value standard deviation) were calculated using Duncan's multiple range test at *P 0.05* (Gomez and Gomez 2010).

Results

Plants acclimatization

Acclimatization was the last stage of the micropropagation protocol. It was the plantlets' adaptation process before moving to a natural environment or commercial plantation (Rabaiolli *et al*. 2017). Red ginger plantlets from *in vitro* culture could be well acclimatized at the greenhouse using a sterile growth medium (soil + manure + husk) and the survival percentage was high (90%). There was no difference between subcultures on plant survival and growth.

Growth components

The plant growth started with the development of new shoots. After the plants had been in the greenhouse for four weeks, new shoots started to develop. The plant's stems and leaves were dark green and healthy, with no signs of pathogens. The number of new shoots increased with healthy plant vigor at six months (Fig. 2).

Observation of the growth components in the greenhouse showed significant differences between the sub-culture treatments and control plants on the number of shoots, plant height, and stem diameter, except for leaf length and width (Fig. 3). There was no significant difference in the leaf length and width between plants derived from the third and fourth subcultures. This performance may be related to the short duration between the third and fourth sub-cultured (two months), so the plant's performance in the greenhouse was not affected.

Rhizome yield

The yield of red ginger derived from *in vitro* culture was

Fig. 1: Acclimatization of red ginger derived from *in vitro* culture: **a**) red ginger plantlets after washing with tap water, **b**) red ginger plantlets planted at the growth media, **c**) red ginger plants after four weeks acclimatized, **d**) red ginger plant derived from *in vitro* culture at three-month-old

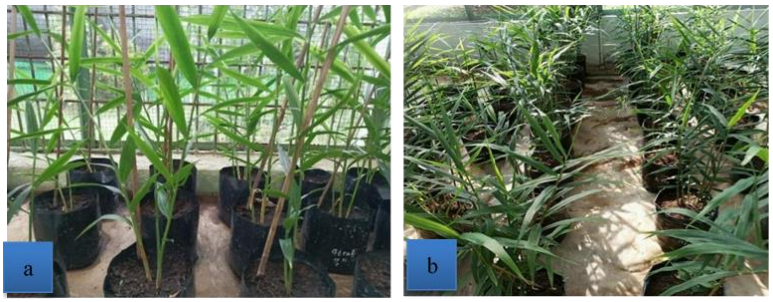

Fig. 2: Performance of red ginger derived from *in vitro* culture at the greenhouse: **a**) The red ginger from the third and fourth subcultures at four months after acclimatization, **b**) The red ginger from the third and fourth subculture at seven months after acclimatization

observed twelve months after acclimatization. Red ginger rhizomes from *in vitro* culture were harvested longer than conventional rhizomes because they needed more time for the acclimatization process at the greenhouse. Commonly, the conventional rhizome was harvested ten to twelve months after planting. The first generation of red ginger from *in vitro* culture revealed a lower rhizome weight than conventional rhizome (Fig. 4 and 5).

Plant health

The ginger plants derived from *in vitro* culture were healthy, with no sign of attack by pathogens. The production of red ginger planting materials from *in vitro* culture was one of the alternatives to eliminate the pathogen and this study proved the method. So, the main advantage of producing rhizomes through *in vitro* propagation was the high percentage of healthy seeds. The red ginger rhizome resulted from in vitro being free from leaf spot and bacterial wilt disease, unlike the conventional rhizome. This condition was valuable because the seeds did not contain any pathogens for use in the subsequent plantings.

Chloroplast content

The number of chloroplasts in the stomata guard's cells of red ginger was not significantly different between the control plant and the *in vitro* culture (Fig. 6 and 7). The average number of chloroplasts per cell in tissue-cultured red ginger stomata guard cells was 24.4–25.2. The number of chloroplasts in each cell was not substantially different from the control plant (21.2).

