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Dose-Dependent Effects of Sodium Azide on Seed Germination, Seedling Survival, and Vegetative Growth Traits in Pomegranate (*Punica granatum* L. cv. 'Bhagwa')

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Abstract

Pomegranate (*Punica granatum* L. cv. 'Bhagwa') has a relatively narrow genetic base, necessitating the development of novel genetic variability for crop improvement. The present study was conducted at the ICAR–National Research Centre on Pomegranate, Solapur, India, to evaluate the effects of sodium azide (SA) on seed germination, seedling survival, and early vegetative growth traits, and to identify a biologically effective working dose for mutation breeding in pomegranate. Freshly harvested seeds of cv. Bhagwa were treated with ten sodium azide concentrations (1–10 mM) for 1, 2, and 3 h in a Completely Randomized Design with three replicates of 25 seeds each. Significant treatment effects were observed for seed germination, seedling survival, plant height, and secondary branches. The mean germination percentages were 73.91%, 72.73%, and 74.36% at 1, 2 and 3 h of exposure, respectively, while the corresponding seedling survival percentages were 75.91%, 71.30%, and 67.87%. Plant height varied considerably among treatments, indicating differential growth responses to mutagen exposure. Seedling survival after 2 h of exposure declined from 76.98% in the control to 60.18% at 5 mM SA, indicating moderate biological injury while maintaining adequate viability. Based on overall biological responses and population survival, 5 mM sodium azide for 2 h was identified as a biologically effective working dose for mutation breeding in pomegranate cv. 'Bhagwa'.

Abbreviations: SA- Sodium azide; mM- millimolar; h- hours; LD_{50}- Lethal dose required to kill 50% of the population; cv.- cultivar

***Key words*:** *Punica granatum*, Sodium azide, Mutation breeding, Induced variability, Seedling survival, Biologically effective dose

Pomegranate (*Punica granatum* L.) is an economically important fruit crop widely cultivated in arid and semi-arid regions because of its nutritional value, medicinal properties, and adaptability to water-limited environments [1-2]. Recent advances in pomegranate genetic resource conservation and breeding have underscored the need to broaden the genetic base of elite cultivars to sustain productivity, improve fruit quality, and achieve long-term improvements in crops [3]. In India, the cultivar 'Bhagwa' is the most commercially popular pomegranate variety because of its attractive rind color, soft-seeded arils, superior fruit quality, and export acceptability [4-5]. However, the large-scale cultivation of a limited number of elite cultivars has reduced their genetic variability and increased their vulnerability to biotic stress. Among these, bacterial blight caused by *Xanthomonas axonopodis* pv. *Punicae* is one of the most destructive diseases affecting pomegranate cultivation, significantly reducing its productivity and export potential [6-8]. Conventional genetic improvement of pomegranates is constrained by their perennial growth habit, prolonged juvenile phase, high heterozygosity, and complex inheritance patterns [9-10]. Hybridization-based breeding programs often require several years to evaluate and stabilize the desirable recombinants. Therefore, alternative breeding approaches that generate novel variations while preserving the genetic background of elite cultivars are increasingly important. Induced mutagenesis has emerged as an effective complementary strategy for crop improvement and has significantly contributed to the development of improved

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cultivars across several agricultural and horticultural species [11-12]. Mutation breeding enables the creation of novel allelic variations without extensive alterations to the existing genetic background. Recent reviews have highlighted the growing importance of induced mutagenesis in fruit crops for improving yield, stress tolerance, disease resistance, plant architecture, and fruit quality [13]. Among chemical mutagens, sodium azide (SA) is widely used for its effectiveness in inducing point mutations and generating useful phenotypic variability in plants [14-15]. The biological response to SA is strongly influenced by the genotype, mutagen concentration, treatment duration, and physiological condition of the treated material [16]. Excessive exposure to SA can cause severe biological injury, reduce germination, and increase seedling mortality, whereas moderate treatment can induce useful variability while maintaining sufficient plant viability for subsequent selection. Therefore, optimizing the mutagen dose is a critical prerequisite for successful mutation breeding programs. Early biological parameters, such as seed germination, seedling survival, plant height, and branching behavior, are commonly used as reliable indicators of mutagen sensitivity and treatment efficacy [17-18]. Determining a biologically effective working dose is particularly important in perennial fruit crops, where excessive mortality can substantially reduce breeding populations and delay progress in selection.

Although sodium azide-induced mutagenesis has been extensively studied in annual crops, information on dose–duration responses in perennial fruit crops is limited. In pomegranate, particularly in the commercially important cultivar 'Bhagwa', systematic studies to identify an appropriate biologically effective working dose are limited. Establishing cultivar-specific treatment thresholds is essential to maximize the induced variability while maintaining an adequate population size for mutant recovery. Therefore, the present study was undertaken to (i) evaluate the dose-dependent effects of sodium azide on seed germination and seedling survival, (ii) assess its effects on early vegetative growth and branching characteristics, and (iii) identify a biologically effective working dose suitable for mutation breeding in pomegranate cv. 'Bhagwa'. These findings provide baseline information for developing mutant populations and support future genetic improvement programs aimed at broadening the genetic variability of this commercially important fruit crop.

MATERIALS AND METHODS

Plant material and experimental site

Uniform, healthy, and fully mature pomegranate (*Punica granatum* L.) seeds of cv. 'Bhagwa' were obtained from the Fruit Culture Laboratory of the ICAR–National Research Centre on Pomegranate (NRCP), Solapur, Maharashtra, India (17°10′ N latitude, 74°42′ E longitude; 483.5 m above mean sea level). This cultivar was chosen for its commercial importance and export potential. The experiment was conducted under controlled laboratory conditions at 25 ± 2°C and 54 ± 5% relative humidity. Germinated seedlings were maintained in a protected nursery environment to ensure consistent growth conditions during early vegetative assessment.

