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GENETIC VARIABILITY DEVELOPED THROUGH MUTATION IN RED GINGER (*ZINGIBER OFFICINALE*) BASED ON MORPHOLOGICAL TRAITS, RHIZOME YIELD, AND 6-GINGEROL CONTENT

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SUMMARY

This study explored the impact of sodium azide (NaN3) treatment on red ginger (*Zingiber officinale* var. rubrum) 'Jahira-2,' a variety known for high yield but low 6-gingerol content. NaN3 application sought to increase genetic variability, tested at four concentrations (0, 100, 300, and 500 mg l^{-1}) on rhizomes, before growing in polybags. After initial treatment and harvest, replanting the M1V1 rhizomes continued to produce the second generation (M1V2). Six months after planting proceeded to morphological and rhizome characteristics' assessment, with the 6-gingerol content measured nine months after planting using a TLC (thin-layer chromatography) scanner. Genetic analysis using RAPD with 10 primers confirmed variations among mutants, with the NaN3 treatment enhancing 6-gingerol content. A significant correlation occurred between rhizome yield and morphological traits. The principal component analysis identified 11 components, with four (eigenvalue >1) accounting for 82.83% of the total variability. Heatmap analysis clustered nine mutants, revealing distinct genetic variations. Phylogenetic analysis grouped M1V2 mutants into three clusters with a 0.62 similarity coefficient, indicating enhanced genetic diversity. These findings underscore the potential of NaN3 treatment in breeding programs to enhance red ginger's genetic diversity and phytochemical profile.

Keywords: Cluster analysis, genetic variability, M1V2 generation, morphological traits, NaN3, red ginger (*Z. officinale* var. rubrum), rhizome yield, 6-gingerol content

Key findings: The NaN3 treatment increased the 6-gingerol content in the red ginger (*Z. officinale* var. rubrum) mutants.

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INTRODUCTION

Red ginger (*Zingiber officinale* var. rubrum), a potential medicinal plant belonging to the Zingiberaceae family, is increasingly popular in Asia, particularly in China, Indonesia, and Malaysia. Chemical compounds associated with its pharmacological and biochemical properties in ginger are gingerols, shogaols, homovanillyl alcohol, alkaloids, flavonoids, saponins, tannins, phenolics, steroids, triterpenoids, and essential oils (Hendra *et al*., 2022; Zhang *et al*., 2022). Sustainable cultivation of red ginger is crucial for producing high-quality raw materials rich in bioactive compounds, enhancing the efficiency of red ginger's medicinal use to meet the rising demand of the community. 'Jahira-2' is one of the commercial red ginger cultivars with a potential yield of around 460.20 ± 117.41 g plant^{-1}; however, its 6-gingerol content is below 1.5% (Kementerian Pertanian, 2007). The 6-gingerol is one of the most attractive pharmacological properties of red ginger due to its medicinal potential and least toxicity (Magdy *et al*., 2020).

Since red ginger is a vegetatively propagated crop, its genetic variability is narrow (Wahyuni *et al*., 2003). Furthermore, red ginger rarely produces flowers, and the absence of seed makes conventional breeding, like hybridization, impossible (Dev and Sharma, 2022). Improving the 'Jahira-2' red ginger variety through mutation will broaden its genetic variation; therefore, efforts have progressed to enhance the genetic variability of red ginger using sodium azide (NaN3).

NaN3, a potent mutagen, has been serving to induce genetic variations to enhance rice varieties' yield and other desirable traits (Ikhajiagbe and Omoregie, 2020). However, it is toxic at high doses (Herwibawa and Kusmiyati, 2017). Yafizham and Herwibawa (2018) found low doses of NaN3, ranging from 0 to 1.60 mM, are safe and could improve the genetic diversity of chili peppers.

The promising study aimed to examine the effects of NaN3 concentration on the growth, rhizome yield, 6-gingerol content, and genetic diversity of red ginger mutants in the M1V2 generation. This information will be a valuable tool for improving the red ginger yield and phytochemical contents through breeding.

