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Induced floral morphological variability in pomegranate (*Punica granatum* L. cv. Bhagwa) through sodium azide

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Abstract

Sodium azide mutagenesis is a widely applied technique for creating genetic variability in crop plants, including perennial fruit species. However, its impact on pomegranate flower morphology remains poorly understood. This study evaluated the effects of sodium azide (SA) on the floral traits of pomegranate (*Punica granatum* L., cv. 'Bhagwa'). Seeds were exposed to sodium azide (1–10 mM) for 1–3 h, and the resulting M_1 plants were assessed across two Mrig Bahar seasons (2023–2024). Fully opened hermaphrodite flowers were used for measuring calyx length and width and petal length/width, and analyzed the data using factorial ANOVA. Moderate concentrations (5–7 mM), especially with 2 h exposure, generated the most consistent variations. The maximum values recorded were 40.62 mm (calyx length), 15.35 mm (calyx width), 24.77 mm (petal length), and 20.64 mm (petal width). Most floral traits showed significant treatment effects, whereas higher concentrations reduced the responses. Optimized sodium azide treatment can thus induce useful floral variability, providing valuable baseline information for future pomegranate mutation breeding programs.

Keywords: Bhagwa, floral morphology, induced mutagenesis, mutation breeding, *Punica granatum*, sodium azide.

Pomegranate (*Punica granatum* L.) is one of the oldest cultivated fruit crops and is widely recognized for its nutritional, medicinal, and economic importance. The fruit is an excellent source of antioxidants, phenolic compounds, anthocyanins, vitamins, and minerals, making it a key functional food with growing global demand. India is the world's leading pomegranate producer. Among commercially cultivated cultivars, 'Bhagwa' is the most popular for its bright-red arils, soft seeds, superior fruit quality, excellent export potential, and adaptability to semi-arid agro-climatic conditions (Chandra *et al.,* 2010). Consequently, continuous genetic improvement of 'Bhagwa' is a key objective of pomegranate breeding programs.

Despite its commercial importance, pomegranate genetic improvement is relatively slow because the crop is highly heterozygous, predominantly cross-pollinated, perennial, and has a prolonged juvenile phase. These biological constraints limit the efficiency of conventional breeding and reduce opportunities for rapid genetic gain (Melgarejo *et al.,* 2015). Therefore, alternative breeding strategies that generate novel genetic variability are increasingly important for perennial fruit crops. Induced mutagenesis is an effective approach for broadening the genetic base of crop plants by generating novel heritable variation without substantially altering the desirable characteristics of elite cultivars. Both physical and chemical mutagens have been widely used in breeding programs to improve economically important traits, including plant architecture, flowering behavior, yield, fruit quality, and resistance to biotic and abiotic stresses (Mba, 2013; Oladosu *et al.,* 2016; Shu *et al*., 2012; Londhe *et al.,* 2025).

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Among chemical mutagens, sodium azide (SA) is considered one of the most efficient because it predominantly induces point mutations and causes fewer chromosomal aberrations than many other mutagenic agents (Kumar and Rai, 2019; Wani *et al.,* 2020). Sodium azide has been successfully used to induce genetic variability in several agricultural and horticultural crops, including cereals, legumes, ornamentals, and vegetables. Previous studies have shown that chemical

mutagen treatments can modify plant growth, flowering characteristics, reproductive traits, and yield components by inducing stable genetic changes (Bado *et al.,* 2015; Datta, 2017; Rodge *et al.,* 2024). However, information on its effects on pomegranate floral morphology remains scarce.

Flower morphology is fundamental to pomegranate reproductive biology, pollination efficiency, fruit set, and productivity. The species is andromonoecious, producing both functional male (staminate) and hermaphroditic flowers on the same plant (Wetzstein *et al.,* 2011). Only hermaphroditic flowers can set fruit, whereas staminate flowers typically abscise after anthesis. Floral traits, such as calyx and petal length and width, are important reproductive traits that may influence flower development, pollinator attraction, fertilization efficiency, and subsequent fruit production. Therefore, evaluating floral morphology provides valuable information for identifying desirable phenotypes in mutation breeding programs.

