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Development of Chrysanthemum Mutants Adapted to Lowland Area using Ethyl Methanesulphonate

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ABSTRACT

Chrysanthemum is a subtropical ornamental plant traditionally cultivated in highland regions. Increasing temperatures caused by climate change are causing a decrease in flower production and quality, thus encouraging the availability of chrysanthemum varieties adapted to warmer lowland environments. This study aimed to induce genetic and phenotypic variations in the Indonesian chrysanthemum variety 'Marina' for potential lowland adaptation using Ethyl Methanesulphonate (EMS) combined with Paclobutrazol (PBZ). EMS concentrations of 0 and 0.77% and PBZ concentrations of 100 and 200 ppm were used as treatment. Morphological parameters, including plant height, number of leaves, number of nodes, diameter of stem, number of flowers, diameter of flowers, and percentage change in flower colour, were evaluated under natural lowland heat stress. Genetic distance was confirmed using RAPD and SSR molecular markers. The results showed that the application of 0.77% EMS and 200 ppm PBZ successfully induced significant phenotypic and genetic variations in the 'Marina' chrysanthemum. Applying 0.77% EMS mutagen has produced several promising putative mutant lines, specifically KM11, KM20, and KM22, which have distinct leaf shapes, bright flower colours, and reduced the time of flowering compared to the parent. Molecular analysis showed significant genetic polymorphisms, with KM11 showing the highest genetic distances (56% similarity) from the parent, while KM22 showed the lowest. These results provide a valuable genetic background for developing lowland-tolerant chrysanthemums, although further analysis of the next generations is required to confirm the stability of these traits for commercial release.

Keywords: Chrysanthemum, ethyl methanesulphonate, lowland area, molecular marker, paclobutrazol

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INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium*), belonging to the Asteraceae family, is an ornamental plant with a high economic value in domestic and global markets. It is an important Indonesian export commodity, ranked among the top 20 in the world and ranks third in ASEAN (Arumta et al., 2019).

In tropical areas, chrysanthemum is usually grown commercially in highlands between 700 and 1200 m above sea level, due to environmental adaptation to its temperate origins (Kurniati et al., 2013). The plant can also grow optimally at temperatures of 20-26ºC in the growth phase and 16-18ºC in the flowering phase, with humidity of 70-80% (Sanjaya et al., 2018). However, these conditions greatly limit their production, owing to various factors.

The increasing demand for chrysanthemums and limited land in the highlands have caused farmers to expand chrysanthemum cultivation to the lowlands. The problem in cultivating plants in this area is that the air temperature is relatively higher than in the highlands, especially with global climate change, which impacts the quality and production of chrysanthemum flowers. Temperature above 25ºC has been reported to inhibit the flower initiation and delay flower formation in chrysanthemums (Nozaki & Fukai, 2008; Sembiring et al., 2021). Besides that, high temperatures also cause flowers to be dull, pale, and faded. Using chrysanthemum varieties adapted for lowland areas is an effective and economical solution, but until now, we have not obtained Indonesian chrysanthemums with this character. Lowland adaptation is defined not merely as survival, but as the plant's ability to maintain high-value ornamental characteristics, such as stable flower diameter and colour intensity, while completing a normal reproductive cycle under temperatures exceeding the optimal 25ºC threshold.

The efficiency of ethyl methanesulphonate (EMS) in inducing mutations in *C. indicum indicum* var. *aromaticum* has been proven in the experiments of Chen et al. (2020) and Nasri et al. (2022). This method has also been used to increase plant tolerance to abiotic stress. Application of 0.5% EMS for 60 to 120 minutes successfully increased the tolerance of Petunia to stress (Krupta et al., 2017), drought tolerance in rice plants (Naser et al., 2024), and wheat tolerance in lowlands (Tarigan et al., 2023).

Previous reports indicate that chrysanthemums grown in lowland exhibit etiolation, weakness, and extended flowering. Paclobutrazol is a recommended plant growth retardant and has been widely recommended for suppressing the growth of elongated shoots or internodes. The application of PBZ on chrysanthemum has been proven to be effective in reducing plant height, as well as thickening leaves and stems (Chauhan et al., 2021; Lailaty et al., 2021; Salih & Hussen, 2020). Meng et al. (2022) stated that PBZ could reduce the deleterious effects of high temperature stress on peony growth by reducing lipid membrane peroxidation, activating photosynthesis, and protecting cell structures.