MATERIALS AND METHODS

Plant material

The red ginger variety 'Jahira-2' came from the Institute for Spice and Medicinal Crops Standardization, Bogor, Indonesia, at 6° 26' S latitude and 106° 48' E longitude, with an elevation of 245 m asl**.**

Cutting the rhizomes consisted into sections of 2–3 buds. The NaN3 solution preparation in the laboratory included concentrations of 0, 100, 300, and 500 mg l^{-1}, with the pH adjusted to 3.6, following Srivastava *et al*. (2011). The rhizomes (M1) sustained NaN3 treatments for 24 h before planting in growth media and remaining for two weeks in a greenhouse. The treated rhizomes' planting in 30 cm × 40 cm polybags underwent field maintenance. The experiment, designed randomly, had four treatments (0, 100, 300, and 500 mg l^{-1} of NaN3 solution), with three replications, each replication consisting of five plants. After nine months, harvesting the mutant rhizomes (M1V1) continued re-planting to produce the second generation (M1V2), following the ginger cultivation operational procedures (Bermawie *et al*. 2015).

Morphological traits

The recording of morphological characteristics of the M1V2 generation ensued after six months of transplanting into polybags, whereas recording for 6-gingerol resulted from the rhizomes harvested after nine months. According to Bermawie *et al*. (2015), the morphological characteristics assessed were the number of tillers, plant height, leaf length and width, stem diameter, and rhizome characteristics (weight, length, width, and rhizome thickness).

6-gingerol content analysis

The use of thin-layer chromatography (TLC) analyzed the 6-gingerol content of the rhizomes (Sugiarti *et al*., 2011). Approximately 0.25 g of red ginger powder (100 mesh), poured into a 25 ml volumetric flask, reached filling with one-third of 95% ethanol. The mixture, shaken with the shaker for 2 h, remained at room temperature for 24 h. The extract filtration used ordinary filter paper, with its 5 µl placed onto a 20 cm × 20 cm aluminum silica gel plate, and heated in an oven at 105 °C for 30 min. The study spotted a 5 µl 6-gingerol standard (Sigma, USA) with a concentration of 500 ppm for comparison. Placing the plate in the hexane eluent chamber, eluting 30 ml of diethyl ether in a 3:7 ratio continued for 45–60 min until the elution limit was at ±15 cm. Then, air drying the plate, its measurement with a TLC-Scanner was at λ = 282.

RAPD analysis

Genomic DNA extraction proceeded using a modified CTAB method (Doyle and Doyle, 1990). This method involves adding 2% (w/v) PVP (polyvinylpyrrolidone) and 0.3% (w/v) sodium bisulfite to the extraction buffer and repeating the extraction process using a chloroform, i.e., isoamyl alcohol (24:1) solution twice. The DNA from each mutant sample then acquired dilution to the concentration of 10 ng µl $^{-1}$. Each DNA sample's amplification used 10 RAPD primers, as presented in Table 1.

Each reaction contained 10 µl template DNA at a concentration of 10 ng µl^{-1} with 2 µl; 2x My Taq H.S. (Bioline, UK) with 5 µl RAPD primers at a concentration of 10 pmole with 0.5 µl; and sterile ddH_2O. The performed PCR reaction was in a T100 Thermal Cycler (Biorad, USA) with the following PCR profile: initial denaturation performed at 94 °C for 4 min, followed by 45 cycles of denaturation process at 94 °C for 30 s, hybridization at 36 °C for 1 min, and extension at 72 °C for 2 min. The PCR reaction ended with the final extension stage at 72 °C for 5 min. The PCR products' separation used 1.5% agarose gel in a tank containing 1x TAE buffer at 90 V for 45 min. The gel, stained with ethidium bromide (10 mg ml), gained visualization using a U.V. Transilluminator (Biorad, USA).

Data analysis

The morphological data underwent statistical evaluation using one-way ANOVA in SPSS statistical software (version 23.0). Significant differences between means (expressed as mean value ± standard deviation) reached assessment using Duncan's multiple range test at a significance level of $p < 0.05$ (Gomez and Gomez1984). The estimates of phenotypic variances (σ^2p) came from the variance of the red ginger mutant population, while the environmental variance (σ^2e) estimation from the variance of the red ginger wild-type population. The genotypic variance (σ^2g) estimates employed the formula of $\sigma^2g = \sigma^2p - \sigma^2e$ (Singh and Chaudhary, 1979).