Mutagen source and preparation of treatment solutions

Sodium azide (NaN_3; analytical grade, 99–105% purity) was procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Fresh sodium azide solutions were prepared immediately before use at concentrations ranging from 1 to 10 mM. The required quantity of sodium azide was dissolved in a freshly prepared 0.1 M phosphate buffer, and the pH was adjusted to 3.0. The acidic medium was maintained to facilitate the formation of the active mutagenic species derived from sodium azide during seed treatment. All solutions were prepared immediately before treatment and protected from direct light throughout the experimental period. Due to the hazardous nature of sodium azide, all handling procedures were performed using appropriate laboratory safety measures, including protective gloves, laboratory coats, and safety eyewear. Residual treatment solutions were disposed of in accordance with the institutional biosafety and chemical safety guidelines.

Seed pre-treatment and mutagenic treatment

Fully mature and healthy seeds of pomegranate (*Punica granatum* L. cv. 'Bhagwa') were thoroughly washed with running tap water to remove adhering pulp and surface contaminants from the fruit. Seeds were surface-sterilized in a 0.1% mercuric chloride ($HgCl_2$) solution for 5 min and then washed repeatedly with sterile distilled water to remove residual sterilant. Prior to mutagen treatment, seeds were soaked in distilled water for 24 h at room temperature (25 ± 2 °C) to enhance seed hydration and mutagen uptake. The pre-soaked seeds were subsequently exposed to sodium azide concentrations of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM for treatment durations of 1, 2, and 3 h. Treatments were conducted in conical flasks maintained on a rotary shaker at 120 rpm to ensure uniform contact between seeds and the mutagen solution. Untreated seeds soaked in distilled water were used as the control. Following treatment, seeds were thoroughly rinsed under running tap water for 10 min to eliminate residual sodium azide before further germination and growth assessment.

Experimental design and germination procedure

The experiment was conducted using a completely randomized factorial design (CRD) with the following factors:

- *Factor A*: Sodium azide concentration (11 levels, including a control)
- *Factor B*: Exposure duration (three levels: 1h, 2h, and 3h)

Each treatment combination was performed in triplicate, with 25 seeds per replicate (75 seeds per treatment). The treated seeds were placed in sterilized Petri dishes lined with Whatman No. 1 filter paper. A carbendazim-based fungicide (0.1%) was applied to prevent fungal contamination. The Petri dishes were incubated under controlled laboratory conditions in a growth chamber. Germination was recorded when the radicles emerged (≥2 mm). Subsequently, the germinated seedlings were carefully transplanted into pro-trays filled with sterilized coco-peat medium for further growth assessment and survival monitoring for up to 30 days after sowing (DAS).

Observations and data recording (30 DAG)

Seed germination percentage

The germination percentage was calculated based on the visible radicle emergence as follows:

Daily observations were recorded until germination was complete.

$$\text{Germination \%} = \frac{\text{No. of seeds germinated}}{\text{Total No. of seeds}} \times 100$$

Seedling survival percentage

Seedling survival was recorded 30 days after germination (DAG) to assess post-germination mortality due to mutagenic stress and was calculated as follows:

$$\text{Survival \%} = \frac{\text{No. of surviving seedlings at 30 DAG}}{\text{Total No. of seeds germinated}} \times 100$$

The reduction in survival relative to that of the control was used to assess mutagenic injury.

Vegetative growth parameters (30 DAS)

The following parameters were recorded:

Plant height (cm): Measured from the collar region to the apical meristem using a graduated scale.

Primary branches: Number of first-order branches arising from the main stem of a plant.

Secondary branches: Number of branches emerging from the primary branches.

Determination of biologically effective working dose

A biologically effective working dose was defined as the sodium azide treatment that produced moderate biological injury while maintaining sufficient seedling survival to recover useful variability in subsequent generations. The seedling survival percentage recorded 30 days after germination (DAG) was used as the primary biological indicator for dose optimization. Survival responses were evaluated across all tested concentrations of sodium azide and exposure durations. The treatment resulting in a measurable reduction in seedling survival while maintaining an adequately surviving population for subsequent selection was considered the biologically effective working dose for mutation breeding purposes. Because the experimental concentration range (1–10 mM) did not encompass the concentration required to achieve 50% mortality relative to the untreated control, the direct estimation of LD_{50} was not considered statistically reliable. Therefore, dose optimization was based on biological response patterns, survival reduction, and maintenance of population viability, rather than on LD_{50} estimation.

Statistical analysis

Data were analyzed using two-way analysis of variance (ANOVA) under a factorial Completely Randomized Design (CRD) using WASP 2.0 statistical software. The model included:

- Fixed effect of sodium azide concentration (Factor A)
- Fixed effect of treatment duration (Factor B)
- Interaction effect (A × B)

Significance was tested at $P \leq 0.05$ and $P \leq 0.01$. Whenever treatment effects were significant, mean comparisons were conducted using the Critical Difference (CD) test at the 5% significance level. The following descriptive statistics were calculated.