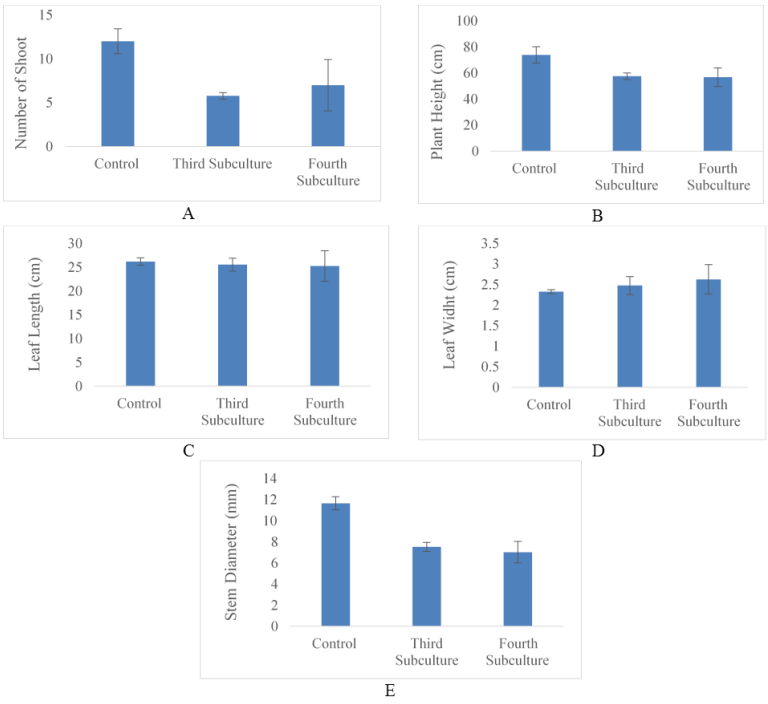

Fig. 3: Growth component of red ginger derived from in vitro culture at six months on number of shoots (**A**), plant height (**B**), leaf length (**C**), leaf width (**D**) and stem diameter (**E**)

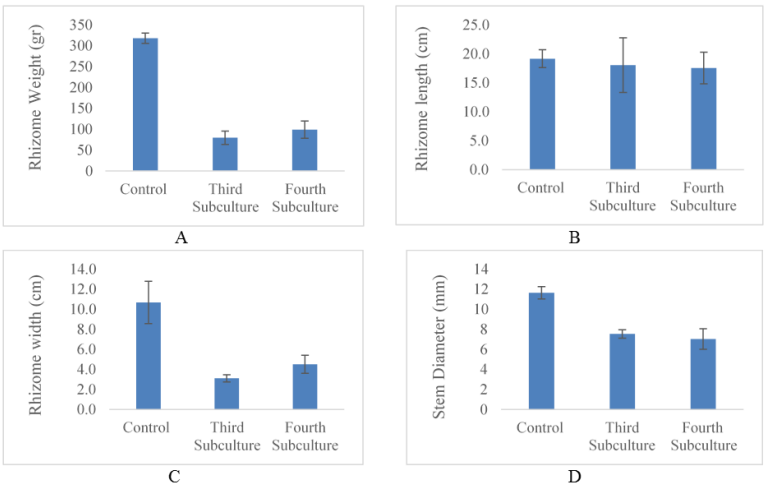

Fig. 4: Yield of red ginger derived from *in vitro* culture, twelve months after acclimatization: rhizome weight (**A**), rhizome length (**B**), rhizome width (**C**) and rhizome thickness (**D**)

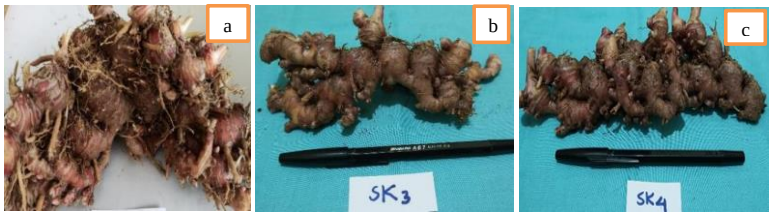

Fig. 5: Yield of red ginger rhizome on eight-months-old derived from *in vitro* culture: **a**) control rhizome from conventional planting material, **b**) rhizome yield derived from third in vitro sub-cultured treatments **c**) rhizome yield derived from fourth sub-cultured treatments

Table 1: Genome size estimation of the red ginger variants nuclear DNA content obtained by flow cytometry