Table 1. List of RAPD primers used in this study.

No	Primer name	Sequence (5'-3')	References
1	OPF-01	ACGGATCCTG	Reflinur *et al*. (2022)
2	OPF-04	GGTGATCAGG	Reflinur *et al*. (2022)
3	OPA-03	AGTCAGCCAC	Al-Saghir and Abdel-Salam (2015)
4	OPA-04	AATCGGGCTG	Al-Saghir and Abdel-Salam (2015)
5	OPA-07	GAAACGGGTG	Al-Saghir and Abdel-Salam (2015)
6	OPA-011	CAATCGCCGT	Al-Saghir and Abdel-Salam (2015)
7	OPP-08	ACATCGCCCA	Reflinur *et al*. (2022)
8	OPP-09	GTGGTCCGCA	Reflinur *et al*. (2022)
9	OPE-06	AAGACCCCTC	Reflinur *et al*. (2022)
10	OPE-20	AACGGTGACC	Reflinur *et a*l. (2022)

The broad sense heritability (h^2bs) approximation used the formula of $h^2bs = \sigma^2g/\sigma^2p$, with the criteria determined as, i.e., high (≥50%), medium (20% to 50%), and low (<20%) (Singh and Chaudhary, 1979; Stansfield, 1991). Obtaining the coefficient of genetic variability (CGV) estimates utilized the genetic variance value with criteria determined as, i.e., wide (σ2g > 2σ2g) and narrow (σ2g < 2σσ2g) (Asghar and Mehdi, 2010).

The Pearson correlation, principal component (PCA), and cluster gram analyses through heatmap continued using R 4.1.2. The molecular analysis scored the amplicon band obtained from agarose gel electrophoresis. Each visible band gained a score of 1, while the invisible band scored 0. In contrast, the unamplified sample received a score of 9, a considered missing data and results in binary data. The scoring data analysis then engaged the Sequential Agglomerative Hierarchical and Nested (SAHN)-UPGMA (Unweighted Pair-Group Method with Arithmetic) program on NTSYS 2.1, presented as a phylogenetic tree (Rohlf, 2000). Meanwhile, the central allele frequency, gene diversity, observed heterozygosity, and Polymorphic Information Content (PIC) estimations employed the Power Marker 3.25 (Liu and Muse, 2005).

RESULTS AND DISCUSSION

Morphological traits

The NaN3 significantly affected morphological traits of red ginger mutants with varying effects on tiller number, leaf length, width, and stem diameter across treatments (Table 2). Plants treated with NaN3 at 100, 300, and 500 mg l^{-1} were notably shorter than the control, with the 300 mg l^{-1} concentration showing the greatest reductions in height, leaf length, tiller number, and rhizome weight. These reductions were significant compared with the control and 500 mg l^{-1} but not different from the 100 mg l^{-1} treatment. Stem diameter varied significantly between the 300 and 500 mg l^{-1}, with the smallest diameter at 500 mg l^{-1}. Similar results were notable in rice (Dewi *et al*., 2016) and chili peppers (Yafizham and Herwibawa, 2018). NaN3 treatments reduced rhizome weight in red ginger mutants compared with the control, with yields ranging from 450 to 1500 g $plant^{-1}$ (Soeparjono, 2016; Azizah *et al*., 2019). In contrast, Srivastava *et al*. (2011) found 200 mg l^{-1} NaN3 improved yield in wheat, suggesting species-specific responses to NaN3. In the M1V2 generation, the reduction may stem from higher NaN3 concentrations and prolonged exposure (24 hours). NaN3, a strong mutagen, induces mutations, but stability may only emerge in later generations (M1V3, M1V4). Optimizing NaN3 concentration and exposure in mutation breeding is essential to improve yield traits (Laskar *et al*., 2018; Goyal *et al*., 2021).