Sodium azide primarily induces point mutations after being metabolized in plant tissues into an active mutagen. These mutations may alter gene function and regulatory pathways that control plant growth and reproductive development, generating useful phenotypic variation (Kumar and Rai, 2019). Nevertheless, the biological response to sodium azide depends heavily on mutagen concentration, exposure duration, genotype, and the physiological condition of the treated material. Moderate doses generally induce useful variability, whereas excessive doses may result in growth inhibition, physiological injury, and reduced reproductive performance (Bado *et al.,* 2015; Wani *et al.,* 2020; Vaithiyalingan *et al.,* 2024). Although induced mutagenesis has been extensively studied in annual field crops, relatively little information is available on its application to perennial fruit crops, particularly floral morphological variation in pomegranate. Most previous studies have emphasized seed germination, vegetative growth, fruit characteristics, and yield-related traits, whereas systematic evaluation of floral morphology after sodium azide treatment has received comparatively little attention. Consequently, we still need to determine whether sodium azide can generate useful phenotypic variation in reproductive organs that may contribute to future genetic improvement of pomegranate. Therefore, this investigation evaluated the effects of different sodium azide concentrations and exposure durations on the floral morphological traits of pomegranate (*Punica granatum* L. cv. 'Bhagwa'). This study specifically aimed to quantify variation in calyx length, calyx width, petal length, and petal width in fully opened hermaphrodite flowers and to identify treatment combinations that generate useful phenotypic variability for future mutation breeding programs.

Experimental site and environmental conditions

The experiment was conducted at the experimental farm of the ICAR–National Research Centre on Pomegranate (ICAR–NRCP), Solapur, Maharashtra, India (17°10′ N, 74°42′ E; 483.5 m above mean sea level). The study was conducted over two consecutive Mrig bahar seasons (2023 and 2024) in a semi-arid Indian climate. The experimental site had medium black clay loam (Vertisol) soils with high water-holding capacity and a slightly alkaline pH. During the flowering period (June–July), mean temperatures ranged from 24 to 38°C, while maximum summer temperatures exceeded 40°C. The total annual rainfall during the experimental period was approximately 545 mm, with monsoon precipitation concentrated between June and September of the same year. All experimental plants were maintained under uniform agronomic practices in accordance with the recommended package of practices of ICAR–NRCP, including regulated water stress before Mrig Bahar, drip irrigation, nutrient management, pruning, and plant protection. Because the treatment responses showed similar trends in both experimental seasons, the data were pooled for statistical analysis.

Plant material

Mature seeds were collected from healthy, true-to-type fruits of the commercially important pomegranate cultivar 'Bhagwa' maintained at the ICAR–National Research Centre on Pomegranate, Solapur. Uniform, healthy and fully developed seeds were selected for mutagenic treatment. The experiment comprised three exposure duration sets (1, 2, and 3 h). Each exposure duration set included an untreated control (0 mM) and ten sodium azide concentrations (1–10 mM), resulting in 33 treatment groups (11 treatments per exposure duration). The treated seedlings were raised to the flowering stage under uniform field conditions, and observations were recorded for the resulting M_1 population.

Mutagenic treatment and field establishment

Seeds were surface-sterilized and pre-soaked in distilled water for 24 h to synchronize physiological activity before mutagen treatment. Sodium azide (NaN3) solution in 0.1 M acidified phosphate buffer (pH 3.0) was freshly prepared, as acidic conditions enhance its mutagenic activity and are widely recommended for mutation-breeding studies. Seeds were treated with sodium azide at concentrations ranging from 1 to 10 mM for 1, 2, and 3 h under continuous mechanical agitation to ensure uniform chemical exposure. Untreated control seeds were exposed to the same acidified phosphate buffer, but without sodium azide.