Variant	Mean value of FCM	2C DNA Content (pg)	1C Genome size (Mbp)	Difference (%) from control
Control	3059.23	1.5768	772.62	-
A1, SC 3	2859.39	1.4738	722.15	-6.53
A2, SC 3	3097.53	1.5965	782.29	+1.25
A3, SC 3	3122.71	1.6095	788.65	+2.07
B1, SC 4	3248.44	1.6743	820.41	+6.18
B2, SC 4	3528.10	1.8184	891.04	+15.33
B3, SC 4	3506.50	1.8073	885.58	+14.62
Glycine max (Standard)	4850.45	2.5	1,225.00	-

Note: A1, SC3, A2, SC3, A3, SC3 were the red ginger variants from the third subculture, and B1, SC4, B2, SC 4, B3, SC 4 were the red ginger variants from the fourth subculture

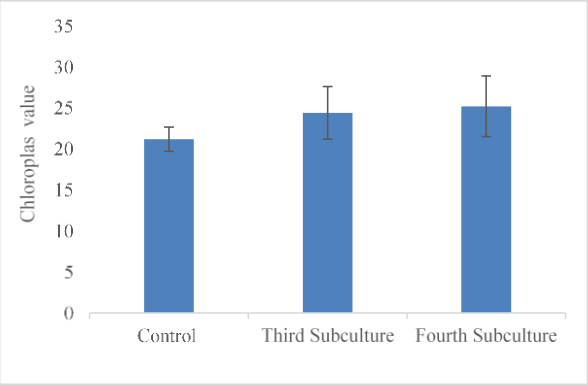

Fig. 6: The average numbers of red ginger chloroplast derived from *in vitro* culture

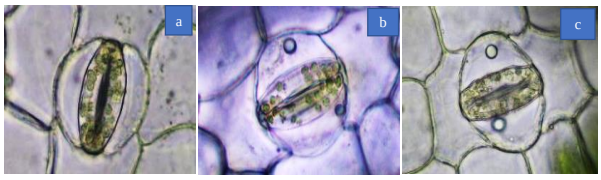

Fig. 7: Chloroplast performance of stomata guard cell of red ginger (x 400) **a**) Control, **b**) Third sub-cultured, **c**) Fourth sub-cultured

Flow cytometry analysis

The results of fluorescence intensity analysis obtained from nucleus staining using propidium iodide on the third subculture (A1 SC 3, A2 SC 3, A3 SC 3), fourth subculture (B1 SC 4, B2 SC, B3 SC 4) and control of red ginger plants using a flowcytometry could be seen on Fig. 8. The histogram represents the G1 peak of each variant that was tested. The G1 peak refers to the phase of the cell cycle where the DNA content of the cells is at its lowest, indicating cells with a single set of chromosomes. Peak G1 of all these variants is in relatively the same channel position. The results were transferred into digital data and then statistically analyzed by a computer program. The G1 peak in all tested variants was relatively at the same channel position. It is estimated that the DNA content of the cells in these variants is similar and they all have a comparable chromosome number. The fluorescence intensity values were converted to the amount of DNA using soybean standard DNA (*Glycine max*) as a reference to analyze the data obtained from the histograms. This conversion enables more accurate comparisons of the DNA content between different samples. The average fluorescence intensity values converted to the DNA amount for each variant are presented in Table 1.

Discussion

In vitro culture techniques play a crucial role in the propagation and conservation of plant species, particularly in the case of economically important and horticulturally valuable varieties like the red ginger Jahira 2 variety. The successful application of *in vitro* culture methods can ensure the production of true-to-type and healthy plants, essential for maintaining the desired characteristics and traits of the variety. However, there are certain benefits and drawbacks to seed production employing *in vitro* cultures.

In this investigation, the Jahira 2 variety was successfully propagated *in vitro*. Acclimatization was one of the challenges of *in vitro* seed development. Transferring plantlets from *in vitro* to greenhouse conditions could be challenging. If not carefully controlled, the acclimatization process, in which plants adjust to external conditions, could result in a high mortality rate. Several factors influence plantlet acclimatization success, including the plantlets' roots, appropriate planting media, and the plantlets' environment. The Jahira 2 red ginger plantlets could be successfully transferred for acclimatization in this study, with a high growth rate (90%). However, it was not possible to reach 100% of the research described by (Zahid *et al.* 2021).