Phenotypic changes in mutants could be easily identified morphologically. However, this method is subjective and is strongly influenced by the environment. Detection of

mutant changes can be done more accurately by using molecular markers, which help to understand the phenotypic effects of mutations (Bhat et al., 2023), as well as the genetic distance between the mutant and the original parent (Kang et al., 2013). Simple Sequence Repeat (SSR) is a molecular marker which commonly used to identify non-coding regions with short repetitive base sequences at specific loci (Weising et al., 2005). SSR markers offer various advantages, such as being easy to use, highly informative, possessing specific loci, reading codominant traits, and applicable for genetic diversity analysis (Choudhary et al., 2023). This study aims to induce genetic and phenotypic variations in the Indonesian chrysanthemum variety 'Marina', which can adapt to lowlands using Ethyl Methanesulfonate (EMS) combined with Paclobutrazol (PBZ).

MATERIALS AND METHODS

Plant Materials

Plant material used for mutation induction was rooted cuttings (8-10 cm long) of the Marina variety. Marina is an Indonesian chrysanthemum variety officially released by the Ministry of Agriculture of the Republic of Indonesia based on decree number 051/Kpts/SR.120/D.2.7/7/2014. Marina is a popular commercial variety with high aesthetic value and yellow flowers, but is traditionally restricted to highland production (1000 m above sea level). The cuttings were transferred to a screen house at the Universitas Pembangunan Nasional Veteran Jawa Timur, at an altitude of ± 5 m above sea level. The minimum temperature was recorded at 24°C and a maximum of 30°C, with an average temperature of 27.2°C. The humidity level was 75% to 95%.

The material used for molecular analysis included three chrysanthemum mutant genotypes (KM22, KM20, and KM11) and the original chrysanthemum as a control (KN1). Young leaves of EMS-induced mutants and the original chrysanthemum were used for DNA extraction using the CTAB method.

EMS and PBZ Treatment

EMS stock solution 1% (v/v) was used to prepare 0.77% (v/v) solutions using 0.1 M phosphate buffer (pH 7.2), which were then filter-sterilised with an SFCA-PF 0.2µm filter (Corning, NY, USA). The concentration of 0.77% EMS was adopted based on its proven efficacy in previous chrysanthemum mutation studies (Latado et al., 2004; Rahmah et al., 2011), ensuring a high probability of viable mutation induction.

The cuttings were immersed in 0.77% EMS solution and sterile distilled water (0% EMS) as a control for 90 min. Explants were washed with sterile distilled water four to five times and transplanted using a mixture of cocopeat, soil, and cow manure in a ratio of 1:1:1. PBZ at 100 and 200 ppm was applied 30, 37, 44, and 52 days after planting, by spraying over the entire surface of the leaves and stems in the morning.

Observations were made on quantitative characteristics (plant height, number of leaves, number of internodes, stem diameter, number of flowers, diameter of flower, percentage change in flower colour) and qualitative characteristics (leaf colour, leaf shape, flower colour, and flower shape). The colour of the leaves and flowers was assessed using the Munsell Colour Charts, and the results were described in terms of hue, chroma, and value.

Data Analysis

The experiment was arranged as a factorial design based on a Completely Randomised Design (CRD. EMS (0, 0.77%) and PBZ (0, 100, and 200 ppm) were considered the first and second factors, respectively. Each treatment was repeated 4 times, resulting in 24 experimental units. Each experimental unit consisted of 10 plants. Data were subjected to an analysis of variance (ANOVA). Significant differences were further analysed using the Fisher's Least Significant Difference (LSD) at the 5% level.

DNA Extraction

Total plant genomic DNA was extracted using the small-scale CTAB method (mini preparation) developed by Doyle (1991) with minor modifications. The chrysanthemum leaf samples were ground from each genotype and mixed with liquid nitrogen in a mortar until they became a powder. The crushed powder was then placed in a 2 ml Eppendorf tube, followed by the addition of 700 µL of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2% (w/v) CTAB (cetyltrimethylammonium bromide), and 2% (w/v) PVP (Polyvinylpyrrolidone) to each sample. Furthermore, 5 µL of mercaptoethanol was added to each sample, followed by homogenisation of the mixture by carefully inverting the tube, and this was incubated at 65 ºC for 15 min in a water bath. The mixture was homogenised again during incubation by inverting the microtube every 5 min.