Following treatment, the seeds were thoroughly washed under running tap water to remove the residual mutagen. The treated and control seeds were initially germinated in Petri dishes under laboratory conditions and subsequently transplanted into nursery pro-trays. Healthy seedlings were later established in the experimental field of ICAR–NRCP, Solapur, India, where all plants were maintained under identical agronomic and plant protection practices until the flowering stage. Sodium azide concentrations

(1–10 mM) and exposure durations (1, 2, and 3 h) were selected based on preliminary standardization experiments, previous mutation breeding studies, and the biological responses of pomegranate seedlings observed under these treatments. This concentration range encompassed low to relatively high mutagenic doses to evaluate dose-dependent phenotypic responses while minimizing excessive physiological damage. Previous observations on seed germination and seedling survival indicated that these treatment combinations were biologically suitable for inducing phenotypic variability in pomegranates.

Data collection and flower characterization

Observations were recorded during the flowering period of the Mrig bahār season. Fully opened hermaphrodite flowers at uniform anthesis were selected for floral characterization to eliminate variation associated with flower type, as pomegranate is an andromonoecious species bearing both staminate and hermaphrodite flowers. Because only hermaphrodite flowers can develop into fruit hence, selected them for quantitative floral measurements. The staminate-to-hermaphrodite flower ratio was not quantified because the objective was to evaluate only floral morphometric traits. Three to six hermaphrodite flowers were randomly sampled from each plant in each treatment for measurement.

Quantitative floral traits, namely calyx length (mm), calyx width (mm), petal length (mm), and petal width (mm), were measured using a digital Vernier caliper (accuracy ±0.01 mm). Calyx length was measured from the base of the receptacle to the apex of the longest calyx lobe, whereas calyx width was measured across the widest portion of the calyx. Petal length was measured from the point of attachment at the base to the tip of the petal, and petal width was measured at the widest portion of the petal lamina. Qualitative observations including flower type and petal colour were recorded using standard descriptors for pomegranates, with minor modifications appropriate to the objectives of the present study (Melgarejo *et al*., 2015; Wetzstein *et al*., 2011). The mean values from individual flowers were used for statistical analysis.

Statistical analysis

The experiment was analyzed using a factorial randomized complete block design (Factorial RCBD) in the Web Agri Stat Package (WASP version 2.0). Sodium azide concentration and exposure duration were the main treatment factors. Data from the two consecutive Mrig bahār seasons (2023 and 2024) were pooled after confirming similar treatment response patterns across years.

Analysis of variance (ANOVA) was used to assess the significance of treatment effects, sodium azide concentration, exposure duration, and their interactions. Treatment means were compared using the Critical Difference (CD) test at the 5% probability level ($P \leq 0.05$). We computed the standard error (SE), coefficient of variation (CV), and other descriptive statistics to assess experimental precision. Because the observations were recorded from the M_1 generation, we interpreted the detected phenotypic variations as putative mutagen-induced responses; confirming their genetic stability and heritability will require evaluation in subsequent generations or vegetatively stabilized mutant lines.

Effect of sodium azide on calyx length

Sodium azide treatment significantly influenced calyx length in pomegranates (**Table 1, Table 2 and Table 3; Fig. 1 and Fig. 5**). The pooled mean value for calyx length ranged from 34.16 mm (8 mM) to 36.75 mm (5 mM), whereas the untreated control recorded 34.32 mm in the same period. Among the exposure durations, the 2 h treatment (Set B) produced the highest length (36.57 mm), whereas the maximum individual calyx length (40.62 mm) was recorded with 10 mM sodium azide after 2 h of exposure. Analysis of variance revealed significant effects of sodium azide concentration, exposure duration, and their interaction on calyx length. The enhanced calyx length observed at moderate sodium azide concentrations suggests that optimized mutagenic treatments can promote floral variability, whereas higher concentrations or prolonged exposure may reduce trait expression due to physiological stress (Bado *et al*., 2015). Since the observations were recorded from seed-derived M_1 plants, the detected variation should be considered as putative mutagen-induced phenotypic responses and should be confirmed in subsequent generations. Similar alterations in floral morphology following sodium azide treatment have also been reported in other crop species. Sodium azide-induced mutagenesis has been shown to modify flower size, floral architecture, and reproductive characteristics in marigolds (*Tagetes erecta*), ornamental crops, lentils (*Lens culinaris*), and several cereal species. These studies suggest that moderate mutagen doses generate useful phenotypic variability, whereas higher doses often suppress floral development due to increased physiological stress (Datta, 2017; Kumari *et al*., 2018; Laskar and Khan, 2017; Bado *et al*., 2015).

