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Genetic Diversity Analysis of Sodium Azide-Induced Mutants of Ornamental Banana (*Musa ornata* Roxb.) Using ISSR Markers

Nattapong Srisamoot

Department of Biotechnology, Faculty of Agricultural Technology, Kalasin University, Mueang Kalasin, Kalasin, Thailand

E-mail: nattapong2.sr@ksu.ac.th

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Abstract

Chemical mutagenesis is widely used to generate genetic variation in clonally propagated crops where conventional hybridization is limited. This study evaluated the mutagenic effects of sodium azide (NaN_3) on *Musa ornata* Roxb. and assessed the resulting genetic diversity using inter simple sequence repeat (ISSR) markers. In vitro–propagated plantlets were treated with NaN_3 concentrations ranging from 0.1 to 4.0 mM, and survival responses were used to determine optimal mutagenic doses. Plant survival decreased linearly with increasing NaN_3 concentration ($y = -20.92x + 93.59$, $R^2 = 0.938$), with estimated LD_{30} and LD_{50} values of 1.13 mM and 2.08 mM, respectively. Sublethal NaN_3 treatments induced measurable morphological variation in regenerated plantlets, including changes in fresh weight, leaf number, leaf length, and root development. Molecular analysis using ten ISSR primers generated 81 scorable ISSR bands, of which 36 were polymorphic (43.02%). Genetic diversity indices indicated moderate variation among mutants (PIC = 0.28, Nei's gene diversity = 0.24, Shannon's index = 0.34). Cluster analysis based on UPGMA grouped the plantlets into three major genetic clusters, with pairwise genetic dissimilarities ranging from 34.7% to 68.4%. The clustering pattern showed partial correspondence with observed morphological variation, suggesting that NaN_3-induced genomic changes contributed to phenotypic diversification. These findings demonstrate that the combination of *in vitro* sodium azide mutagenesis and ISSR marker analysis is an

effective approach for generating and identifying novel variation in *M. ornata*. The resulting lines represent valuable genetic resources for the improvement of ornamental bananas through mutation breeding.

Keywords: *Musa ornata,* sodium azide, ISSR markers, mutation breeding, genetic diversity

Introduction

Ornamental bananas (*Musa ornata* Roxb.) are widely appreciated for their bright pink bracts, upright inflorescences, and compact growth habit, making them popular ornamental plants for landscapes and container gardening (Horsburgh & Noller, 2005; Karunakaran et al., 2024). Despite their horticultural value, genetic improvement of ornamental bananas remains challenging because many *Musa* species exhibit sterility, parthenocarpy, and low seed production, which restrict conventional hybridization and limit the generation of new cultivars with improved ornamental traits (Lamo et al., 2017). Consequently, alternative approaches capable of generating novel genetic variation are essential for the breeding and improvement of these vegetatively propagated plants.

Mutation breeding has emerged as an effective strategy for generating genetic variation in clonally propagated crops, where conventional hybridization is often limited. Chemical mutagens such as sodium azide (NaN_3) are particularly effective because they primarily induce point mutations through base substitutions while causing relatively limited chromosomal damage (Al-Quariny & Khan, 2009; Khan et al., 2009). Previous studies in several crops and ornamentals have shown that NaN_3 treatments can generate useful phenotypic variation, including reduced plant height, altered leaf morphology, and changes in pigmentation—traits that are highly desirable for ornamental plant development (Adeosun et al., 2020; Ganesan et al., 2005). In many species, moderate mutagenic doses produce the most useful mutants because they induce sufficient genomic alterations while maintaining plant survival and regeneration capacity (Abdullah et al., 2009; Adeosun et al., 2020).

At the molecular level, induced mutations are commonly verified using DNA marker systems. Among them, inter simple sequence repeat (ISSR) markers are widely used for detecting genome-wide polymorphisms due to their high reproducibility, simplicity, and ability to reveal variation without prior genomic information (Bornet & Branchard, 2001). ISSR analysis has successfully been applied to evaluate genetic diversity in various crops and ornamentals, including banana mutants and other vegetatively propagated species (Khatri et al., 2011; Ramzan et al., 2020). Typical polymorphism levels reported in mutagenized plant populations range from approximately 40–70%, reflecting moderate to substantial genomic variation generated through induced mutagenesis (Beyramizadeh et al., 2023; Hamdan, 2024).

Despite these advances, a clear knowledge gap remains regarding chemical mutagenesis and molecular verification of induced diversity in *Musa ornata*. While mutation breeding and molecular marker analyses have been conducted in several *Musa* species and ornamental plants, studies specifically combining sodium azide mutagenesis with ISSR-based diversity analysis in *M. ornata* are still lacking. As a result, the extent to which NaN_3 can generate measurable genetic variation in this ornamental banana species remains unclear.

Therefore, this study aimed to evaluate the mutagenic effects of NaN_3 on *in vitro*–propagated *M. ornata* and to characterize the resulting genetic diversity using ISSR markers. Two testable hypotheses were proposed: (1) increasing NaN_3 concentration would significantly affect plant survival and produce measurable genetic polymorphism detectable by ISSR markers; and (2) moderate (sublethal) NaN_3 concentrations would generate higher levels of genetic diversity indices compared with very low or highly toxic doses.