In the next stage, 700 µL of chloroform isoamyl alcohol (24:1 v/v) solution was added. The mixture was homogenised using a vortex or centrifuged at 12.000 rpm for 10 min, and the supernatant of each sample was carefully transferred into a new 1.5 mL microtube. Subsequently, 1/10 times the volume of the supernatant was added to each sample, comprising a solution of 3 M sodium acetate (pH 5.2), followed by a cold isopropanol solution, which equalled the volume of the supernatant. The samples were then incubated in a freezer at -20 ºC for one hour, and centrifuged at 12.000 rpm for 10 min until a DNA pellet precipitate was formed. The supernatant liquid was discarded, and the DNA pellet was washed with 500 µL of 70% ethanol solution. The mixture was centrifuged again at 12.000 rpm for 5 min, and the supernatant was discarded. The washed DNA pellet was air-dried until the remaining ethanol was depleted. The dried DNA pellet was dissolved in TE solution (10 mM Tris, pH 8.0, 1 mM EDTA) supplemented with RNase (10 mg/mL). The stock DNA solution was then incubated at 37 ºC for 1 h and stored at -200 ºC until ready.

PCR Analysis with RAPD Primers

The stock DNA solution, which had been tested for quality and quantity, was then diluted into a working solution at a concentration of 2 ng/µL. The diluted DNA from each sample was then amplified using 7 (seven) RAPD primers: OPA-08 (5'-GTGACGTAGG-3'), OPA-02 (5'-TGCCGAGCTG-3'), OPA-03 (5'-AGTCAGCCAC-3'), OPA-07 (5'-GAAACGGGTG-3'), OPA-04 (5'-AATCGGGCTG-3'), OPA-09 (5'-GGGTAACGCC-3'), and OPA-10 (5'-GTGATCGCAG-3'). DNA amplification was carried out in a total volume of 20 µL consisting of 2 µL of template DNA with a concentration of 2 ng/µL, 10 µL of My Taq HS (Bioline, UK), 1 µL of RAPD primer with a concentration of 5 µM, and sterile ddH2O.

Amplification was performed on a PCR Thermocycler machine (Biorad, USA) with the following profile: an initial denaturation stage at 94°C for 5 minutes, followed by 45 cycles of denaturation at 94°C for 1 minute, annealing at 37°C for 1 minute, and elongation at 72°C for 1 minute. The PCR reaction was terminated with a final elongation stage at 72°C for 5 minutes. The amplified DNA was then electrophoresed on a 1.2% agarose gel in a tank containing 0.5x TBE buffer at 90 V for 35 minutes. The gel was then immersed in ethidium bromide solution (10 mg/ml) for 10 minutes. The stained gel was then rinsed with distilled water for further visualisation on a UV Transilluminator (Biorad, USA).

PCR Analysis with SSR Primers

DNA was amplified using 5 (five) SSR primers, namely PIF 107 (5'-ATGGTGAAAGAGGAAGCATG-3'), PIF 98 (5'-TGA ACT ATG GTG ACG GCG GT-3'), PIF 7 (5'-TGA GAT CTT TTT TAC CGC GTG TTT-3'), cmGFR 01 (5'-GGTGGAAACGCTCAGCT-3'), and cmGFR 02 (5'-CACGAGCAAGCCATCAG-3').

The Polymerase Chain Reaction (PCR) reaction was carried out in a total reaction volume of 25 µL. The SSR marker-based PCR reaction composition consisted of 10 ng/µl DNA from each test sample, ddH2O, 2X MyTaq DNA Polymerase, and 0.5 µM primer. The PCR profile used included the following stages, namely initial denaturation at 94 °C for 7 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, attachment of markers at a specific annealing temperature (depending on the annealing temperature of each SSR marker) for 30 seconds, and polymerisation at 72°C for 1 minute. The PCR analysis ended with a final extension stage at 72°C for 7 minutes.

Data Analysis

Data analysis for RAPD and SSR was performed by scoring the DNA bands observed in the electrophoresis results. Each visible band was considered one allele, and DNA bands with the same migration rate were considered at the same locus. Scoring was done in a binary manner: visible bands were scored as 1, absent bands as 0, while unamplified samples

were scored as 9 and considered missing data, resulting in binary scoring data. The binary scoring data were analysed using the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean)-SAHN (Sequential Agglomerative Hierarchical and Nested) programme in NTSYS version 2.1 software. The analysis results were presented as a dendrogram and a genetic similarity matrix.