- Mean
- Standard deviation (SD)
- Standard error [$SE = SD/\sqrt{n}$]
- Coefficient of variation (CV%)

RESULTS AND DISCUSSION

Effect of sodium azide on seed germination

Sodium azide treatment significantly influenced seed germination in the pomegranate cv. 'Bhagwa'. The germination percentage generally declined with increasing mutagen concentrations, indicating a concentration-dependent inhibitory effect. The mean germination percentages across the exposure durations were 73.91% (1 h), 72.73% (2 h), and 74.36% (3 h). Two-way ANOVA revealed a significant main effect of sodium azide concentration ($P \leq 0.05$), whereas exposure duration and concentration × duration interaction were not statistically significant. Germination inhibition became more pronounced at higher concentrations, particularly beyond 6 mM (1 h), 5 mM (2 h), and 4 mM (3 h) (Table 1, Fig 1). Although minor fluctuations were observed in some intermediate treatments, the overall response showed reduced germination as mutagenic stress increased. Such variability is commonly observed in mutagenesis experiments and may reflect the biological heterogeneity among individual seedlings

Table 1 Effect of sodium azide concentration and treatment duration on seed germination, seedling survival, and growth parameters in pomegranate cv. 'Bhagawa'

Sodium Azide Treatments	Seed germination (%)			Seedling survival (%)			Plant height (cm)			Primary branches			Secondary branches		
(Conc.)	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h
Control	84	73	85	78.33	76.98	63.69	29.7	26.7	16.17	6.1	7.2	6.2	28.7	37.3	23.8
1 mM	84	73	88	84.51	84.9	71.45	31.9	35.3	17.9	7.3	6.2	4.9	24.7	38.4	22.3
2 mM	79	79	81	95.38	73.5	71.74	30.4	35.9	19.9	7.1	5.2	5.7	30.5	29.1	27
3 mM	77	71	73	91.03	81.03	74.67	30.4	31.5	19.5	6.3	5.7	5.8	33.1	26.9	32
4 mM	73	63	61	90.48	75.88	69.44	28.3	31.9	18.2	6.3	6	5.2	30.8	30.4	26.3
5 mM	65	60	72	68.83	60.18	85.71	26.6	31.4	16.1	6	6.1	5.4	30.8	28.5	25.8
6 mM	60	72	63	61.5	64.3	64.62	29.5	27.3	15.2	5.7	6.2	5.8	32.9	29.7	31.2
7 mM	64	77	72	62.73	77.83	76.81	28.5	29.4	13.4	5.8	6.1	5.2	35.1	27	28.6
8 mM	79	72	80	82.16	65.32	50.67	27.8	27.3	22.3	5.9	6.2	5.6	35.3	31.5	38
9 mM	72	75	71	48.06	71.56	50.4	24.7	19.7	16	5.8	5.1	5.5	34.6	26.3	30
10 mM	76	85	72	71.96	52.78	67.36	23.5	26.8	17.5	5.5	7	6	26.8	29.4	23

Effect on seedling survival

Seedling survival recorded 30 days after sowing exhibited greater sensitivity to sodium azide treatment than seed germination. The mean survival percentages were 75.91%, 71.30%, and 67.87% for 1, 2, and 3 h of exposure, respectively. Analysis of variance indicated a significant effect of sodium azide concentration on seedling survival ($P \leq 0.05$), whereas exposure duration and concentration × duration interaction effects were not significant (Table 3). Although a general decline in survival was observed as the sodium azide concentration increased, several treatments exhibited survival values comparable to or greater than those of the untreated control. These deviations from a strictly monotonic dose–response pattern may reflect biological variability among surviving seedlings, treatment-induced selection effects, or experimental heterogeneity. Similar inconsistencies have been reported in mutation-breeding studies using chemical mutagenesis. Under the 2 h exposure treatment, survival

decreased from 76.98% in the control to 60.18% at 5 mM sodium azide, indicating moderate biological injury while maintaining an adequate survival population. Therefore, this treatment was considered a biologically effective working dose for subsequent mutation-breeding applications rather than a true LD_{50} treatment (Table 1, Fig 2).

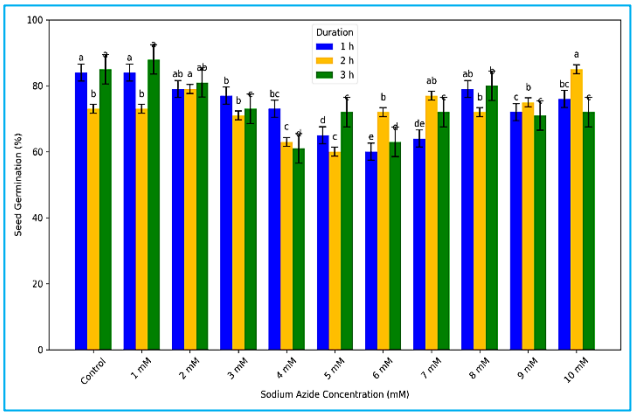

Fig 1 Effect of sodium azide concentration and exposure duration on seed germination (%) of pomegranate cv. 'Bhagawa'. Error bars represent the standard error of the mean (SE). Bars sharing at least one common letter are not significantly different at P ≤ 0.05 according to the Critical Difference (CD) test following two-way ANOVA, whereas bars with different letters indicate significant differences among treatments

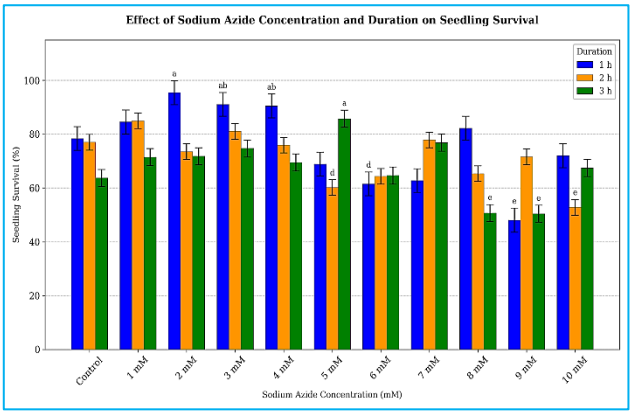

Fig 2 Influence of sodium azide concentration and exposure duration on seedling survival (%) of pomegranate cv. 'Bhagawa' was recorded 30 days after germination. Error bars represent the standard error of the mean (±SE). Different lowercase letters above the bars indicate significant differences among treatment means at P ≤ 0.05 according to the Critical Difference (CD) test following two-way ANOVA, whereas means sharing at least one common letter are not significantly different

Table 2 Descriptive statistics for seed germination, survival, and growth traits recorded under different SA treatment durations