Slow growth speed was one of the limitations of *in vitro* culture seed production. The growth characteristics obtained in this study in Jahira 2 red ginger were much lower than in control conventional plants. Significant differences in the number of tillers, plant height, and stem diameter of plants grown from *in vitro* culture with control may be linked to the differences in their rhizome as a source of energy for growth. Red ginger plantlets formed from tissue culture have not yet developed rhizomes. Therefore, they have limited energy sources to begin their growth, whereas planting material from control treatments are rhizomes with sufficient carbohydrates as energy sources. Nonetheless, one of the key challenges in *in vitro*

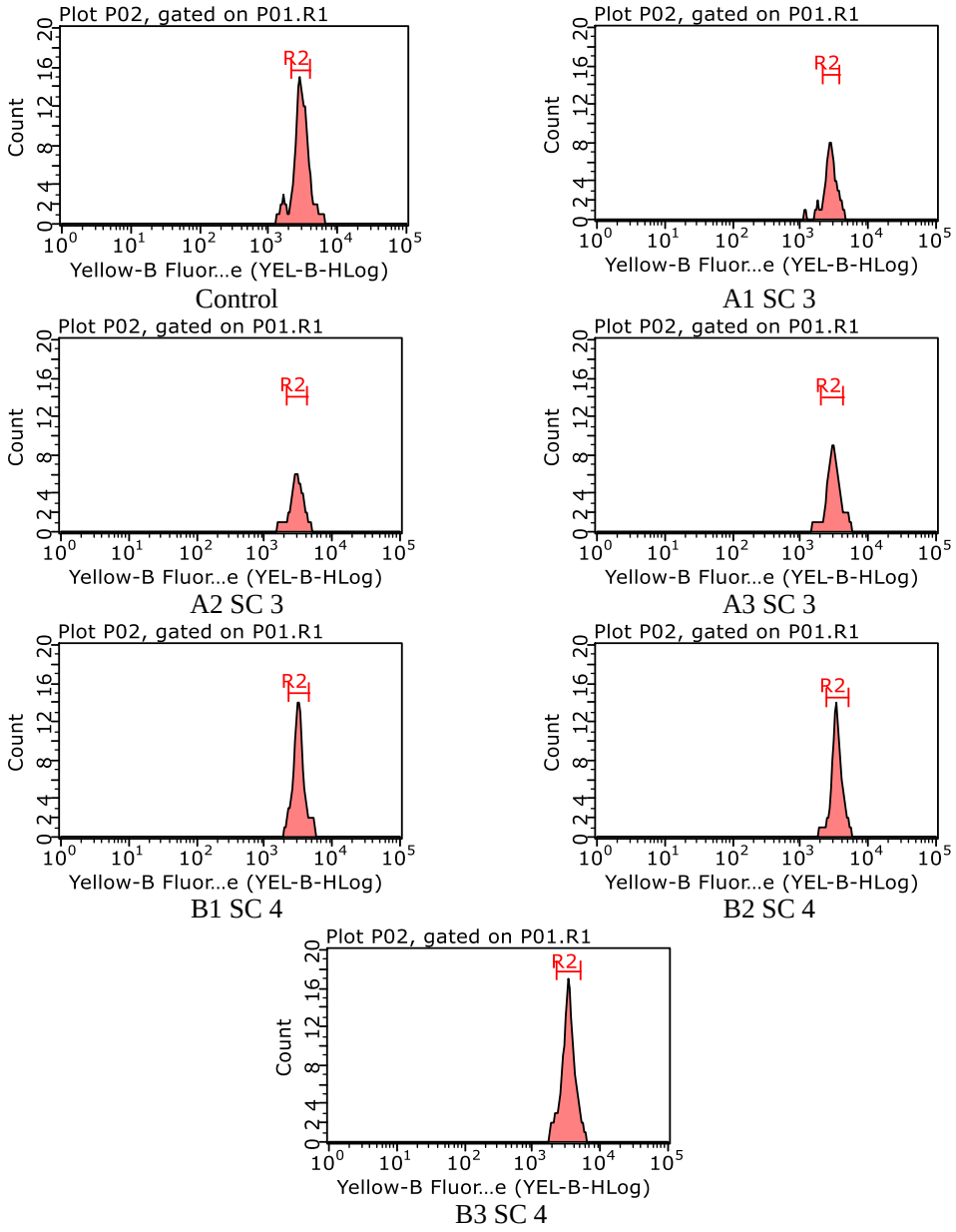

Fig. 8: Flow cytometry analysis of red ginger derived from in vitro culture.

propagation of the Zingiberaceae family was delayed rhizome formation in field-grown plantlets compared to traditionally propagated plants (Chithra *et al.* 2004).

Furthermore, tissue-cultured plantlets grown under complete nutrient supply, highly regulated, and sterile circumstances differ significantly from typical plants' natural environment. When these tissue-cultured plantlets are released into the environment, they must adjust to new conditions, such as changes in light, temperature, humidity, and soil nutrients. This change might be stressful and hinder their growth. It is crucial to note that while tissue-cultured plantlets may initially grow slowly, they can catch up and perform well over time with good care and management. To boost development rates, growers frequently take steps to gradually convert tissue-cultured plants to natural settings and give necessary nutrients (Hazarika 2003).

Somaclonal variation is another issue that arises from *in vitro* culture technology for seed propagation. Slight genetic changes might arise during the culture period, resulting in disparities in plant performance. Maintaining genetic stability in *in vitro* cultures is thus a concern. Somaclonal variations are common due to factors such as high hormone concentration and a high frequency of subcultures. Therefore, subcultures were limited only up to four times in this investigation. The number of chloroplasts in the stomata guard cells was counted to check if