RESULTS AND DISCUSSION

Morphological Analysis

Quantitative Characteristics

Statistical analysis showed no effect of interaction between EMS and PBZ on plant height, number of leaves, number of internodes, stem diameter, number of flowers, and diameter of flowers. However, a single factor (EMS or PBZ) was influenced at 126 days after planting (Table 1).

Table 1
Plant height, number of leaves, number of nodes, stem diameter, number of flowers, and diameter of flowers of chrysanthemum at 126 days after planting

Treatments	Plant height (cm)	Number of leaves	Number of internodes	Stem diameter (cm)	Number of flowers	Diameter of flowers (cm)
EMS (%)						
0	70.48^{a}	29.03^{a}	23.93^{b}	0.56^{b}	1.04^{a}	10.00^{a}
0.77	69.78^{a}	29.14^{a}	20.26^{a}	0.56^{b}	1.06^{a}	10.79^{b}
PBZ (ppm)						
0	81.38^{b}	33.09^{b}	24.93^{b}	0.50^{a}	1.04^{a}	9.86^{a}
100	67.13^{ab}	27.51^{ab}	21.84^{ab}	0.60^{bc}	1.04^{a}	10.57^{b}
200	61.90^{a}	26.66^{a}	19.51^{a}	0.58^{b}	1.08^{a}	10.75^{b}

Note. Means followed by the same letter in the same column are not significantly different at $p<0.05$

Applying 0.77% EMS produced mutant chrysanthemums with fewer internodes and significantly different from the control. However, the plant height, number of leaves, stem diameter, and number of flowers were not significantly different between the mutant and control (Table 1). The effect of EMS, which reduces the number of internodes, was also observed in the research of Rime et al. (2019), who applied EMS at a concentration of 0.2-1.0% to mango plants.

The application of EMS increased the flower diameter of mutants, which was significantly different from that of the control. EMS causes point mutations; if mutations occur in the genes that regulate cell growth and elongation, or hormone-forming pathways,

they produce phenotypes with larger growth characteristics, such as increased flower diameter and internode length (Subramaniam & Kumar, 2023). Statistical analysis showed a significant interaction between EMS and PBZ on flowering time. Treatment of 0.77% EMS combined with 200 ppm PBZ resulted in the earliest flower bud initiation at 75.55 days, significantly earlier than the control (0% EMS without PBZ), which required 82.25-83.05 days (data not presented). The reduced flowering time (approximately 7 days earlier than the control) without compromising flower quality further validates the adaptive capacity of the mutant. This suggests that the synergistic effect of EMS and PBZ application optimises the plant's ability to cope with high temperature stress.

PBZ was often used to inhibit plant growth and enhance plant vigour, as shown in Table 1, where PBZ treatment (100 and 200 ppm) decreased plant height, leaf number, and internodes. These sizes decreased as the concentration of PBZ increased, due to the inhibition of gibberellin production by hindering the oxidation of kaurene to kaurenoic acid. Consequently, it could further reduce the speed of cell division, reduce vegetative growth, and indirectly redirect assimilates toward reproductive growth for flower formation (Desta & Amare, 2021; Soumya et al., 2021). PBZ also increased stem and flower diameters, enhancing the chrysanthemum's appearance. Numerous studies have shown the effectiveness of PBZ in reducing chrysanthemum plant height (Elhassan et al., 2021; Chauhan et al., 2020) and increasing stem diameter (Salih & Hussein, 2020). These morphological changes induce anatomical alterations, such as increased leaf thickness and stem diameter (Lailaty & Nugroho, 2021).

Qualitative Characters

Leaf Colour and Shape

Plant colour can be described by three factors: hue, saturation, and lightness, or XYZ colour-matching functions (Kasajima, 2019). Hue is a type of colour, such as red, green, and blue. In the Munsell classification system, the degree to which a colour is expressed by 'saturation' or chroma. Lightness represents the brightness of the colours or values. The results showed that EMS and PBZ did not have much effect on leaf colour. All leaves had a green-yellow colour with a hue value of 5GY. However, there was a gradual change in leaf colour (Figure 1). The leaf colour of the wild-type had a lightness value of 7 and a chroma of 10 (bright yellowish green). Meanwhile, EMS or EMS+PBZ chrysanthemum leaves had a slightly darker green leaf colour with the codes "hue", "chroma," and" value," namely 5GY 6/6, 5GY 7/8, 5GY 6/10, and 5GY 6/8, respectively.