Effect of sodium azide on calyx width

Significant variation in calyx width was observed among the sodium azide treatments (**Table 1, Table 2 and Table 3; Fig. 2 and Fig. 5**). The pooled mean calyx width ranged from 12.35 mm (9 mM) to 13.37 mm (6 mM), whereas the untreated control recorded a mean of 12.41 mm. The 1 h and 2 h exposure durations produced similar value (13.22 mm), whereas the 3 h exposure resulted in a comparatively lower mean (12.53 mm). The maximum individual calyx width (15.35 mm) was recorded at 10 mM sodium azide after 2 h of treatment. Analysis of variance indicated that sodium azide concentration and the concentration × exposure duration interaction significantly influenced calyx width, whereas exposure duration alone was not significant. The increase in calyx width at moderate sodium azide concentrations

Table 1. Effect of sodium azide concentration and exposure duration on floral morphological traits of pomegranate (*Punica granatum* L. cv. 'Bhagwa') pooled data from two consecutive 2023–2024 Mrig Bahar seasons

Treatment	Sodium azide concentration (mM)	Calyx length (mm) 1 h	Calyx length (mm) 2 h	Calyx length (mm) 3 h	Calyx length (mm) Pooled mean	Calyx width (mm) 1 h	Calyx width (mm) 2 h	Calyx width (mm) 3 h	Calyx width (mm) Pooled mean	Petal length (mm) 1 h	Petal length (mm) 2 h	Petal length (mm) 3 h	Petal length (mm) Pooled mean	Petal width (mm) 1 h	Petal width (mm) 2 h	Petal width (mm) 3 h	Petal width (mm) Pooled mean
T1 (Control)	0 mM	34.32	34.32	34.32	**34.32**	12.41	12.41	12.41	**12.41**	21.71	21.71	21.71	**21.71**	18.35	18.35	18.35	**18.35**
T2	1 mM	33.78	35.34	34.72	**34.61**	12.51	12.71	12.57	**12.60**	22.99	21.90	23.41	**22.77**	19.60	17.03	20.10	**18.91**
T3	2 mM	35.26	36.98	34.69	**35.64**	13.46	13.13	12.91	**13.17**	23.25	22.94	21.27	**22.49**	20.63	19.04	18.99	**19.55**
T4	3 mM	35.77	36.90	35.72	**36.13**	13.81	13.45	12.21	**13.16**	23.32	22.23	21.79	**22.45**	19.06	18.76	18.34	**18.72**
T5	4 mM	35.15	35.88	35.10	**35.38**	14.04	12.46	12.73	**13.08**	22.61	22.03	23.20	**22.61**	20.64	18.28	18.86	**19.26**
T6	5 mM	37.33	37.15	35.77	**36.75**	13.81	13.93	12.34	**13.36**	22.60	22.41	21.88	**22.30**	19.37	18.45	18.83	**18.88**
T7	6 mM	36.01	37.78	34.45	**36.08**	14.35	12.88	12.88	**13.37**	23.46	23.01	21.45	**22.64**	19.78	18.24	18.98	**19.00**
T8	7 mM	34.93	37.50	33.77	**35.40**	13.16	13.77	13.10	**13.34**	23.44	23.91	21.95	**23.10**	20.24	19.44	19.03	**19.57**
T9	8 mM	33.78	35.81	32.90	**34.16**	13.01	13.30	12.13	**12.81**	21.52	22.47	21.86	**21.95**	19.85	18.52	19.91	**19.43**
T10	9 mM	35.73	33.95	34.78	**34.82**	12.58	12.04	12.42	**12.35**	21.92	21.91	20.94	**21.59**	18.25	17.99	18.42	**18.22**
T11	10 mM	34.43	40.62	34.13	**36.39**	12.25	15.35	12.10	**13.23**	21.17	24.77	21.41	**22.45**	18.51	18.26	19.28	**18.68**

Note: Values represent mean observations recorded under 1 h, 2 h, and 3 h sodium azide exposure durations. Pooled mean represents the mean value across the three exposure durations.