By integrating *in vitro* mutagenesis with molecular marker analysis, this research provides a framework for detecting and selecting novel mutants in ornamental bananas and contributes to the development of improved cultivars in vegetatively propagated crops.

Materials and methods

Plant materials and experimental design

In vitro plantlets of ornamental banana (*Musa ornata* Roxb.) were used as plant materials in this study. The plantlets were maintained in the Plant Tissue Culture Laboratory under controlled environmental conditions prior to mutagenesis treatment. Mutation breeding using chemical mutagens is widely applied in clonally propagated crops to generate genetic variability when conventional hybridization is limited (Amano, 2004; Lamo et al., 2017).

The experiment was arranged in a completely randomized design (CRD). Seven NaN_3 concentrations were tested: 0.0 (control), 0.1, 0.5, 1.0, 2.0, 3.0, and 4.0 mM. For each treatment, 30 explants were used, and the experiment was repeated three independent experimental runs, resulting in 90 explants per treatment.

The experimental unit for survival analysis was defined as an individual plantlet. For molecular analysis, ten individual mutant lines per treatment were randomly selected for ISSR fingerprinting.

Culture medium and growth conditions

Plantlets were cultured on Murashige and Skoog (MS) basal medium containing full-strength MS salts and vitamins supplemented with 30 g L^{-1} sucrose, 2.0 mg L^{-1} 6-benzylaminopurine (BAP), 0.5 mg L^{-1} naphthaleneacetic acid (NAA), and solidified with 7 g L^{-1} agar. The pH of the medium was adjusted to 5.8 ± 0.02 prior to autoclaving at 121 °C for 20 min. The cultures were maintained in a growth room at 25 ± 2 °C under a 16 h light/8 h dark photoperiod with a light intensity of approximately 40–50 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. Murashige and Skoog medium is widely used as a standard nutrient medium for plant tissue culture due to its balanced macro- and micronutrient composition supporting plant regeneration and growth (Murashige & Skoog, 1962).

Sodium azide mutagenesis treatment

Sodium azide solutions were prepared at concentrations of 0.0 (control), 0.1, 0.5, 1.0, 2.0, 3.0, and 4.0 mM using sterile distilled water. The pH of the mutagen solution was adjusted to 3.0 using phosphate buffer, which is known to enhance NaN_3 mutagenic activity. The pH was verified both before and after NaN_3 addition using a digital pH meter to ensure stability during treatment. Sodium azide is metabolically

activated in plant cells and induces mutations primarily through base substitutions during DNA replication (Owais & Kleinhofs, 1988; Velemínský & Angelis, 1987).

In vitro plantlets were immersed in the NaN_3 solutions for 1 hour with gentle shaking. After treatment, the explants were thoroughly rinsed three times with sterile distilled water to remove residual mutagen and then transferred to fresh MS medium for recovery and growth.

Survival assessment and LD_{30}–LD_{50} estimation

Plant survival was evaluated four weeks after mutagen treatment, when plantlets had resumed growth and new leaves were visible. Survival percentage was calculated as the number of surviving plantlets divided by the total number of treated plantlets multiplied by 100.

To estimate the lethal doses causing 30% and 50% mortality (LD_{30} and LD_{50}), survival data were subjected to regression analysis between NaN_3 concentration and survival percentage. The regression equation and coefficient of determination (R^2) were obtained using Microsoft Excel statistical analysis tools. The LD_{30} and LD_{50} values were determined from the regression equation by substituting survival values of 70% and 50%, respectively. Determination of LD values based on survival response is commonly used in mutation breeding studies to identify optimal mutagen doses that balance mutagenic effectiveness and plant survival (Mostafa, 2011; Vinithashri et al., 2020).

Morphological screening of mutant plantlets

Morphological responses of *M. ornata* plantlets were evaluated eight weeks after NaN_3 treatment to determine the effects of mutagen concentration on plant growth and development. Surviving plantlets from each treatment were carefully removed from the culture vessels, gently rinsed to remove residual medium, and subjected to morphological measurements.

The evaluated traits included fresh weight (g), leaf number, leaf length (cm), root number, and root length (cm). Fresh weight of each plantlet was measured using a digital analytical balance. Leaf number was determined by counting fully expanded leaves per plantlet, while leaf length was measured from the leaf base to the leaf tip using a digital ruler. Root number was counted as the total number of visible roots per plantlet, and root length was measured as the length of the longest root.

All measurements were recorded for individual plantlets, and the mean values for each treatment were calculated. The data were expressed as mean ± standard deviation (SD). Statistical differences among NaN_3 treatments were analyzed using analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at the 0.05 probability level to compare treatment means. The coefficient of variation (CV) was also calculated to evaluate the variability among measured traits.

Plantlets exhibiting noticeable morphological deviations compared with the untreated control, such as altered leaf size, changes in biomass accumulation, or differences in root development, were considered putative mutants and were selected for subsequent molecular analysis using ISSR markers.