Rhizome length and width showed no considerable variation, except thickness, which was most reduced at 500 mg l^{-1} of NaN3. This contrasts with Raina *et al*. (2022), who found remarkable growth and yield changes with NaN3. The inhibitory effect in the M1V2 generation could result from enzymatic disruption, mitotic interference, and direct gene alterations by NaN3 (Liamngee *et al*., 2017).

Table 2. Plant growth parameters and rhizome attributes of red ginger derived from sodium azide treatments.

Treatments	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Tiller number	Stem diameter (cm)	Rhizome weight (g)	Rhizome length (cm)	Rhizome width (cm)	Rhizome thickness (cm)
Control	79.17 a	27.24 a	2.64 a	27.11 a	0.60 ab	200.67 a	20.00 a	8.55 a	2.02 a
100 mg^{l-1}	50.33 bc	18.17 bc	1.76 c	17.00 b	0.58 ab	116.67 b	17.44 a	6.63 a	1.77 a
300 mg^{l-1}	41.55 c	17.14 c	1.82 bc	14.22 b	0.68 a	84.56 b	16.50 a	6.5 0 a	1.89 a
500 mg^{l-1}	55.5 b	20.85 b	2.14 b	27.33 b	0.57 b	97.33 b	15.44 a	5.94 a	1.34 b

Table 3. Pearson correlation coefficient among the quantitative characters in red ginger mutant populations.

Characters	Plant height	Leaf number	Leaf length	Leaf width	Tiller number	Stem diameter	Rhizome weight	Rhizome length	Rhizome width	Rhizome thickness
Plant height	1.00									
Leaf number	0.87**	1.00								
Leaf length	0.81**	0.73**	1.00							
Leaf width	0.73**	0.68**	0.93**	1.00						
Tiller number	0.53**	0.54**	0.29	0.34**	1.00					
Stem diameter	0.57**	0.49**	0.65**	0.57**	-0.07	1.00				
Rhizome weight	0.48**	0.41**	0.43**	0.49**	0.46**	0.07	1.00			
Rhizome length	0.54**	0.52**	0.55**	0.59**	0.44**	0.34**	0.74**	1.00		
Rhizome width	0.50**	0.44**	0.45**	0.50**	0.35**	0.32**	0.76**	0.83**	1.00	
Rhizome thickness	0.55**	0.49**	0.60**	0.63**	0.35**	0.34**	0.66**	0.87**	0.73**	1.00

This study showed no substantial improvement in red ginger's desirable yield traits (rhizome weight and size), suggesting the need for more effective mutation methods. Previous research with gamma irradiation in ginger also reported mixed results, with some cultivars responding positively and others showing no improvement or even decreases (Bermawie *et al*., 2015; Abdullah *et al*., 2018). Since this study focused on the Jahira-2 cultivar, mutation outcomes could vary across cultivars, underscoring the importance of cultivar selection, mutation method, mutagen dose, and treatment duration.

In mutation breeding, analyzing trait correlations is a key to understanding yield-related attributes, given the yield's complex polygenic inheritance (Raina *et al*., 2022). Pearson's correlation analysis showed significant correlations between most quantitative traits and rhizome characteristics (weight, length, width, and thickness), except for stem diameter, which did not correlate with rhizome weight. Leaf number, leaf length, plant height, and leaf width correlated with rhizome traits, while a negative correlation appeared between stem diameter and tiller number (Table 3). Previous studies linked plant height, tillers, leaf area, stem girth, rhizome traits, and oil content with fresh rhizome yield in ginger (Ravi *et al*., 2017; Anargha *et al*., 2020).

This research found genetic variability in morphological traits, with coefficients of variation offering more precision than range estimates (Dev and Sharma, 2022). NaN3 treatments (100, 300, and 500 mg l^{-1}) increased variability in plant height, as well as, 100 and 500 mg l^{-1} doses enhancing the variability in rhizome width. However, variations remained limited for leaf number, size, tiller number, stem diameter, rhizome weight, length, and thickness. High broad-sense heritability was evident for plant height and rhizome width, consistent with CGV results (Table 4), supporting targeted breeding strategies (Saha *et al*., 2019).