Table 2. Duration-wise mean ± standard error (SE) for floral traits

Exposure duration	Calyx length (mm)	Calyx width (mm)	Petal length (mm)	Petal width (mm)
Set A (1 h)	35.14 ± 0.61	13.22 ± 0.42	22.55 ± 0.48	19.48 ± 0.50
Set B (2 h)	36.57 ± 1.06	13.22 ± 0.53	22.66 ± 0.54	18.40 ± 0.35
Set C (3 h)	34.58 ± 0.48	12.53 ± 0.20	21.90 ± 0.44	19.01 ± 0.33

Table 3. Factorial analysis of variance for floral morphological traits of sodium azide-treated pomegranate (*Punica granatum* L. cv. 'Bhagwa') plants during the 2023-2024 Mrig Bahar season

Floral trait	Source of variation	F value	Significance	CD at 5%	CD at 1%
Calyx length	Sodium azide concentration	20.38	***	0.65	0.86
	Exposure duration	3.97	*	1.22	1.63
	Concentration × duration	2.59	*	2.13	2.83
Calyx width	Sodium azide concentration	6.60	**	0.44	0.59
	Exposure duration	1.66	NS	—	—
	Concentration × duration	1.96	*	1.45	1.93
Petal length	Sodium azide concentration	4.07	*	0.58	0.77
	Exposure duration	1.36	NS	—	—
	Concentration × duration	1.69	NS	—	—
Petal width	Sodium azide concentration	4.73	*	0.71	0.94
	Exposure duration	0.91	NS	—	—
	Concentration × duration	0.60	NS	—	—

Note: The factorial analysis was performed using plant-level observations from sodium azide-treated plants during the 2023-2024 Mrig Bahar season. Untreated control plants were used as set-wise descriptive reference plants and were not included in the factorial analysis. NS, non-significant; *, significant at P ≤ 0.05; **, significant at P ≤ 0.01; ***, significant at P ≤ 0.001. CD, critical difference.

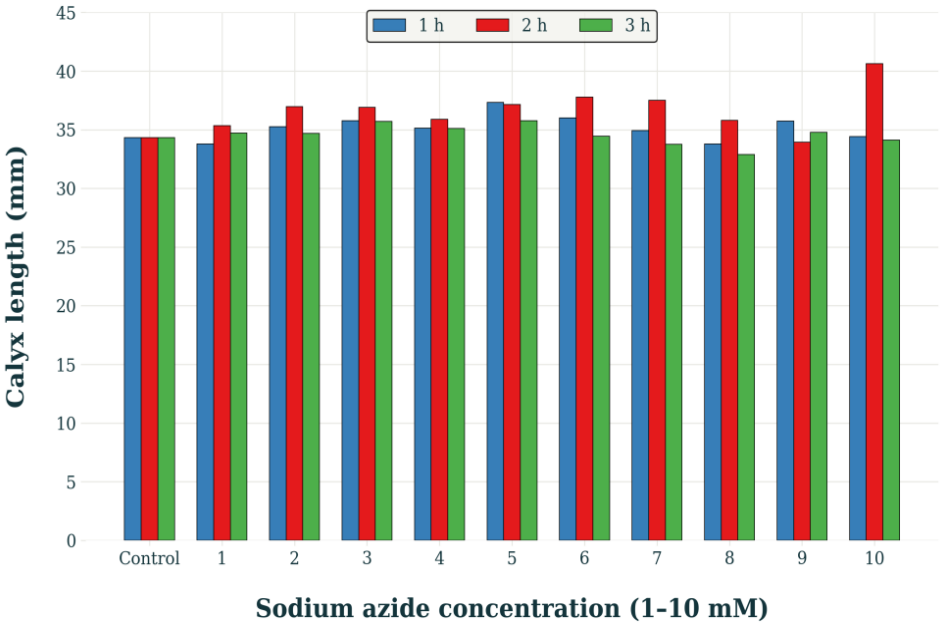


Fig. 1. Effect of sodium azide concentration and exposure duration on calyx length (mm) of pomegranate (*Punica granatum* L. cv. 'Bhagwa') during the 2023-2024 Mrig Bahar season. Values represent treatment means