DNA extraction and ISSR-PCR amplification

Genomic DNA was extracted from young leaves of selected plantlets using the CTAB extraction method. DNA concentration and purity were determined using a NanoDrop spectrophotometer, and DNA integrity was verified by electrophoresis on 1% agarose gel. A total of ten ISSR primers were used to analyze

genetic variation among NaN_3-induced mutants. ISSR markers are widely used in plant genetic diversity studies due to their reproducibility and ability to amplify multiple bands without prior sequence information (Bornet & Branchard, 2001).

PCR amplification was carried out in a 25 µL reaction mixture containing 1× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM ISSR primer, 1 U *Taq* DNA polymerase, and 50 ng template DNA. The amplification was performed in a thermal cycler with an initial denaturation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 45 s, annealing at 50–55 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. The amplified products were separated by electrophoresis on 2% agarose gel in 1× TBE buffer and visualized using a gel documentation system. To ensure reproducibility, each primer was amplified in two independent PCR runs, and only bands that consistently appeared in both replicates were recorded for subsequent analysis.

Data scoring and genetic diversity analysis

Clear and reproducible DNA bands were scored as binary data, where the presence of a band was recorded as 1 and the absence of a band as 0. Genetic diversity parameters were calculated to evaluate the level of polymorphism among the analyzed genotypes. The polymorphic information content (PIC) was calculated as PIC = 2f(1 – f), where *f* represents the frequency of band presence. Nei's gene diversity (h) was calculated as $h = 1 - \sum p_i^2$, while Shannon's information index (I) was calculated as $I = -\sum p_i \ln(p_i)$. Genetic diversity parameters (PIC, Nei's gene diversity, and Shannon's index) were calculated using Microsoft Excel based on binary marker data. These indices are commonly used in molecular diversity studies to quantify genetic variability within and among populations (Ramzan et al., 2020; Yang et al., 2023).

Genetic similarity among genotypes was calculated using the Jaccard similarity coefficient. Cluster analysis was performed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm, and the resulting matrix was used to construct a phylogenetic dendrogram for NaN_3-treated *M. ornata* populations using the online dendrogram construction utility DendroUPGMA (http://genomes.urv.es/UPGMA/) (Garcia-Vallvé et al., 1999).

Results and discussion

Effect of sodium azide on survival rate and LD estimation

Exposure of *M. ornata* plantlets to increasing concentrations of sodium azide resulted in a progressive reduction in survival rate (Figure 1). Survival declined from 100 ± 0.0% in the control to complete mortality at 4.0 mM NaN_3. Intermediate concentrations showed gradual reductions in viability, with mean survival values of 86.3 ± 5.8%, 75.1 ± 6.2%, 66.4 ± 4.9%, and 52.8 ± 5.5% at 0.5, 1.0, 2.0, and 3.0 mM NaN_3, respectively. The presence of standard deviation (SD) indicates moderate variability among experimental replicates.

Regression analysis demonstrated a strong negative relationship between NaN_3 concentration and plantlet survival, described by the equation, $y = -20.92x + 93.59$ ($R^2 = 0.938$), indicating that nearly 94% of survival variation was explained by mutagen concentration. From this regression model, the lethal dose

values were estimated as LD_{30} = 1.13 mM and LD_{50} = 2.08 mM, corresponding to concentrations predicted to cause approximately 30% and 50% mortality, respectively.

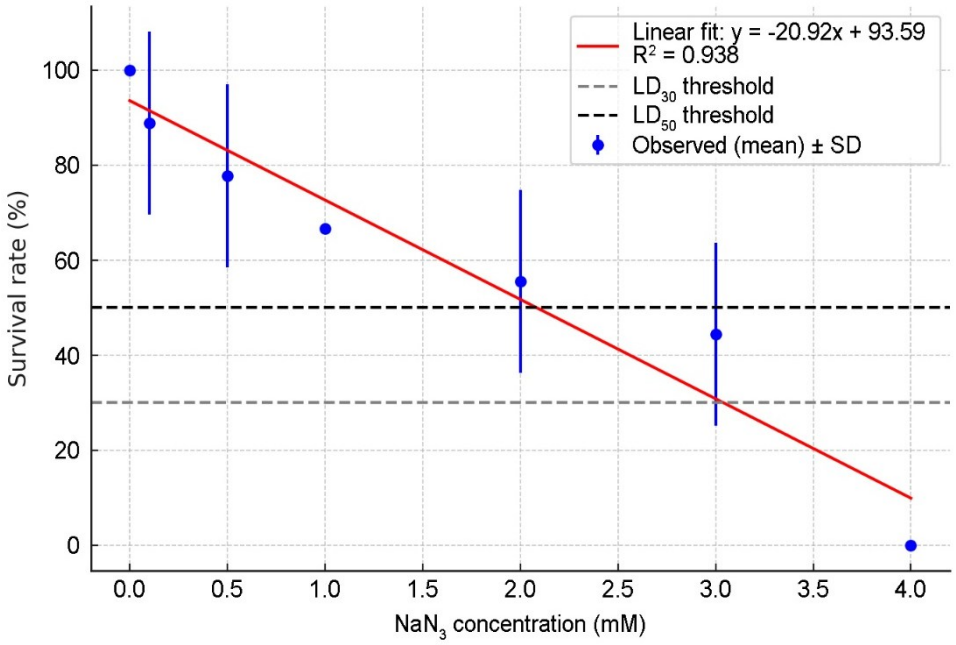


Figure 1 Survival response of *M. ornata* plantlets exposed to different concentrations of NaN_3. Survival percentage was recorded four weeks after mutagen treatment. Error bars represent standard deviation (SD) of three independent experimental runs (n = 30 explants per treatment per run). The regression line describes the relationship between NaN_3 concentration and plant survival, and was used to estimate the lethal dose causing 30% and 50% mortality (LD_{30} and LD_{50}).