Parameter	Statistic	1 h	2 h	3 h
Seed germination (%)	Mean	73.91	72.73	74.36
	Standard Deviation	7.96	11.46	8.51
	Standard Error	2.4	3.46	2.57
	Coefficient of Variation (%)	10.77	15.76	11.44
Seedling survival (%)	Mean	75.91	71.3	67.87
	Standard Deviation	14.7	9.58	10.51
	Standard Error	4.43	2.89	3.17
	Coefficient of Variation (%)	19.37	13.43	15.49
Plant Height (cm)	Mean	28.3	29.38	17.57
	Standard Deviation	2.54	4.57	2.44
	Standard Error	0.77	1.38	0.74
	Coefficient of Variation (%)	8.96	15.55	13.9
Primary Branches	Mean	6.16	6.09	5.61
	Standard Deviation	0.57	0.65	0.38
	Standard Error	0.17	0.19	0.12
	Coefficient of Variation (%)	9.21	10.42	6.78
Secondary Branches	Mean	31.2	30.4	28.15
	Standard Deviation	3.45	4	4.84
	Standard Error	1.04	1.21	1.46
	Coefficient of Variation (%)	11.04	13.16	17.2

Note: Descriptive statistics were calculated for each sodium azide concentration for each exposure duration
SD = standard deviation; SE = standard error (SD/√n); CV (%) = coefficient of variation expressed as a percentage
Observations were recorded at 30 DAG for seedling survival (%) and at 3 months for growth parameters

Effect on plant height

Plant height was measured in 3-month-old plants and varied significantly among the sodium azide treatments, with mean height of 28.30 cm (1 h), 29.38 cm (2 h), and 17.57 cm (3 h) (Table 2). A two-way analysis of variance revealed highly significant effects of concentration ($F = 3.35$, $P = 0.004$) and duration ($F = 81.65$, $P < 0.001$); however, the interaction effect was not significant ($P = 0.30$). The relatively low coefficient of variation (CV = 13.51%; Table 2) indicates good experimental precision. A biphasic response was observed in the present study. Moderate concentrations (1–2 mM) enhanced plant height relative to the control, with maximum values recorded at 1 mM (1 h): 31.9 cm; 2 mM (2 h): 35.9 cm; and 2 mM (3 h): 19.9 cm. However, higher concentrations (≥ 7 mM) significantly suppressed elongation, reflecting the inhibitory effects of excessive mutagenic stress (Table 1, Fig 3).

Effect on primary branching

The number of primary branches per plant exhibited relatively low variation across the sodium azide treatments (Table 1, Fig 4). The mean primary branch numbers were 6.16, 6.09, and 5.61 at 1, 2, and 3 h exposure durations, respectively (Table 2). The highest number of primary branches was recorded at 1 mM under 1 h exposure (7.3 branches per plant),

whereas the lowest value was observed at 1 mM under 3 h exposure (4.9 branches per plant). Analysis of variance indicated that sodium azide concentration had no significant effect on primary branch development (F = 0.86, P = 0.58), and the concentration × duration interaction was also non-significant (P = 0.17). However, exposure duration significantly influenced the primary branch number (F = 4.32, P = 0.02) (Table 3). The coefficient of variation (11.40%) indicates acceptable experimental precision. Overall, the results suggest that primary branching at the early seedling stage was relatively stable and less responsive to sodium azide treatment than the other growth parameters evaluated in the present study.

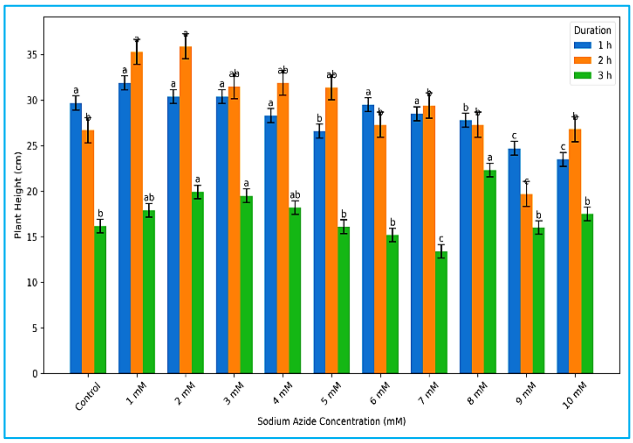

Fig 3 Effect of sodium azide concentration and exposure duration on plant height (cm) of pomegranate cv. 'Bhagawa'. Error bars represent the standard error of the mean (±SE). Different lowercase letters above the bars indicate significant differences among treatment means at P ≤ 0.05 according to the Critical Difference (CD) test following two-way ANOVA, whereas means sharing at least one common letter are not significantly different

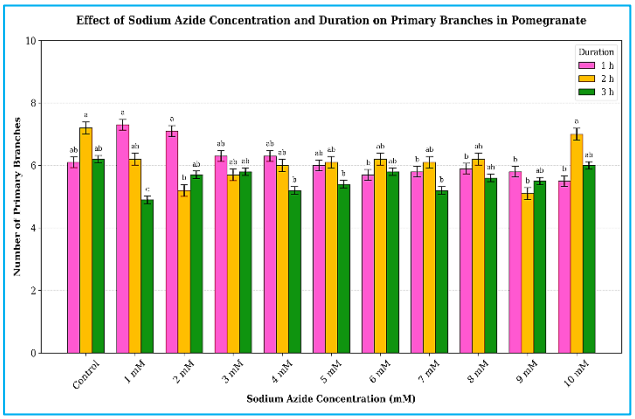

Fig 4 Effect of sodium azide concentration and exposure duration on the number of primary branches per plant in pomegranate cv. 'Bhagawa'. Error bars represent the standard error of the mean (±SE). Different lowercase letters above the bars indicate significant differences among treatment means at P ≤ 0.05 according to the Critical Difference (CD) test following two-way ANOVA, whereas means sharing at least one common letter are not significantly different

Effect on secondary branching

Secondary branching showed pronounced variability across all treatments. The mean secondary branch numbers were 31.20 (1 h), 30.40 (2 h), and 28.15 (3 h) (Table 2). ANOVA revealed significant effects of concentration (F = 4.25, P = 0.001), duration (F = 7.99, P = 0.002), and their interaction (F = 5.28, P = 0.001) (Table 3). A low CV (8.80%; Table 2) indicates a high experimental reliability. Enhanced secondary branching relative to the control was observed for specific treatments, notably 8 mM (1 h), 1 mM (2 h), and 8 mM (3 h). The significant interaction effect indicates that the response of secondary branching depends on the combined influence of concentration and exposure duration (Table 1, Fig 5).