The leaves showed variations between mutants, specifically in the sinuses and sinus side between the two lateral lobes (Figure 1). The leaf shape was long, ovate, slender, and elongated, with large serrated leaf edges. The sides of the sinus between the two lateral lobes diverged (opening) and opened wider in EMS without PBZ, and the shape of the

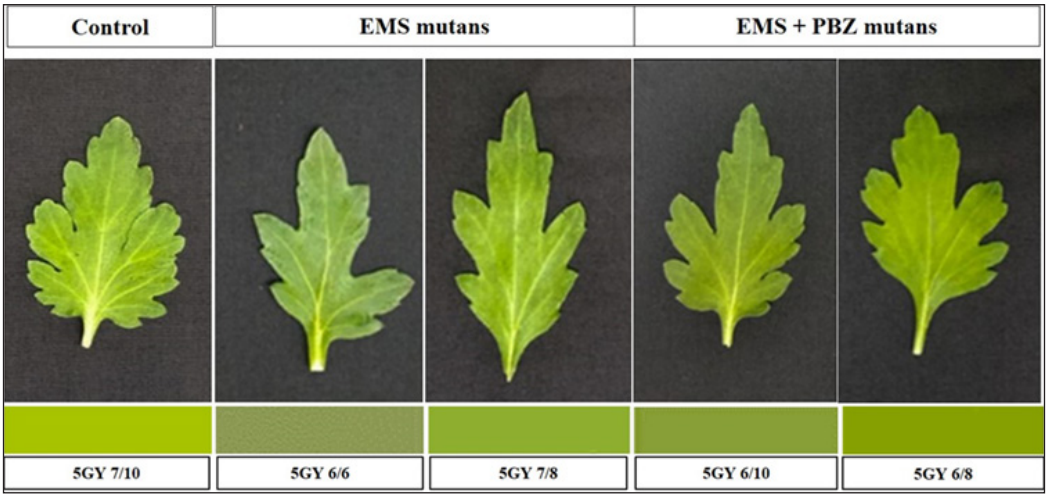

Figure 1. The shape and colour of control and mutant chrysanthemum leaves

sinus between the two lateral lobes was acute (tapered). The control had an ovate leaf shape with converging sides and rounded sinuses (Figure 1). Song et al. (2021) classified chrysanthemum leaf morphology into broad, ovate, and long. This study showed that control chrysanthemum leaves were ovate, whereas leaves of mutants were long ovate.

Flower Colour and Shape

Marina variety in the highlands has yellow flowers, with stacked flower crowns. Cultivation of this variety in the lowlands at an altitude of ± 5 m above sea level causes the colour of the flowers to change from yellow to yellowish white (pale), with thin flower crowns, so that the appearance of the flowers is not attractive. This shows that environmental conditions in the lowlands influence the colour and shape of the flowers.

PBZ treatment without EMS application could not induce flower colour changes (Figure 2). The flower appearance of mutant genotypes in the lowlands showed a wide range of flower colour and shape variations, as presented in Table 2 and Figure 2. The application of 0.77% EMS induced a broad spectrum of flower colour variations, comprising greenish-white (37.5%), white (29.1%), pink (20.8%), dark yellow (12.5%), and yellowish-white (0.1%) (Table 2). In contrast, the control exhibited complete uniformity with 100% yellowish-white flowers. In the greenish-white variants, the green pigmentation was specifically observed on the petal margins and/or the central disc of the flower. Furthermore, EMS treatment resulted in a structural modification of the ray florets, transitioning from an irregular to a regular arrangement.

Table 2
Percentage of flower colour in EMS chrysanthemum mutants in the lowlands

Concentration of EMS (%)	Flower colour (%)				
	Yellowish white	Greenish white	White	Dark yellow	Pink
Without EMS (0)	100	0	0	0	0
EMS (0.77)	0.1	37.5	29.1	12.5	20.8