A dose-dependent decline in survival following NaN_3 exposure has been widely documented in various crops including maize, sunflower, and orchids, where increasing mutagen concentration leads to increased cytotoxicity and reduced regeneration capacity (Dahiru et al., 2021; Mostafa, 2011; Wannajindaporn et al., 2016). Sodium azide is known to act as a metabolic mutagen that is converted intracellularly into azidoalanine, which interferes with DNA replication fidelity and cellular respiration (Owais & Kleinhofs, 1988). Such biochemical interference leads to point mutations while limiting large chromosomal aberrations, making NaN_3 suitable for mutation breeding programs in clonally propagated crops.

Morphological responses of NaN_3-treated plantlets

Morphological traits of *Musa ornata* plantlets were evaluated eight weeks after NaN_3 treatment to determine the effects of mutagen concentration on plant growth and development (Table 1). Significant differences among treatments were observed for fresh weight, leaf number, leaf length, root number, and root length (F-test, $p < 0.05$), indicating that NaN_3 exposure influenced several morphological characteristics of the regenerated plantlets.

Fresh weight varied significantly among treatments, ranging from 12.71 to 14.06 g. The highest fresh weight was recorded at 0.1 mM NaN_3 (14.06 ± 0.36 g), which was significantly higher than most other treatments, while slightly lower values were observed in the control (13.01 ± 0.79 g) and in plantlets treated

with 0.5–1.0 mM NaN_3 (12.71–13.09 g). This increase in biomass at a low mutagen concentration may indicate a mild stimulatory response to low-level stress, whereas higher concentrations tended to reduce growth performance. Similar responses have been reported in other NaN_3-treated crops where moderate mutagenic stress resulted in minor growth stimulation before toxic effects became dominant (Adeosun et al., 2020; Türkoğlu et al., 2023).

Leaf number ranged from 2.98 to 3.92 leaves per plantlet. The highest leaf number was observed at 0.1 mM NaN_3 (3.92 ± 0.08), while treatments of 0.5 and 1.0 mM resulted in significantly fewer leaves (3.02 ± 0.43 and 2.98 ± 0.00, respectively). Leaf length showed a similar trend, with the longest leaves recorded in the control treatment (9.89 ± 0.90 cm), followed by 0.1 mM NaN_3 (9.19 ± 0.37 cm). In contrast, plantlets treated with 0.5–2.0 mM NaN_3 exhibited significantly shorter leaves (approximately 8.15–8.29 cm). Such reductions in leaf size have been reported as common morphological responses to chemical mutagenesis due to disruptions in cell division and elongation processes (Ganesan et al., 2005; Ahmad et al., 2007).

Table 1 Morphological traits of *M. ornata* plantlets eight weeks after NaN_3 treatment

NaN_3 (mM)	Fresh weight (g)	Leaf number	Leaf length (cm)	Root number	Root length (cm)
0.0	13.01 ± 0.79 ^{b}	3.68 ± 0.85 ^{ab}	9.89 ± 0.90 ^{a}	5.18 ± 0.15	3.21 ± 0.68 ^{ab}
0.1	14.06 ± 0.36 ^{a}	3.92 ± 0.08 ^{a}	9.19 ± 0.37 ^{ab}	4.48 ± 0.46	4.41 ± 0.85 ^{ab}
0.5	12.71 ± 0.13 ^{b}	3.02 ± 0.43 ^{b}	8.17 ± 0.09 ^{c}	4.65 ± 0.95	3.85 ± 0.78 ^{ab}
1.0	13.09 ± 0.42 ^{b}	2.98 ± 0.00 ^{b}	8.29 ± 0.24 ^{c}	5.22 ± 0.59	2.99 ± 0.89 ^{b}
2.0	13.39 ± 0.48 ^{ab}	3.54 ± 0.65 ^{ab}	8.15 ± 0.36 ^{c}	5.59 ± 0.98	4.59 ± 0.85 ^{a}
3.0	13.56 ± 0.58 ^{ab}	3.83 ± 0.43 ^{ab}	8.61 ± 0.50 ^{bc}	5.07 ± 1.79	3.36 ± 0.86 ^{ab}
4.0	-	-	-	-	-
F-test	**350.82***	**26.01***	**168.90***	**13.96***	**12.19***
CV (%)	**4.1**	**15.57**	**5.99**	**20.83**	**23.78**

Note: Values represent mean ± standard deviation (SD). Means followed by different lowercase letters within the same column are significantly different based on Duncan's Multiple Range Test (DMRT) at $p < 0.05$. A dash (–) indicates that no data were recorded due to complete mortality at the highest NaN_3 concentration.