Principal component analysis (PCA) identified 11 components in red ginger mutants, with four having eigenvalues over 1 (Table 5). PC1 (eigenvalue 3.61) explained 32.82% of the variability, primarily influenced by rhizome length, width, weight, and thickness (Figure 1). PC2 (eigenvalue 2.61) contributed 23.69%, driven by leaf length, width, tiller number, and stem diameter. PC3 (eigenvalue 1.88, 17.07%) consisted of plant height and leaf number, while PC4 (eigenvalue 1.02, 9.25%) reflected stem shape. Multivariate analysis showed similar traits contributed to a variation in ginger germplasm (Kumar *et al*., 2016), with leaf number showing the highest variability (45.39%) across components.

Table 4. Estimating variances, broad sense heritability, and coefficient of genetic variability of the quantitative characters of red ginger mutants treated by sodium azide.

Variance components	Sodium azide treatments		
	100 mg^{l-1}	300 mg^{l-1}	500 mg^{l-1}
	Plant height		
σ^2p	190.16	86.04	129.80
σ^2g	128.21	24.09	67.86
2 $\sigma_{\sigma^2 g}$	27.58	18.55	22.79
h^2bs (%)	67.42^{H}	28.00^{M}	52.28^{H}
CGV	18.50^{W}	8.55^{W}	12.21^{W}
	Leaf number		
σ^2p	7.61	4.40	10.67
σ^2g	0.32	0.00	3.38
2 $\sigma_{\sigma^2 g}$	5.52	4.19	6.53
h^2bs (%)	4.20^{L}	0.00^{L}	31.66^{M}
CGV	3.96^{N}	0.00^{N}	10.02^{N}
	Leaf length		
σ^2p	3.24	10.37	6.53
σ^2g	0.00	0.00	0.00
2 $\sigma_{\sigma^2 g}$	3.60	6.44	5.11
h^2bs (%)	0.00^{L}	0.00^{L}	0.00^{L}
CGV	0.00^{N}	0.00^{N}	0.00^{N}
	Leaf width		
σ^2p	0.07	0.03	0.03
σ^2g	0.00	0.00	0.00
2 $\sigma_{\sigma^2 g}$	0.53	0.36	0.34
h^2bs (%)	0.00^{L}	0.00^{L}	0.00^{L}
CGV	0.00^{N}	0.00^{N}	0.00^{N}
	Tiller number		
σ^2p	27.96	51.06	50.44
σ^2g	0.00	0.00	0.00
2 $\sigma_{\sigma^2 g}$	10.58	14.29	14.20
h^2bs (%)	0.00^{L}	0.00^{L}	0.00^{L}
CGV	0.00^{N}	0.00^{N}	0.00^{N}
	Stem diameter		
σ^2p	0.006	0.02	0.01
σ^2g	0.00	0.00	0.00
2 $\sigma_{\sigma^2 g}$	0.15	0.29	0.23
h^2bs (%)	0.00^{L}	0.00^{L}	0.00^{L}
CGV	0.00^{N}	0.00^{N}	0.00^{N}
	Rhizome weight		
σ^2p	4500.36	967.80	2934.22
σ^2g	0.00	0.00	0.00
2 $\sigma_{\sigma^2 g}$	134.17	62.22	108.34
h^2bs (%)	0.00^{L}	0.00^{L}	0.00^{L}
CGV	0.00^{N}	0.00^{N}	0.00^{N}
	Rhizome length		
σ^2p	11.58	4.94	74.47
σ^2g	5.93	0.00	68.82
2 $\sigma_{\sigma^2 g}$	6.81	4.44	17.26
h^2bs (%)	51.21^{H}	0.00^{L}	92.41^{H}
CGV	13.96^{N}	0.00^{N}	53.71^{H}

σ^2p: phenotype variance, σ^2g: genotype variance, h^2bs: broad sense heritability, CGV: coefficient of genetic variability, H: high, M: medium, L: low, W: wide, N: narrow.

Table 4. (cont'd.)