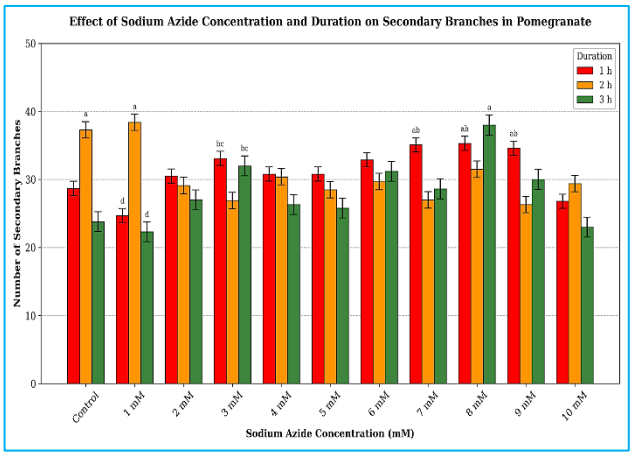

Fig 5 Effect of sodium azide concentration and exposure duration on the number of secondary branches per plant in pomegranate cv. 'Bhagawa'. Error bars represent the standard error of the mean (±SE). Different lowercase letters above the bars indicate significant differences among treatment means at P ≤ 0.05 according to the Critical Difference (CD) test following two-way ANOVA, whereas means sharing at least one common letter are not significantly different

Dose optimization for mutation breeding

Among the evaluated exposure durations, the 2 h treatment produced substantial variability in germination, seedling survival, and vegetative growth traits, while maintaining acceptable plant viability. Therefore, this exposure duration was considered the most suitable for dose optimization (Table 1-2). Seedling survival generally declined with increasing sodium azide concentrations; however, treatment responses were not strictly monotonic across all concentrations. Certain treatments exhibited survival values comparable to or greater than those of the untreated control, indicating biological variability among the surviving seedlings and possible treatment-induced selection effects. Under 2 h exposure, seedling survival decreased from 76.98% in the untreated control to 60.18% at 5 mM sodium azide, representing moderate biological injury while retaining a sufficiently large surviving population (Table 1, Fig 2). This treatment also generated measurable variations in vegetative growth traits without causing excessive mortality. As the tested concentration range did not encompass true LD_{50} conditions, the present study did not attempt to estimate LD_{50}. Based on the overall biological response patterns, survival reduction, and maintenance of adequate plant viability, a 2 h exposure to 5 mM sodium azide was identified as a biologically effective working dose for mutation breeding in pomegranate cv. 'Bhagwa'. This treatment is expected to facilitate the generation of useful variability while maintaining an adequate population size for subsequent selection and evaluation (Table 1, Fig 2).

Mutation breeding has become an important complementary approach to crop improvement, particularly for perennial fruit crops, where conventional breeding is constrained by long juvenile periods, high heterozygosity, and complex inheritance patterns [18-19]. In this study, sodium azide treatment affected seed germination, seedling survival, and vegetative growth traits in pomegranate (*Punica granatum* L. cv. 'Bhagwa'). Responses varied with concentration and exposure duration, indicating that biological traits differ in their

sensitivity to mutagenic treatments. Despite reduced germination and survival at higher concentrations, several treatments maintained satisfactory plant viability while producing measurable changes in growth-related traits. These findings suggest that sodium azide can serve as a useful mutagen to induce phenotypic variability in pomegranate and provide baseline information for developing mutant populations for future selection and breeding programs.

Effect of sodium azide on seed germination and seedling survival

The present study demonstrated that sodium azide treatment affected both seed germination and seedling survival in pomegranate cv. 'Bhagwa'. Although response magnitudes varied among treatments, a general decline in germination and survival was observed as mutagen concentration increased, indicating greater biological stress at higher exposure levels. The reduction in germination observed at elevated sodium azide concentrations may be attributed to physiological disturbances that occur during the early stages of seed metabolism and, consequently, during seedling establishment. Similar reductions in germination following sodium azide treatment have been reported in several crop species and are commonly regarded as indicators of mutagen-induced biological injury [9], [16]. However, the precise cellular and molecular mechanisms responsible for these responses were not investigated in the present study and therefore require further validation through cytological and molecular analyses. Interestingly, some treatments showed germination and survival rates comparable to or exceeding those of the untreated controls. Such deviations from a strictly monotonic dose–response pattern have also been reported in mutation-breeding studies and may reflect biological variability among seedlings, differential seed vigor, treatment-induced selection effects, or experimental heterogeneity. Consequently, these responses should be interpreted cautiously when identifying suitable treatment levels for breeding. The observed reduction in seedling survival at intermediate and higher sodium azide concentrations suggests that mutagenic stress increased with dose, whereas moderate treatment levels maintained sufficient viability to enable the recovery of potentially useful genetic variability in subsequent generations [11-12].