Root development showed relatively high variability among treatments, as indicated by larger coefficients of variation. Root number ranged from 4.48 to 5.59 roots per plantlet, with the highest value observed at 2.0 mM NaN_3 (5.59 ± 0.98). Root length also differed significantly among treatments, with the longest roots observed at 2.0 mM NaN_3 (4.59 ± 0.85 cm), while the shortest roots were recorded at 1.0 mM NaN_3 (2.99 ± 0.89 cm). Enhanced root growth at moderate NaN_3 concentrations may reflect adaptive

responses to mutagen-induced stress, as reported in previous studies on NaN_3-treated horticultural crops (Ganesan et al., 2005; Adeosun et al., 2020).

At the highest concentration tested (4.0 mM NaN_3), no morphological data were recorded because all treated plantlets died, indicating severe cytotoxic effects of the mutagen at this level. High concentrations of sodium azide are known to interfere with cellular respiration and DNA replication, leading to impaired cell division and eventual tissue death (Owais & Kleinhofs, 1988). Similar lethal effects at high NaN_3 doses have been reported in several plant species including cotton, maize, and orchids (Dahiru et al., 2021; Wannajindaporn et al., 2016).

Overall, the results demonstrate that NaN_3 treatment significantly affected morphological development in *Musa ornata* plantlets. Low concentrations (0.1 mM) tended to enhance certain growth parameters such as fresh weight and leaf number, whereas moderate concentrations (0.5–2.0 mM) induced noticeable morphological variations. These findings suggest that sublethal NaN_3 doses can generate phenotypic variability while maintaining plant viability, which is essential for mutation breeding programs aimed at developing new ornamental banana varieties with desirable traits.

ISSR amplification and polymorphism analysis

ISSR markers were used to evaluate genomic variation among *Musa ornata* plantlets regenerated after sodium azide mutagenesis. The ten ISSR primers produced clear and reproducible amplification profiles (Figure 2), generating a total of 81 scorable ISSR bands, of which 36 were polymorphic, corresponding to an overall polymorphism rate of 43.02% (Table 2). This level of polymorphism indicates that NaN_3 treatment induced detectable genomic variation among regenerated plantlets.

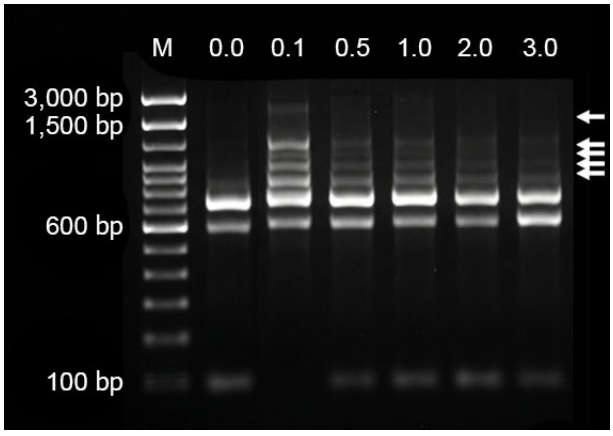


Figure 2 Representative ISSR amplification profile of *M. ornata* plantlets generated using primer P2, which exhibited the highest polymorphism among the tested primers. PCR products were separated on 2% agarose gel electrophoresis. Lanes correspond to plantlets derived from the control and different NaN_3 treatments. M = DNA ladder. White arrows indicate polymorphic bands distinguishing mutant lines from the control genotype.

The number of amplified bands varied among primers, ranging from 6 bands (P3, P6, and P8) to 13 bands (P5). Primers P2, P5, and P7 produced the highest polymorphism levels (71.43%, 61.54%, and 66.67%, respectively), suggesting that these primers were particularly informative for detecting mutation-induced variation within the *Musa* genome. In contrast, primers P6 and P10 exhibited relatively low polymorphism levels, indicating amplification of more conserved genomic regions. Such primer-dependent variation is commonly observed in ISSR analyses because different primers target distinct microsatellite regions distributed throughout the genome (Bornet & Branchard, 2001; Ramzan et al., 2020).

Genetic diversity indices further confirmed the presence of moderate genomic variability within the NaN_3-treated population. The polymorphic information content (PIC) ranged from 0.12 to 0.41 (mean = 0.28), while Nei's gene diversity (h) and Shannon's information index (I) ranged from 0.09 to 0.35 (mean = 0.24) and 0.13 to 0.51 (mean = 0.34), respectively. Similar levels of ISSR polymorphism have been reported in mutagenized populations of vegetatively propagated crops and ornamental plants, reflecting moderate genetic variation induced by chemical mutagens (Khatri et al., 2011; Beyramizadeh et al., 2023).

Table 2 Summary statistics of ISSR polymorphism and genetic diversity indices among *M. ornata* mutants induced by NaN_3. Values represent mean ± standard deviation (SD) calculated across ten ISSR primers.