somaclonal variation occurred in this study. The chloroplast number has been used to estimate ploidy levels (Bat *et al*. 2021). In general, high cell divisions caused by the activity of potent growth regulators (cytokinins and auxins) influence genetic modifications, including changes in ploidy levels. Treatments with increased multiplication rates produced more variations (Duta-Cornescu *et al*. 2023; Zhao *et al.* 2023). In this study, the administration of BA 2.0 mg/L for *in vitro* shoot multiplications and culture maintenance in the laboratory for eight months did not generate somaclonal changes in this experiment, based on the chloroplast number in the stomatal guard cells. The restriction on the number of subcultures to three to four times was intended to prevent somaclonal variation. According to the study, there were no significant variations in the number of chloroplasts between the third and fourth subcultures. This finding suggests that three to four sub-cultured treatments did not influence the quantity of red ginger chloroplasts and ploidy levels of red ginger plants obtained from *in vitro* culture. This result differed from (Zhao *et al*. 2023), who revealed somaclonal changes in the propagation of the large white ginger of Wuling variety in MS + BA 1.0 mg/L + IBA 0.2 mg/L, which attributed to a high frequency of sub-cultures (18 times). Variations were observed in stem morphology and rhizome size.

This research also investigated the possibility of somaclonal variation by assessing the size of the nucleus DNA. The FCM results revealed no variations in DNA size (Table 3). It implies that regardless of how frequently the cells were subcultured, there was no significant impact on the DNA size in the Jahira 2 cell nuclei. The consistent position of the G1 peak in the histograms indicates that the variations and control plants had identical DNA content and size. The absence of somaclonal variation throughout the experiment is suggested by the lack of changes in DNA size among variants and the effect of cultural frequency. Mohanty *et al.* (2008) reported similar results, revealing that cytophotometric analysis indicated a unimodal distribution of DNA content with no somaclonal fluctuation, supporting the genetic uniformity of *in vitro* cultivated *Curcuma aromatica*. These findings are critical for understanding the genetic features and stability of the tested variations, which can have implications for various research and biotechnology applications. Despite some somaclonal differences in the tissue culture plants used in this investigation, they did not manifest at the phenotypic level. Several qualitative morphological characteristics show no morphological variations between the tissue-cultured and conventional plants (color of the leaf shoot base, leaf shape and color, stem color and shape, rhizome shape and color, sheath of the rhizome). According to Ferreira *et al*. (2021), somaclonal variation occurred due to physical factors (medium, plant growth regulator, sub-culture period and environment) influencing DNA replication during cell division during *in vitro* growth.