Variance components	Sodium azide treatments		
	100 mg^{l-1}	300 mg^{l-1}	500 mg^{l-1}
	Rhizome width		
σ^2p	9.63	3.69	17.86
σ^2g	6.74	0.80	14.97
2 $\sigma_{\sigma}{}^2{}_g$	6.21	3.84	8.45
h^2bs (%)	70.00^H	21.66^M	83.82^H
CGV	38.68^W	17.02^N	65.85^W
	Rhizome thickness		
σ^2p	0.00	0.00	0.00
σ^2g	0.00	0.00	0.00
2 $\sigma_{\sigma}{}^2{}_g$	0.00	0.00	0.00
h^2bs (%)	0.00^L	0.00^L	0.00^L
CGV	0.00^N	0.00^N	0.00^N

σ^2p: phenotype variance, σ^2g: genotype variance, h^2bs: broad sense heritability, CGV: coefficient of genetic variability, H: high, M: medium, L: low, W: wide, N: narrow.

Table 5. Eigenvalues, variances, and cumulative variances contributed by principal components observed for various morphological traits.

Principal component (PC)	Eigenvalue	Variances (%)	Cumulative variances (%)
PC 1	3.61	32.82	32.82
PC 2	2.61	23.69	56.51
PC 3	1.88	17.07	73.58
PC 4	1.02	9.25	82.83
PC 5	0.49	4.42	87.26
PC 6	0.41	3.71	90.97
PC 7	0.34	3.10	94.07
PC 8	0.27	2.42	96.49
PC 9	0.16	1.47	97.96
PC 10	0.13	1.20	99.16
PC11	0.09	0.84	100

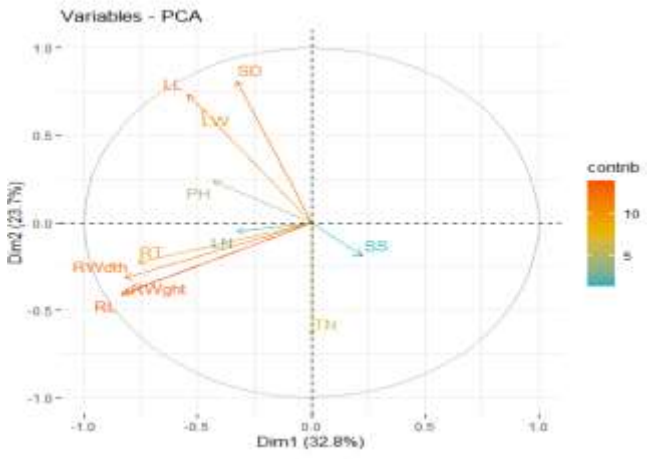

Figure 1. Principal component analysis (PCA) of the morphological traits observed in this study. SD: stem diameter, LL: leaf length, LW: leaf width, PH: plant height, LN: leaf number, SS: stem shape, TN: tiller number, RT: rhizome thickness, RL: rhizome length, RWdth: rhizome width, and RWght: rhizome weight.

Clustergram analysis using a heatmap divided red ginger mutants into two main clusters (Figure 2). Nine treatment groups (100[3], 100[4], 100[8], 300[2], 300[4], 300[6], 300[9], 500[2], and 500[9]) formed the second cluster, showing greater genetic variation than the control, indicating NaN3-induced variability. Genotypes 100[2], 100[5], and 100[6] grouped with the control, reflecting higher similarity. The heatmap also showed correlations among morphological traits, including leaf length, leaf number, rhizome length, tiller number, rhizome width, leaf width, rhizome thickness, stem shape, and diameter clustering. Plant height correlated with rhizome weight, suggesting plant stature selection could enhance rhizome yield.

6-gingerol content

The 6-gingerol is a primary bioactive compound found in ginger. The health benefits of ginger largely depend on its quality, particularly the concentration of bioactive compounds like 6-gingerol. High levels of 6-gingerol in the ginger rhizome can reduce the raw material needed in herbal products, making production more cost-effective.