suggests that optimized mutagenic treatments can enhance floral development in pomegranate, whereas excessive concentrations may reduce trait expression due to physiological stress (Oladosu *et al*., 2016; Wani *et al*., 2020).

Effect of sodium azide on petal length

Petal length varied significantly among the sodium azide treatments (**Table 1, Table 2 and Table 3; Fig. 3 and Fig. 5**). The pooled mean petal length ranged from 21.59 mm (9 mM) to 23.10 mm (7 mM). The 2 h

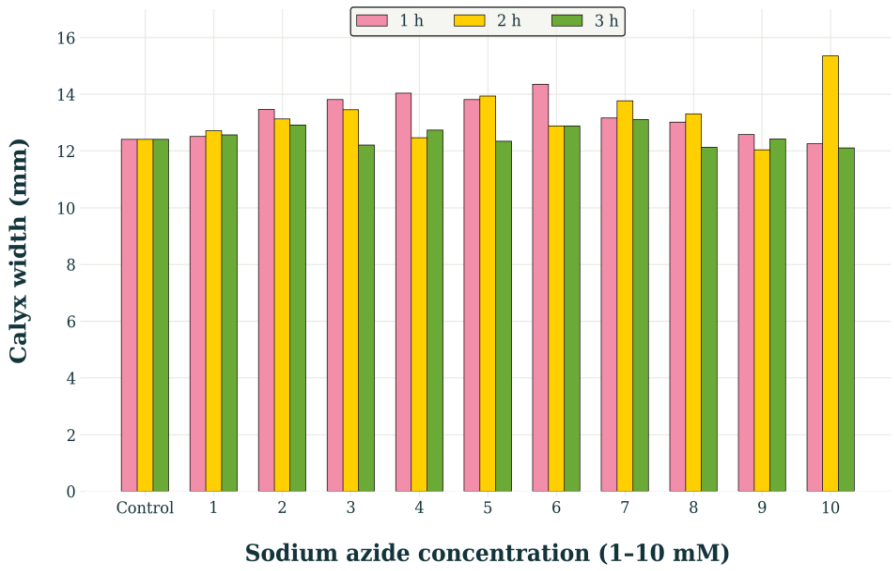


Fig. 2. Effect of sodium azide concentration and exposure duration on calyx width (mm) of pomegranate (*Punica granatum* L. cv. 'Bhagwa') during the 2023-2024 Mrig Bahar season. Values represent treatment means