The study showed that the third and fourth sub-cultured treatments exhibited similar growth responses in various parameters, including tiller number, plant height, leaf length, leaf width and stem diameter. In other words, no significant differences were observed between these two sub-cultured treatments regarding these growth characteristics. It suggests that the third and fourth sub-cultured plants had comparable growth patterns and development, likely because they were derived from the same *in vitro* culture process and had the same genetic backgrounds.

The fact that sub-cultured treatments exhibited similar growth responses, while the plants from conventional rhizomes displayed more robust growth, provides valuable insights into the effects of different propagation methods on the growth and development of red ginger plants. They may have implications for ginger cultivation practices and crop improvement strategies.

The yield of fresh ginger from the first generation of red ginger was significantly lower than the conventional rhizome. In addition, other harvest parameters from subculture treatment, such as rhizome length, rhizome width, and rhizome thickness, were also smaller than those of conventional parents. The lower yield of rhizomes at the first generation of *in vitro* culture compared to those grown from conventional rhizomes is consistent with the results reported by (Zhao *et al.* 2023). These support the notion that *in vitro*-cultured ginger plantlets face challenges in their initial growth due to the absence of rhizomes, as the nutrient reserve is typically provided by conventional rhizomes (Mahender *et al.* 2015). Similarly, Bhagyalakshmi *et al.* (2015) also found that micro-propagated ginger yielded considerably less than conventionally propagated ginger at eight months.

On the other hand, the yield from the first generation of large ginger derived from the first and second subcultures produced normal rhizome thickness but less yield (140 g – 435 g/plant) (Rostiana and Syahid 2007). In contrast, superior growth and yield in micro-propagated plantlets compared to conventionally propagated plants has been demonstrated in turmeric (Salvi *et al.* 2002). Strategies employed in other species, such as *Kaempferia galanga*, involving silver nitrate and high sucrose concentrations *in vitro*, have shown success in countering delayed rhizome development and associated yield reduction (Chithra *et al*. 2004).

Despite these challenges, *in vitro* culture methods offer advantages such as the production of disease-free plants, the potential for year-round seedling production, and the ability to conserve and propagate specific genotypes. In our experiment, all ginger plants derived from *in vitro* culture were healthy, with no sign of attack by pathogens, as also found by Zhao *et al.* (2023) and Zahid *et al.* (2021). This result indicates that producing Jahira 2 red ginger planting materials through *in vitro* culture could be an effective method for pathogen elimination. In conventional cultivation using rhizomes as planting material, there is a

high risk of carrying pathogens from the mother plants to the new generation, leading to disease outbreaks and economic loss. It was shown that the control plants grown from conventional rhizomes displayed leaf spot disease symptoms caused by the fungus *Pyricularia zingiberi*. This validates the importance of disease-free planting materials and the potential risks of using conventional rhizomes that may carry pathogens for subsequent plantings. The production of red ginger planting materials through *in vitro* culture is a promising alternative for pathogen elimination and ensuring healthy seeds.

Conclusion

The tissue culture propagation of the Jahira 2 variety resulted in healthy planting materials. There were no significant differences in the chloroplast number on the stomata guard cells, and they showed similar genome size on the nuclear DNA content measured by flow cytometry, suggesting the absence of somaclonal variation. The methods used for Jahira 2 micropropagation were low BA concentration, less frequent subcultures, and using a shoot tip as an explant source. The age of the culture and the less frequent subcultures reduce the possibility of somaclonal variations. Therefore, it is essential to use methods that favor less somaclonal variation to obtain true-to-type and healthy planting materials.

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Author Contributions

SFS, NB and DS designed and supervised the research. SFS, NLWM and TA conducted data collection. NLWM, TA and DS conducted statistical analysis. SFS and NB writing the original manuscript. SFS, NB, and DS writing-review editing. SFS, TA and NLWM made illustrations. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All authors declare no conflicts of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper

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