Primer	Total bands	Polymorphic bands	%Polymorphism	PIC	h	I
P1	8	3	37.50	0.28	0.23	0.34
P2	7	5	71.43	0.41	0.35	0.51
P3	6	3	50.00	0.31	0.27	0.39
P4	9	4	44.44	0.29	0.24	0.35
P5	13	8	61.54	0.39	0.33	0.48
P6	6	1	16.67	0.12	0.09	0.13
P7	9	6	66.67	0.40	0.34	0.49
P8	6	2	33.33	0.22	0.18	0.26
P9	8	3	37.50	0.27	0.23	0.33
P10	9	1	11.11	0.14	0.10	0.15
Total	**81**	**36**	**-**	**-**	**-**	**-**
Mean ± SD	**8.10 ± 2.13**	**3.60 ± 2.22**	**43.02 ± 20.08**	**0.28 ± 0.10**	**0.24 ± 0.09**	**0.34 ± 0.13**

Importantly, the molecular polymorphism detected by ISSR markers corresponded with the morphological variation observed among NaN_3-treated plantlets (Table 1). For example, plantlets treated with 0.1 mM NaN_3 exhibited increased fresh weight and leaf number compared with the control, whereas treatments of 0.5–1.0 mM produced reduced leaf length and altered growth characteristics. Meanwhile, plantlets exposed to 2.0 mM NaN_3 showed enhanced root development relative to several other treatments. These results suggest that some of the observed phenotypic differences may be associated

with mutation-induced genomic changes. Similar relationships between ISSR polymorphism and morphological variation have been reported in mutation breeding studies of banana and other horticultural crops (Owais & Kleinhofs, 1988; Khatri et al., 2011).

It should be noted that the total number of scorable ISSR band obtained in this study (81 bands) was lower than the larger datasets often recommended for population-level diversity analyses, where approximately 150–200 polymorphic bands are considered desirable for maximizing statistical robustness. Nevertheless, in mutation breeding studies involving vegetatively propagated crops or relatively small mutant populations, ISSR datasets containing approximately 60–100 bands have frequently been sufficient to detect mutation-induced polymorphism and genetic differentiation among regenerated lines (Bornet & Branchard, 2001; Ramzan et al., 2020; Tunç et al., 2025). Therefore, the present dataset provides useful preliminary insight into genomic variation within the NaN_3-treated population, although additional primers or complementary marker systems such as SSR or SNP markers could further enhance the resolution of genetic diversity analysis in future studies.

Overall, the ISSR analysis demonstrated that sodium azide mutagenesis generated detectable genomic polymorphism in *M. ornata*. When combined with morphological evaluation and cluster analysis, these molecular markers provide an effective framework for identifying genetically distinct mutant lines with potential value for ornamental banana breeding programs.

Genetic relationships among mutant lines

Genetic relationships among NaN_3-treated *M. ornata* plantlets were further evaluated using a genetic distance matrix derived from ISSR marker data (Table 3). Pairwise genetic dissimilarity values ranged from 34.736% to 68.373%, indicating moderate genomic divergence among the analyzed mutant populations. In ISSR-based diversity studies, genetic distances within the range of approximately 30–70% are generally interpreted as moderate differentiation, reflecting mutation-induced genomic variation rather than large-scale structural genome alterations.

Table 3 Genetic dissimilarity values among NaN_3-treated populations of *M. ornata* based on ISSR markers

	NaN_3 treatments (mM)					
NaN_3 (mM)	**0.0**	**0.1**	**0.5**	**1.0**	**2.0**	**3.0**
0.0	—	48.093	65.428	66.489	55.079	61.606
0.1	48.093	—	59.373	34.736	53.327	49.717
0.5	65.428	59.373	—	64.062	52.173	68.373
1.0	66.489	34.736	64.062	—	55.776	63.545
2.0	55.079	53.327	52.173	55.776	—	59.351
3.0	61.606	49.717	68.373	63.545	59.351	—

The smallest genetic distance was observed between the 0.1 mM and 1.0 mM treatments, suggesting a relatively close genetic relationship between these two mutant groups. This pattern was consistent with their similar morphological responses, particularly in leaf-related traits (Table 1). In contrast, the largest genetic distance occurred between the 0.5 mM and 3.0 mM treatments, indicating substantial genomic divergence that corresponded with contrasting growth characteristics among these plantlets.

Cluster analysis using the UPGMA algorithm further illustrated the genetic structure of the NaN_3-treated populations (Figure 3). The dendrogram separated the six treatments into three major clusters, demonstrating that sodium azide mutagenesis generated detectable genomic divergence among regenerated plantlets. Cluster I included the control (0.0 mM) and the 3.0 mM treatment, whereas Cluster II grouped plantlets treated with 0.1 mM and 1.0 mM NaN_3, and Cluster III contained the 0.5 mM and 2.0 mM treatments.

Interestingly, the clustering pattern did not follow a strict linear relationship with mutagen concentration. Instead, treatments with different NaN_3 levels were grouped according to genetic similarity, suggesting that sodium azide induces stochastic mutations distributed throughout the genome rather than dose-dependent genomic changes. Similar non-linear clustering patterns have been reported in NaN_3-induced mutants of wheat and other crops (Srivastava et al., 2011; Türkoğlu et al., 2023).