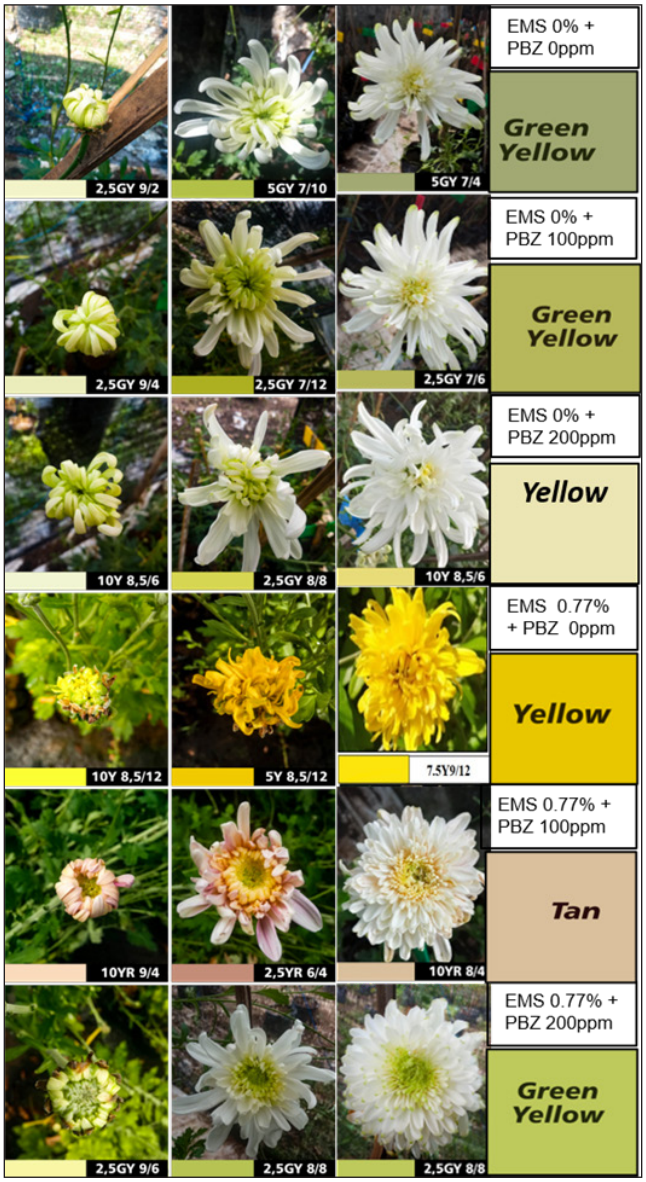

Figure 2. Differences in the shape and colour of flowers between the control and the mutants

EMS and PBZ treatments caused changes in flower shape, flower colour, and flower corolla thickness (Figure 2). EMS application caused a change in the flower colour from yellowish white (7.5 GY 9/4) (wild-type) to various colour variations, such as a dark yellow flower with the colour code 2.5Y 8/12, pink (2.5R 9 /6), greenish-white (2.5 GY 9/2), and pure white (N/9). Changes in flower colour that occurred during EMS+ 100 mg/l PBZ treatment were light yellow (7.5Y 9/12), pink (2.5R 9/2), greenish-white (2.5GY 9/4), and pure white (N/9). The flower colours produced in the EMS + 200 mg/l PBZ treatment were pink (5R 9/2), greenish-white (2.5GY 9/4), and pure white (N/9). Besides causing changes in flower colour, EMS treatment or its combination with PBZ also caused changes in flower shape and corolla thickness. Flowers of the same colour can have different crown thicknesses (Figure 2).

The Marina variety used in this study has a standard type of flower with a decorative shape. The characteristic feature of decorative flowers was a round corolla tightly stacked, and the petals were short in the middle and elongated at the edges. The results showed that chrysanthemum flowers resulting from the EMS mutation had irregular and regular decorative flower shapes (Figure 2). Chrysanthemum flowers in the EMS+ PBZ treatment had a decorative flower shape characterised by long, almost neat, and regular petals, where the length of the innermost petals did not exceed the outermost petals. It appeared that PBZ helped the EMS mutant to form flowers with regular corollas. Chrysanthemum flowers without EMS at all PBZ concentrations had a non-decorative shape with irregular petal lengths from the inside of the flower corolla to the outside of the flower. Although PBZ effectively regulated decorative flowers in EMS mutant plants, it did not occur in control plants or plants without EMS.