Table 3 Two-way factorial ANOVA for the effect of different doses of SA on seed germination, seedling survival, plant height, and primary and secondary branches of pomegranate cv. 'Bhagawa'

Source of variation	F-value	P-value	CD (5%)	CD (1%)
Seed germination %				
Treatment	1.71	0.03*	–	–
Factor A (Concentration)	2.43	0.02*	11.66	15.49
Factor B (Duration)	1.23	0.30ns	6.09	8.09
A × B	1.4	0.16ns	20.2	26.84
CV (%)	17	–	–	–
Seedling survival %				
Treatment	1.62	0.05*	–	–
Factor A (Concentration)	2.4	0.02*	17.15	22.79
Factor B (Duration)	1.95	0.15ns	8.96	11.9
A × B	1.2	0.29ns	29.7	39.47
CV (%)	25.71	–	–	–
Plant height (cm)				
Treatment	6.91	<0.001**	–	–
Factor A (Concentration)	3.35	0.004**	3.99	5.36
Factor B (Duration)	81.65	<0.001**	2.08	2.8
A × B	1.22	0.30ns	6.9	9.28
CV (%)	13.51	–	–	–
Primary branches				
Treatment	1.15	0.15ns	–	–
Factor A (Concentration)	0.86	0.58ns	0.8	1.07
Factor B (Duration)	4.32	0.02*	0.42	0.56
A × B	1.46	0.17ns	1.38	1.86
CV (%)	11.4	–	–	–
Secondary branches				
Treatment	5.13	<0.001**	–	–
Factor A (Concentration)	4.25	0.001**	3.1	4.16
Factor B (Duration)	7.99	0.002**	1.62	2.17
A × B	5.28	0.001**	5.36	7.21
CV (%)	8.8	–	–	–

Note: Two-way factorial ANOVA was performed under a Completely Randomized Design (CRD) using WASP 2.0 statistical software. Factor A = sodium azide concentration (11 levels, including the control); Factor B = exposure duration (1, 2, and 3 h); A × B = interaction between concentration and duration. CD = critical difference; CV (%) = coefficient of variation. * Significant at P ≤ 0.05; ** Significant at P ≤ 0.01; ns = non-significant

Effect of sodium azide on plant height

Plant height showed variable responses to sodium azide treatment, indicating the differential sensitivity of vegetative growth to mutagen exposure. While higher concentrations generally reduced plant height, several low and intermediate concentrations produced growth responses comparable to or greater than those of the untreated controls. The enhanced plant height observed at selected lower concentrations may reflect a hormetic response, characterized by the stimulation of biological performance at low stress levels, followed by

inhibition at higher exposure levels. Similar biphasic responses have been reported in mutation-breeding studies using chemical and physical mutagenesis methods [14], [17]. However, the present study did not include physiological or biochemical measurements to confirm the mechanisms underlying these responses. The reduction in plant height at higher sodium azide concentrations may reflect the increased biological injury and metabolic disruption associated with mutagen exposure. Nevertheless, the considerable variation among treatments suggests that plant growth responses were influenced by both mutagen concentration and inherent biological variability among seedlings. The results indicate that moderate sodium azide treatment can influence early vegetative growth without causing severe growth suppression, thereby providing opportunities for the recovery of phenotypically diverse individuals in subsequent generations.

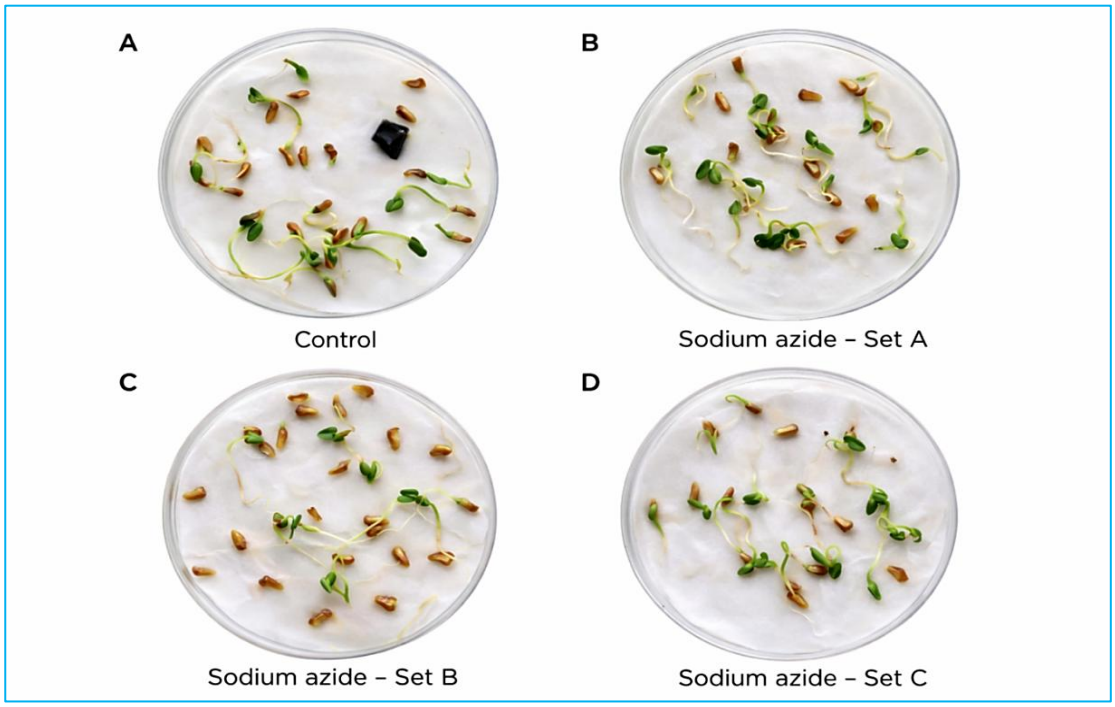

Fig 6 Visual observation of seed germination and seedling development in pomegranate cv. 'Bhagawa' under control and sodium azide (SA) treatments 30 days after germination