From a mutation breeding perspective, the detection of genetically distinct clusters among NaN_3-treated plantlets indicates that chemical mutagenesis successfully broadened the genetic base of *M. ornata*. Treatments that exhibited both molecular divergence and morphological variation, particularly those within Clusters II and III, may represent promising sources of novel mutants for ornamental improvement. These lines could potentially possess desirable horticultural traits such as modified plant architecture, compact growth, or altered leaf morphology, which are valuable characteristics for ornamental banana cultivation.

Because the ISSR dataset was derived from dominant marker data and a limited number of mutant lines, the genetic distance matrix should be interpreted primarily as a descriptive measure of genomic divergence rather than an inferential statistical comparison. Nevertheless, the combined analysis of genetic distance, ISSR polymorphism, and cluster structure provides useful insight into mutation-induced diversity within the NaN_3-treated population.

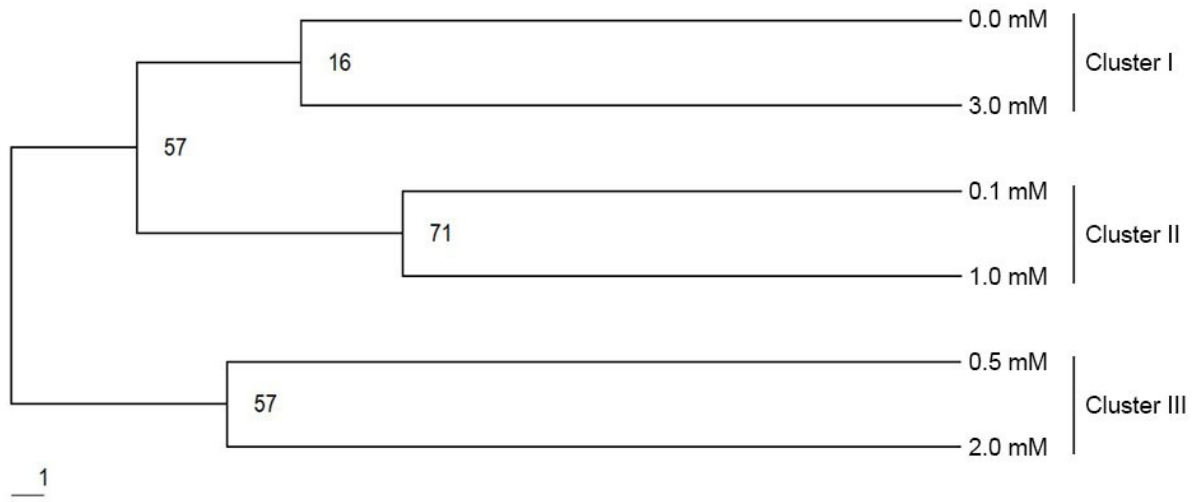


Figure 3 UPGMA dendrogram showing genetic relationships among genotypes based on ISSR markers. Genetic dissimilarity was calculated using the Jaccard coefficient and clustering was performed using the UPGMA method. The dendrogram resolved the analyzed treatments into three major clusters, indicating mutation-induced genomic divergence among NaN_3-treated plantlets.

Implications for Mutation Breeding of Ornamental Banana

The present study integrated survival analysis, morphological evaluation, and ISSR-based molecular characterization to investigate the mutagenic effects of NaN_3 on *M. ornata*. Together, these results provide complementary evidence that NaN_3 treatment successfully generated both phenotypic and genetic variation in this ornamental banana species, demonstrating the potential of chemical mutagenesis as a breeding strategy for vegetatively propagated ornamentals.

The survival analysis indicated a clear dose-dependent response of *M. ornata* plantlets to NaN_3 exposure. Increasing mutagen concentration significantly reduced plant survival, allowing the estimation of effective mutagenic doses such as LD_{30} and LD_{50}. These sublethal doses are widely considered optimal for mutation breeding because they balance mutation induction with plant viability. Similar survival patterns have been reported in NaN_3 mutagenesis studies in crops such as wheat, maize, and cowpea, where moderate mutagen doses produced the highest frequency of useful mutations while maintaining regeneration capacity (Srivastava et al., 2011; Dahiru et al., 2021; Raina et al., 2022). The LD range identified in this study therefore provides an important baseline for future mutagenesis experiments in *M. ornata* and related ornamental banana species.

Morphological evaluation of surviving plantlets further confirmed that NaN_3 treatment induced measurable phenotypic variation. Differences were observed in several growth parameters, including fresh weight, leaf number, leaf length, and root development. Low concentrations of NaN_3 tended to maintain or slightly enhance vegetative growth, whereas intermediate concentrations generated noticeable morphological variation such as reduced leaf size or altered root growth. In contrast, the highest concentration caused complete mortality, indicating strong cytotoxic effects. Comparable morphological responses to NaN_3 have been reported in other plant species, where the mutagen affects cell division,

metabolic activity, and chlorophyll synthesis, resulting in diverse growth phenotypes (Ganesan et al., 2005; Ahmad et al., 2007; Adeosun et al., 2020). Such phenotypic variability is particularly valuable in ornamental plants, where traits such as compact growth, altered leaf morphology, or distinctive coloration may enhance horticultural value.