EMS treatment significantly affected leaf colour, leaf shape, and flower colour. Lethin et al. (2020) stated that EMS mutagenesis has been applied to various plants to produce morphological diversity and induce the formation of ideal characteristics. Gao et al. (2022) showed that Chinese cabbage plants treated with 0.8% EMS produced changes in leaf colour, shape, and head. Several researchers have reported that the induction of mutations in chrysanthemum plants through the application of EMS produces more flower variations (Ghormade et al., 2020; Din et al., 2023). The use of EMS could increase genetic diversity in plants and make it possible to improve only one desired character, without changing other characters (Purnamaningsih & Hutami, 2016). Nasri et al. (2022) produced mutants of the "Fariba2" cultivar, in which their colour varied from light yellow to orange, pink, and red, while the control plants had yellow-coloured ray florets. Chrysanthemum mutants generated with 0.125% and 0.5% EMS had white and light-yellow floret colours from the cultivar "Homa", yellow colour. Suryawati et al. (2023) reported that chrysanthemums treated with EMS through an in vitro technique caused the flower colours to change from red-purple (70B) to purple (70A) and red-purple (60A). In this study, the application of

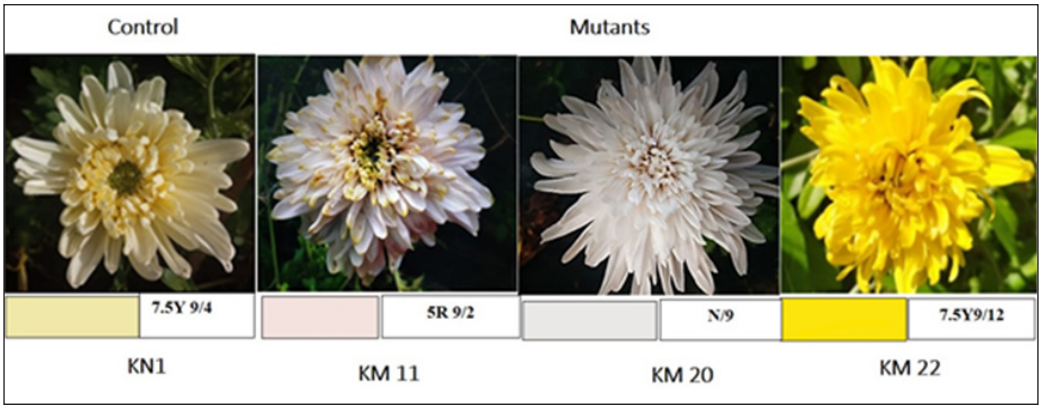

Figure 3. Three candidate chrysanthemum mutant genotypes adapted to the lowlands

EMS and paclobutrazol were proven to have produced mutant plants that could adapt to lowlands and produce flowers of varying colours with normal flower sizes, thick and regular crowns.

Applying 0.77% EMS to the Marina variety (KN1) produced three promising chrysanthemum mutant genotypes in lowlands. These genotypes had good growth performance with attractive flower shapes and colours that were different from those of their parents. Marina variety (control) in lowlands had yellowish-white flowers (7.5Y 9/4), while the mutants had yellow, white, and pink flowers, which included KM22 (yellow 7.5 Y 9/12), KM20 (white N9), and KM11 (pink 2.5R 9/2) (Figure 3).

Distinct phenotypic changes were observed among the selected lines. The flower colour of the KM11 mutant changes from light yellow to magenta-red, characterised by a large diameter and symmetrical ray florets with light green margins. The KM20 mutant exhibited a colour shift to white, featuring regular, symmetrical florets and an increased flower diameter. In contrast, the flower of the KM22 genotype displayed a change to dark yellow, and its florets remained irregular in arrangement. Overall, the synergistic treatment of 0.77% EMS and PBZ (100 and 200 ppm) yielded flowers with enhanced petal thickness and improved structural symmetry. These traits represent significant potential for the commercial development of chrysanthemum varieties tailored for lowland tropical environments (Figure 3). The application of EMS and PBZ represents a strategic cultivation package for lowland production. While EMS induces stable genetic variations for heat tolerance, PBZ acts physiologically to counteract heat-induced etiolation.

RAPD and SSR Molecular Analysis

The genetic similarity matrix using combined RAPD and SSR markers in Table 3 shows the genetic distances between the four chrysanthemum genotypes. Genetic distances ranged from 0.56 to 0.73 across the four genotypes tested. The genetic similarity matrix value

was negatively correlated with the genetic distance, meaning that the lower the value, the higher the genetic distance. The highest genetic distance was observed between the KN1 genotype (control) and the KM11 genotype (pink flowers), and the lowest was the KM22 genotype (yellow flowers). KM11 was the most divergent genotype compared to the control, while KM22 was the most similar.