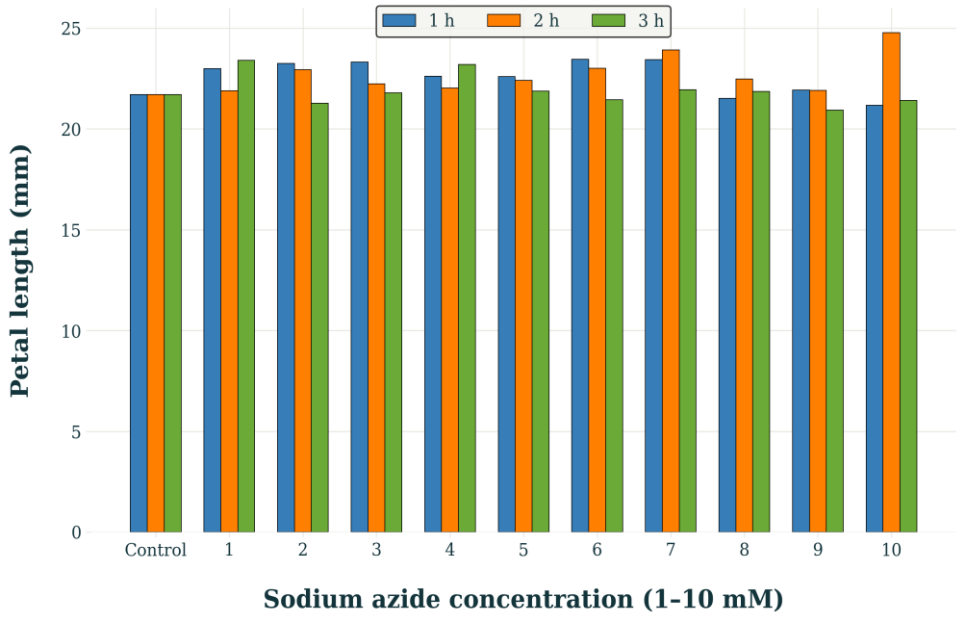


Fig. 3. Effect of sodium azide concentration and exposure duration on petal length (mm) of pomegranate (*Punica granatum* L. cv. 'Bhagwa') during the 2023-2024 Mrig Bahar season. Values represent treatment means

exposure produced the highest length (22.66 mm), while the maximum individual petal length (24.77 mm) was recorded under 10 mM sodium azide with a 2 h exposure time. Analysis of variance showed that sodium azide concentration significantly affected petal length, whereas exposure duration and the interaction effect were not significant. Petal morphology contributes to pollinator attraction and reproductive success; therefore, the enhanced petal length observed at moderate sodium azide concentrations may have a biological significance. Similar responses have been reported in mutation breeding studies on ornamental crops (Datta, 2017; Kumari *et al*., 2018).

Effect of sodium azide on petal width

Petal width also differed significantly among the sodium azide treatments (**Table 1, Table 2 and Table 3; Fig. 4 and Fig. 5**). The pooled mean petal width ranged from 18.22 mm (9 mM) to 19.57 mm (7 mM). The 1 h exposure recorded the highest value (19.48 mm), whereas the maximum individual petal width (20.64 mm) was observed under 4 mM sodium azide with 1 h exposure. Analysis

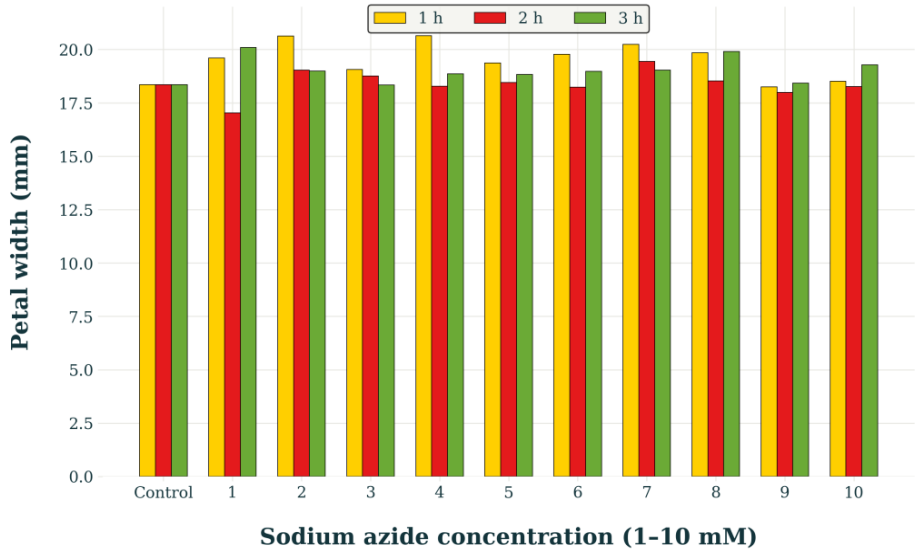


Fig. 4. Effect of sodium azide concentration and exposure duration on petal width (mm) of pomegranate (*Punica granatum* L. cv. 'Bhagwa') during the 2023-2024 Mrig Bahar season. Values represent treatment means