The ISSR analysis provided molecular evidence supporting the presence of induced genomic variation among the NaN_3-treated plantlets. The moderate polymorphism level detected across ISSR primers demonstrated that the mutagen treatment produced detectable DNA-level variation while maintaining overall genome stability. ISSR markers are particularly effective for detecting mutation-induced polymorphisms because they target microsatellite regions that are sensitive to small sequence changes such as base substitutions or insertions. Previous studies have similarly demonstrated the usefulness of ISSR markers in identifying genetic variation among mutagenized plants and vegetatively propagated crops (Bornet & Branchard, 2001; Srisamoot & Padsri, 2018; Ramzan et al., 2020).

Integration of ISSR polymorphism data with cluster analysis revealed distinct genetic relationships among NaN_3-treated populations. The dendrogram grouped treatments into three major clusters, indicating that NaN_3 induced measurable genomic divergence among plantlets. Interestingly, the clustering pattern did not strictly follow mutagen concentration, suggesting that NaN_3 induces random mutations throughout the genome rather than linear or dose-dependent genomic changes. This stochastic mutation pattern has also been observed in other sodium azide mutagenesis studies, where genomic alterations occur independently of treatment gradients (Türkoğlu et al., 2023). Importantly, several genetically distinct groups also exhibited corresponding morphological differences, indicating that some of the observed phenotypic traits may be associated with underlying genomic polymorphisms induced by the mutagen.

From a breeding perspective, the combination of morphological screening and molecular marker analysis provides a powerful framework for identifying promising mutant lines. Treatments producing both phenotypic variation and molecular polymorphism, particularly those derived from sublethal NaN_3 concentrations, represent valuable candidates for further selection and evaluation. In ornamental banana breeding, traits such as compact plant architecture, altered leaf shape, improved vigor, or unique coloration patterns are highly desirable for landscape and container cultivation. Mutant lines generated through controlled chemical mutagenesis therefore represent a valuable alternative to conventional breeding approaches, which are often limited in *Musa* species due to sterility and parthenocarpy.

Overall, the results of this study demonstrate that sodium azide mutagenesis can effectively generate genetic diversity in *M. ornata*, and that ISSR markers are suitable tools for detecting and characterizing this induced variation. The integration of survival analysis, morphological evaluation, and molecular diversity assessment provides a comprehensive approach for identifying and selecting novel ornamental banana mutants. These findings contribute to the development of mutation breeding strategies aimed at expanding the genetic variability and ornamental value of *Musa* species.

Future research should focus on evaluating the stability of promising mutant lines across subsequent clonal generations and under greenhouse or field conditions. In addition, the integration of other molecular marker systems—such as SSR or SNP markers—may further improve the resolution of genetic diversity

analysis and facilitate the identification of genomic regions associated with desirable ornamental traits. Such approaches would strengthen the application of mutation breeding in the improvement of ornamental bananas and other vegetatively propagated horticultural crops. Because the present study evaluated early-generation mutants regenerated from treated explants, further evaluation across subsequent clonal generations will be necessary to confirm the stability and potential heritability of the observed traits.

Conclusions

This study demonstrated that sodium azide mutagenesis effectively induced both phenotypic and molecular variation in *M. ornata* plantlets cultured *in vitro*. Increasing NaN_3 concentrations resulted in a clear reduction in plant survival, enabling the estimation of optimal mutagenic doses (LD_{30} = 1.13 mM and LD_{50} = 2.08 mM). Sublethal concentrations (0.1–2.0 mM) produced measurable morphological variation in several traits, including fresh weight, leaf number, leaf length, and root development, indicating that moderate mutagenic stress can generate phenotypic diversity while maintaining plant viability.

ISSR marker analysis confirmed the presence of induced genomic variation among NaN_3-treated plantlets. Ten ISSR primers produced 81 scorable ISSR bands, of which 36 were polymorphic, corresponding to an overall polymorphism level of 43.02%. Genetic diversity indices (PIC, Nei's gene diversity, and Shannon's information index) indicated moderate levels of molecular variation among the analyzed plantlets. Cluster analysis further revealed three distinct genetic groups among treatments, suggesting that sodium azide induced detectable genomic divergence within the population.

Importantly, the observed ISSR polymorphism corresponded with morphological differences among treated plantlets, indicating that some phenotypic variations may be associated with underlying mutation events. However, because the present study was conducted at the early mutant generation, these variants should be regarded as putative mutant lines with distinct phenotypic characteristics and ISSR profiles, rather than confirmed stable or heritable mutations.

Overall, the integration of *in vitro* mutagenesis and ISSR marker analysis provides an effective framework for detecting and characterizing genetic variation in *M. ornata*. The mutant lines identified in this study represent promising genetic resources for further evaluation and selection in ornamental banana improvement programs. Future research should focus on evaluating the stability and horticultural performance of these putative mutants across subsequent clonal generations and under greenhouse or field conditions.

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