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Research Article

Unveiling New Insights Into Faba Bean Sensitivity and Genetic Responses to the Mutagen Agent EMS (Ethyl Methanesulfonate)

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Mutagenesis breeding via ethyl methanesulfonate (EMS) has been successfully used in faba bean to improve some economically important traits. However, there is a knowledge gap of the factors/mechanisms related to its sensitivity/tolerance to EMS treatment toxicity. It was hypothesized that the seed size could influence the response of the diverse botanical varieties of faba bean. Consequently, we conducted a comprehensive assessment of the sensitivity of six faba bean varieties: three major varieties (Aguadulce superlonga, Reina mora, and Yasmine) and three minor varieties (Zina, Alfia 05, and Alfia 17), to three increasing concentrations (0.05%, 0.5%, and 1%, along with a control 0%) of EMS. Analyses included various germination parameters (germination percentage [GP], germination energy at seven and 14 days [GE7 and GE14], germination rate index [GRI], and vigor index [VI]) across different EMS concentrations. To further explore mechanisms involved in sensitivity to EMS, we measured coat thickness and assessed antioxidant activity. Our findings revealed that the seed size variation did not significantly affect EMS sensitivity. Different varieties showed significant responses to increasing EMS concentrations ($p \leq 0.05$) for most parameters, except for root length. This challenges the assumption that the seed size influences EMS sensitivity in faba bean. Coat thickness was uniform, suggesting similar EMS absorption. DPPH assay revealed significant antioxidant activity differences between nontreated and EMS-treated groups. Antioxidant activity correlated significantly with germination parameters under EMS treatment. The study indicates factors beyond seed size contribute to EMS responses. Examining antioxidant systems may explain plants' ability to counteract EMS-induced oxidative stress.

Keywords: antioxidant activity; germination parameters; mutagenesis; seed sensitivity; seed size; *Vicia faba*

Summary

- Significant differences observed in faba bean varieties' responses to increase concentrations of EMS across various germination parameters.
- Contrary to the hypothesis, the variation in the seed size did not significantly affect sensitivity to EMS in faba bean.
- Uniformity in coat thickness indicated similar patterns of EMS absorption across different faba bean varieties.
- Significant variations in antioxidant activity between nontreated and EMS-treated groups were observed, suggesting adaptations in antioxidant defense mechanisms.

1. Introduction

Faba beans (*Vicia faba* L.), commonly known as broad beans, are an essential legume with significant global importance. They serve as a vital source of plant-based protein, with protein content ranging from 20% to 41%, surpassing many other pulses such as lima, pinto, and red kidney beans [1]. Beyond their nutritional value, faba beans play a crucial role in sustainable agriculture due to their exceptional nitrogen-fixing ability. They can derive up to 96% of their nitrogen from the atmosphere [2], enriching soil fertility, reducing dependency on synthetic nitrogen fertilizers, and mitigating greenhouse gas emissions associated with fertilizer production and application.

Despite their numerous benefits, faba bean cultivation faces considerable challenges due to susceptibility to various biotic and abiotic stresses, including pests, diseases, drought, and salinity. These constraints limit productivity and necessitate the development of improved cultivars with enhanced resistance.

Among various breeding approaches, mutation breeding has emerged as a powerful tool for crop improvement [3]. By inducing novel genetic variations, it enables the selection of desirable traits. Ethyl methanesulfonate (EMS), one of the most widely used chemical mutagens, is particularly effective in inducing point mutations—primarily G/C to A/T transitions—thereby generating beneficial genetic variability [4, 5].

EMS has been extensively employed in mutation breeding programs to enhance key agronomic traits in various crops, including legumes such as faba beans. It has played a crucial role in developing improved plant varieties, contributing to 2947 mutant cultivars worldwide, including 486 legume varieties, 20 of which are faba bean cultivars [6]. Whether through chemical or physical mutagenesis, mutation breeding has significantly advanced crop improvement across diverse species, leading to increased yield, enhanced disease resistance, and improved adaptability.