(A) Control (distilled water) showing normal radicle emergence and seedling vigor. (B) Seedlings from SA treatments with a 1 h exposure duration (Set A), (C) seedlings from SA treatments with a 2 h exposure duration (Set B), and (D) seedlings from SA treatments with a 3 h exposure duration (Set C), illustrating the effects of SA on seed germination and early seedling growth

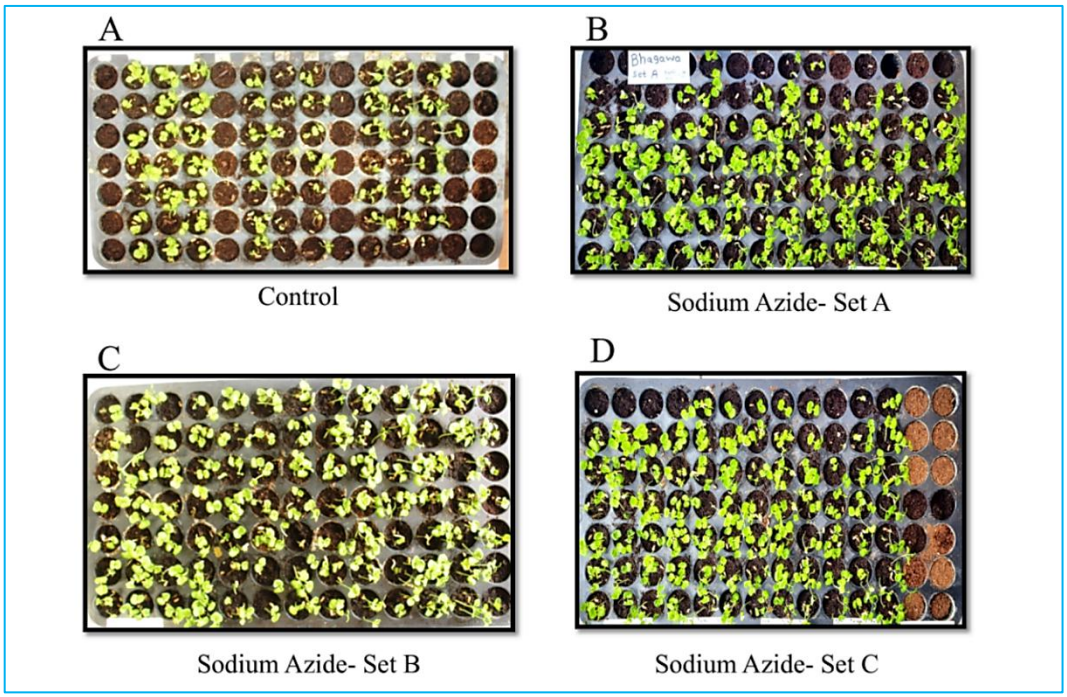

Fig 7 Effect of sodium azide (SA) on seedling establishment and vigor of pomegranate (*Punica granatum* L. cv. 'Bhagawa') in pro-trays 30 days after sowing

(A) Control, showing uniform seedling growth and establishment in the cocopeat medium. (B) Seedlings from sodium azide treatments with a 1 h exposure duration (Set A). (C) Seedlings from sodium azide treatments with a 2 h exposure duration (Set B). (D) Seedlings from sodium azide treatments with a 3 h exposure duration (Set C) showed variations in seedling survival, vigor, and leaf development