Table 3
Genetic similarity matrix of four chrysanthemum genotypes using UPGMA-SAHN methods

Genotype	KN1	KM22	KM20	KM11
KN1	1.00			
KM22	0.73	1.00		
KM20	0.67	0.67	1.00	
KM11	0.56	0.67	0.65	1.00

Based on phylogenetic analysis using the NTSYS tool, the four chrysanthemum genotypes used were separate and had different genetic backgrounds, with a genetic similarity coefficient of 0.73 (Figure 4). KM22 had the closest genetic distance to control (KN1) with a genetic similarity level of 75%, then KM20 had a genetic similarity of 67% to KN1, and KM11 had the furthest genetic distance with a genetic similarity value of 56%. The results obtained showed that the four chrysanthemum genotypes were different individuals. This result was supported by the genetic similarity matrix of the four chrysanthemum genotypes analysed, where the four genotypes had genetic similarities below 73%.

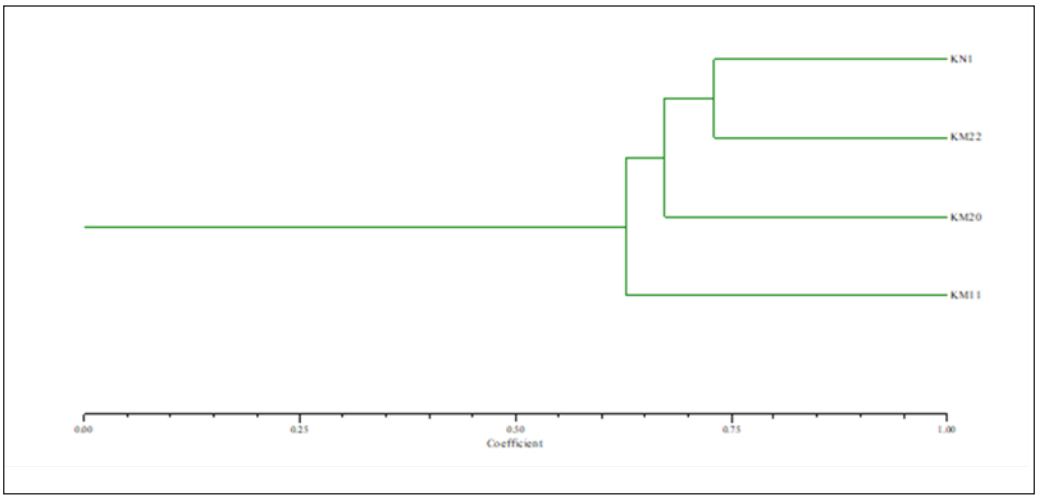

Figure 4. Phylogenetic dendrogram of chrysanthemum mutant genotypes based on the UPGMA-SAHN method

The identification of DNA polymorphisms using RAPD and SSR markers in the first-generation provides significant preliminary evidence of successful genetic induction by EMS. While this study represents the initial phase of selection, the presence of distinct genomic variations suggests that the observed traits, such as increased flower diameter

and reduction in flowering time, are linked to structural changes in the DNA rather than transient phenotypic plasticity, although further verification in subsequent generations is essential to confirm the long-term heritability of these traits.

Some studies on chrysanthemum mutants have shown that RAPD and SSR markers can efficiently identify changes at the genetic level. SSR markers are also potent for studying genetic relationships and could be valuable tools for identifying and classifying chrysanthemum (Chang et al., 2018). Thakur et al. (2022) obtained two prominent genotypes using SSR markers and verified the mutant behaviour of newly evolved chrysanthemum genotypes at the molecular level. These results indicate that the four chrysanthemum genotypes are genetically distinct individuals, confirming that the mutation method successfully produced broad genetic diversity and high potential for developing commercial chrysanthemum varieties in the lowlands.

CONCLUSION

The application of 0.77% EMS and 200 ppm PBZ successfully induced significant phenotypic and genetic variations in the 'Marina' chrysanthemum. Three promising genotype mutants were identified, exhibiting improved floral quality and reducing the time of flowering under lowland conditions. These results proved that chemical mutagenesis is a viable approach to increasing the genetic base of heat-tolerant chrysanthemums. Further evaluation in subsequent generations is required to confirm the stability of these improved traits before they can be officially categorised as stable mutants.

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