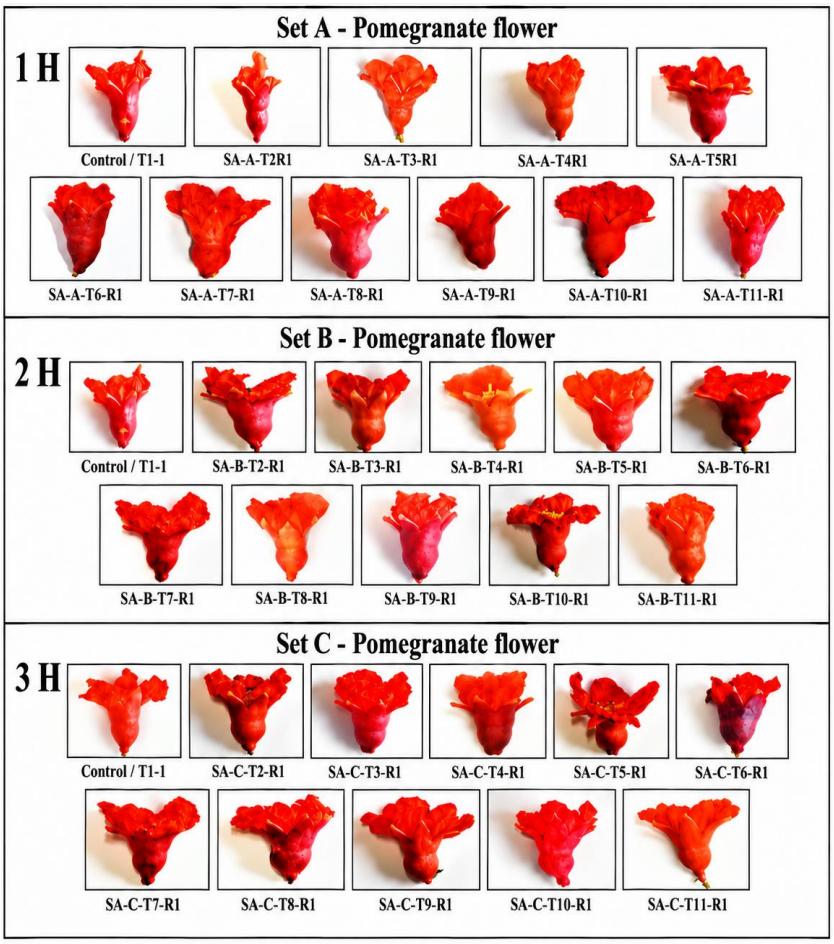


Fig. 5. Representative floral phenotypic variation among seed-derived M[1] pomegranate (*Punica granatum* L. cv. 'Bhagwa') plants following sodium azide treatment during the 2023-2024 Mrig Bahar season. Panels A, B, and C show representative flowers from Set A (1 h), Set B (2 h), and Set C (3 h), respectively. Photographs illustrate variation in calyx size, petal dimensions, and floral morphology

of variance revealed that sodium azide concentration significantly influenced petal width, whereas exposure duration and interaction effects were not significant. Moderate sodium azide concentrations were generally associated with greater petal width compared to higher concentrations, indicating that floral organs are differentially sensitive to chemical mutagenesis.

The present investigation demonstrated that sodium azide treatment significantly altered the floral morphological traits of pomegranate cv. 'Bhagwa'. Overall, moderate sodium azide concentrations (5–7 mM), particularly with a 2 h exposure, produced the most consistent floral responses, although some individual traits attained their maximum values at 10 mM sodium azide concentration. Significant differences were observed in calyx length, calyx width, petal length, and petal width, indicating that floral organs responded differentially to sodium azide concentration and exposure duration. As the present study was conducted on seed-derived M_1 plants, some of the observed variations may reflect natural segregation in this highly heterozygous species. Therefore, the floral modifications reported here should be regarded as putative mutagen-induced responses that require confirmation through the evaluation of subsequent generations and molecular characterization.

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