Study results revealed at the control, the 6-gingerol content was 2.29% and increased to 6.02% at 100 mg l^{-1} of NaN3 treatment. However, the values decreased at 300 and 500 mg l^{-1} of NaN3 treatments, i.e., 2.26% and 3.27%, respectively. The 6-gingerol content at 100 mg l^{-1} exceeds the 4%–5% content, as also reported by Azizah *et al*. (2019).

Chemical mutagens like NaN3 typically cause base pair substitutions, particularly converting G-T to A-T, leading to amino acid variations altering protein functions without eliminating them (Rao and Rao, 1983). The exact mechanism remains unknown, but the NaN3 likely interfered with plant genes. The presented study suggested NaN3 can enhance the gingerol content in red ginger. Other studies have demonstrated NaN3's effectiveness in improving oleate in peanuts (Wang *et al*., 2011) and enhancing nutritional density in cowpeas (Raina *et al*., 2022). However, its application in ginger still needs further reporting.

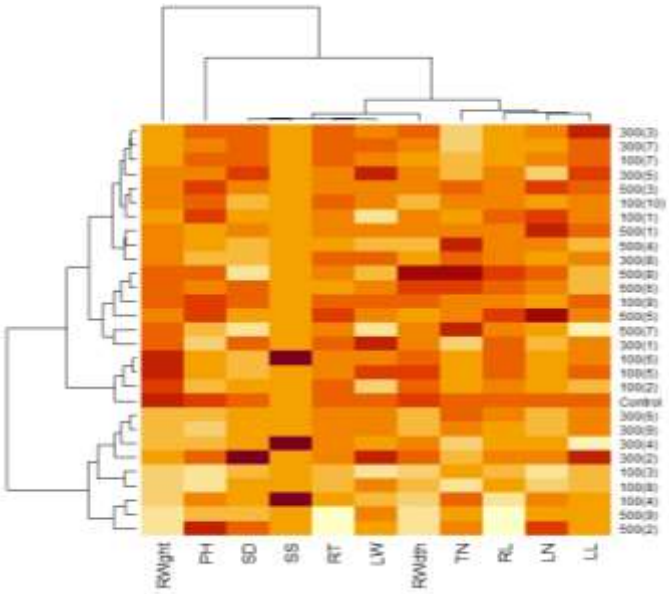

Figure 2. Clustergram analysis using the heatmap method based on morphological characters observed in this study. SD: stem diameter, LL: leaf length, LW: leaf width, PH: plant height, LN: leaf number, SS: stem shape, TN: tiller number, RT: rhizome thickness, RL: rhizome length, RWidth: rhizome width, and RWght: rhizome weight.

RAPD analysis

The RAPD markers generated 75 amplicon bands in this study, including 63 polymorphic bands (Table 6). This total was lower than the 196 bands, with 171 polymorphic bands in 80 ginger accessions, using 26 RAPD primers detected by Baruah *et al*. (2019). It was fewer than the polymorphic bands in 27 ginger genotypes with 30 RAPD markers reported by Akshitha *et al*. (2022). However, this study outperformed Mia *et al*. (2014), who identified total and polymorphic bands in 24 ginger genotypes using only three RAPD markers. Variations in the number of genotypes, markers used, and genetic background can influence the total and polymorphic band counts in studies. In the presented investigations, the obtained major allele frequency ranged from 0.33 (OPP-09) to 0.50 (OPF-04, OPA-07, OPA-11, OPA-04, and OPE-06), with an average of 0.46 (Table 6). The major allele frequency ranged from 0.33 (OPP-09) to 0.50 (multiple markers), averaging 0.46, indicating alleles present in over 50% of genotypes (Table 6).

In the timely study, the gene diversity values ranged from 0.54 (OPF-01) to 0.84 (OPE-20), with an average of 0.72, while the PIC values ranged from 0.43 (OPF-01) to 0.83 (OPE-20), averaging 0.68. The markers used exhibited a higher average PIC than those reported by Baruah *et al*. (2019) and Akshitha *et al*. (2022). The PIC value indicates a marker's ability to detect polymorphism among individuals, serving as an important indicator of marker quality in genetic studies (Serrote *et al*., 2020). Nine RAPD markers demonstrated high informativeness levels with PIC values greater than 0.5 and authenticated them as applicable for future selection in red ginger breeding programs (Botstein *et al*., 1980).