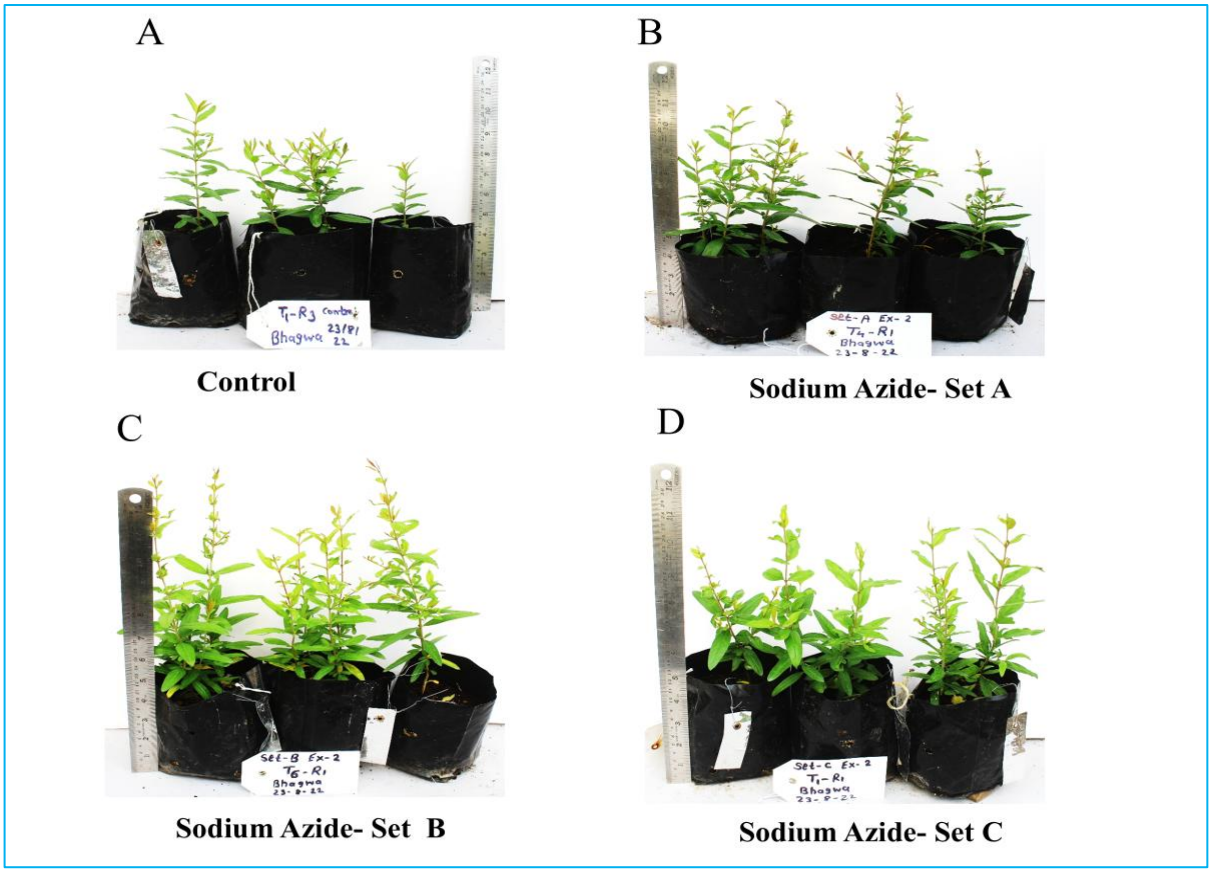

Fig 8 Effect of sodium azide (SA) on plant growth parameters of pomegranate (*Punica granatum* L. cv. 'Bhagawa') 3-month-old plants (A) Control, (B) seedlings from sodium azide treatment with 1 h exposure duration (Set A), (C) seedlings from sodium azide treatment with 2 h exposure duration (Set B), and (D) seedlings from sodium azide treatment with 3 h exposure duration (Set C), showing variation in plant height and primary and secondary branching characteristics

Effect on primary and secondary branch development

Branching behavior is an important architectural trait that influences canopy structure, light interception, and fruit-bearing capacity in perennial fruit crops. In the present study, sodium azide treatment produced measurable changes in both primary and secondary branch numbers, indicating that vegetative architecture is responsive to the exposure to mutagens. The responses of the branching traits were not uniform across all treatment levels. Some concentrations yielded branch numbers comparable to or exceeding those of the untreated control, whereas others moderately reduced the branch numbers. Such variability is commonly observed in mutation-breeding experiments and may reflect differences in the physiological status of individual seedlings, treatment-induced selection effects, or genetic variability induced by mutagen exposure [11], [14]. Because this study was conducted at an early stage of development, the differences in branching should be viewed as initial signs of induced variability, rather than permanent genetic changes. To confirm whether these traits are inherited and stable, further testing in more advanced mutant generations is necessary. Nevertheless, the observed variation in branching characteristics demonstrates the potential of sodium azide treatment to generate architectural diversity in pomegranate, which may prove valuable for future selection and breeding programs.

Dose optimization and implications for mutation breeding

The identification of an appropriate mutagen treatment is a critical prerequisite for successful breeding programs in plants. An effective treatment should induce sufficient biological stress to generate genetic variability while maintaining an adequately surviving population for subsequent selection and evaluation [12], [17]. In this study, sodium azide treatment had measurable effects on germination, seedling survival, plant height, and branch characteristics. Among the evaluated treatment combinations, a 2 h exposure to 5 mM sodium azide caused moderate biological injury while maintaining satisfactory seedling survival and vegetative growth levels. Therefore, this treatment was considered a biologically effective working dose for mutation breeding in the pomegranate cv. 'Bhagwa'. It is important to note that the experimental concentration range used in this study did not encompass the conditions required to directly estimate the true LD_{50}. Consequently, the selected treatment should not be interpreted as an LD_{50} dose but rather as a practical working dose based on the overall patterns of biological response and maintenance of population viability. The findings of the present study provide baseline information for developing mutant populations in pomegranates and may facilitate the selection of desirable variants in the future. Nevertheless, confirmation of induced genetic variability will require the evaluation of subsequent mutant generations using morphological, physiological, and molecular analyses. In the present study, we primarily evaluated the biological responses to sodium azide treatment in the M_1 generation. Therefore, the observed variations in germination, survival, plant height, and branching characteristics should be considered preliminary indicators of induced variability rather than confirmed heritable mutations. Further evaluation of subsequent generations (M_2 and beyond) is necessary to determine the stability, inheritance, and breeding values of the observed traits. Such investigations may also facilitate the estimation of mutagenic effectiveness and efficiency, thereby providing a more comprehensive assessment of sodium azide-induced variability in pomegranate breeding programs [12], [17], [20].

CONCLUSION

The present study demonstrated that sodium azide treatment significantly influenced seed germination, seedling survival, plant height, and branching characteristics in pomegranate cv. 'Bhagwa'. Increasing mutagen concentrations generally resulted in greater biological injury; however, treatment responses varied among concentrations and exposure durations. Among the evaluated treatments, 2 h of exposure to 5 mM sodium azide moderately reduced seedling survival while maintaining sufficient plant viability and generating measurable variation in vegetative traits. Therefore, this treatment was identified as a biologically effective working dose for mutagenesis breeding. The observed variability in growth and architectural traits indicates the potential of sodium azide to generate useful phenotypic diversity in pomegranate. However, confirmation of induced genetic changes will require the evaluation of subsequent generations using morphological, physiological, and molecular approaches. The results provide a foundation for developing mutant populations in pomegranate and may inform future breeding programs aimed at broadening the genetic base of elite cultivars, such as 'Bhagwa.'

Author contributions

S. R. B. conceptualized the study, conducted the experiments, performed data curation and statistical analyses, and prepared the manuscript draft. S. P. contributed to the conceptualization and methodology of the study, provided technical guidance, supervised the research activities, and critically reviewed and edited the manuscript. M. N. J. provided academic supervision, research guidance, validation of the study, and critical reviews of the manuscript. A. R. G. and T. H. D. assisted in the execution of the experiments, the collection and recording of data, and the maintenance of experimental materials. R. A. M. provided overall supervision, institutional support, manuscript review and editing, and approved the final version of the manuscript. All the authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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