Phylogenetic analysis revealed red ginger mutants clustered into three major groups with a similarity coefficient of 0.62 (Figure 3), with the control genotype grouped in the first cluster alongside six mutant genotypes treated with NaN3 (100 mg l^{-1}). The second cluster separated from the control and included a mix of mutant genotypes with NaN3 treatments (100, 300, and 500 mg l^{-1}). The third cluster showed the widest separation from the control, predominantly comprising the ginger genotypes with NaN3 (300 mg l^{-1}). The cluster also included mutants from the NaN3 treatments (100 and 500 mg l^{-1}), consistent with the previous morphological cluster analysis.

The genetic similarity matrix indicated genotypes 300(1) and 300(7) had the lowest genetic similarity to the control (59% and 60%, respectively), implying significant genetic variations. Genotypes 100(9) and 500(1) also exhibited low similarity at 62%. These NaN3-induced mutants were like those reported in Dendrobium, where mutants showed admixture in the treatments (Wannajindaporn *et al*., 2016; Setiawan *et al*., 2022).

Table 6. Polymorphism statistic of RAPD markers used in red ginger mutants.

Markers	Total band number	Polymorphic band number	Major Allele Frequency	Gene Diversity	PIC
OPF-01	5	3	0.48	0.54	0.43
OPF-04	6	3	0.50	0.69	0.65
OPA-07	5	3	0.50	0.70	0.67
OPA-11	8	8	0.50	0.64	0.58
OPA-03	11	11	0.44	0.79	0.78
OPP-08	12	12	0.48	0.76	0.75
OPA-04	8	8	0.50	0.74	0.74
OPP-09	7	5	0.33	0.81	0.79
OPE-20	7	7	0.37	0.84	0.83
OPE-06	6	3	0.50	0.67	0.63
Total	75	63			
Average	7.5	6.3	0.46	0.72	0.68

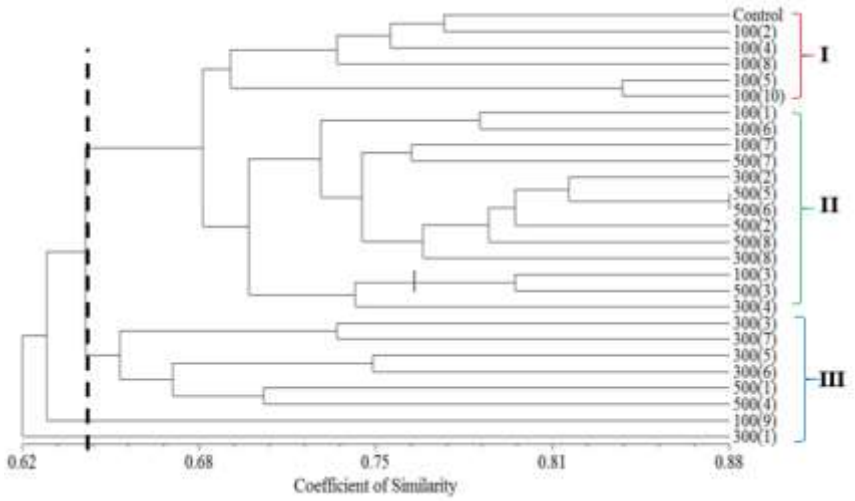

Figure 3. The phylogenetic tree from red ginger mutants used in this study, constructed using the SAHN-UPGMA method.

CONCLUSIONS

NaN3 treatments considerably improved genetic variability in M1V2 mutants in plant height and rhizome width, as indicated by the higher values of CGV and broad-sense heritability. All the quantitative traits, except stem diameter, significantly correlated with rhizome fresh weight. PCA identified 11 components, with four explaining 82.83% of the total variability, and leaf number exhibited the highest variability (45.39%) among these components. The NaN3 treatment at 100 mg l^{-1} reduced the rhizome weight in M1V2 but increased 6-gingerol content. These findings can be beneficial for the further improvement in red ginger through breeding.

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