In legumes, several success stories highlight the impact of induced mutations. For example, in Pakistan, chickpea (*Cicer arietinum*) mutant varieties such as "CM-72," "CM-88," and "CM-98" exhibited resistance to *Ascochyta* blight and *Fusarium* wilt, boosting productivity [7]. Similarly, in India, the urad bean (*Vigna mungo*) mutant variety "TAU-1" was widely adopted in Maharashtra, generating an estimated $64.7 million in additional production between 1989 and 1999 [7]. Beyond legumes, mutagenesis has also led to notable improvements in cereals and industrial crops. One of the most impactful mutant cultivars is Golden Promise barley (*Hordeum vulgare*). Its short-statured, semi-dwarf phenotype enhanced lodging resistance, making it well-suited for high-input farming systems in Europe [8]. Golden Promise also demonstrated superior malting quality, establishing itself as a preferred variety for the brewing industry [7]. Other successful cases include peppermint (*Mentha piperita*) mutant varieties, such as "Todd's Mitcham" and "Murray Mitcham," which provided resistance to *Verticillium* wilt, securing their dominance in the U.S. peppermint oil industry [9]. In fruit crops, the Rio Red grapefruit, known for its deep red flesh color and enhanced flavor, is another example of the commercial success of mutation breeding [10].

Published studies indicate a wide spectrum of EMS concentrations used in faba bean mutagenesis. Some studies have reported the use of relatively low EMS concentrations, such as 0.01%–0.05% [11–15] aiming to induce subtle genetic modifications while preserving overall plant viability. In contrast, other investigations have pushed mutagenesis with higher concentrations, surpassing 0.1% and reaching 1% [16–19], provoking more extensive genetic alterations. Moreover, botanical varieties of faba bean are often overlooked in EMS mutagenesis applications. Studies on *Vicia faba* L. and other species have hypothesized that the seed size could influence sensitivity to EMS-induced mutations, with findings showing higher sensitivity in small-seeded varieties compared to the large-seeded varieties [20]. This hypothesis, substantiated across various species in numerous studies, consistently links differences in responses to the seed size of tested varieties [21, 22], suggesting that larger seeds might possess greater genetic buffering capacity, potentially mitigating mutagenic effects and toxicity compared to smaller seeds. In addition, the physical properties of seeds, such as seed coat thickness, could influence mutagenic responses. Tepfer and Leach [23] demonstrated that seed coat thickness affects resilience to radiation, suggesting it may also play a role in chemical mutagenesis [24].

Given these knowledge gaps, this study aims to evaluate the responses of six faba bean varieties, representing both major and minor botanical types, to different EMS concentrations. By assessing germination parameters, the research will examine the correlation between seed size and EMS sensitivity. Furthermore, the study will investigate factors contributing to differential EMS responses, particularly focusing on antioxidant activity and physical properties such as seed coat thickness. The findings will provide deeper insights into the mutagenic behavior of faba beans, contributing to optimized EMS application strategies for faba bean improvement.

2. Materials and Methods

2.1. Experimental Protocol

2.1.1. Plant Materials. In this study, six faba bean varieties were employed (Table 1), comprising three major varieties (Aguadulce superlonga, Reina mora, and Yasmine) and three minor varieties (Zina, Alfia 05, and Alfia 17).

2.1.2. EMS Treatment. The seeds underwent an initial 8 h presoaking in distilled water. Subsequently, they were subjected to three distinct concentrations of EMS (v:v) (0.05%, 0.5%, and 1%), along with a control group (0% EMS). This exposure occurred over 7 h under shaking conditions, utilizing a 2% DMSO solution, following the protocol outlined by Jankowicz-Cieslak and Till in [25]. To halt the treatment, the seeds were washed in a 3% thiosulfate solution for 16 h and subsequently rinsed three times with distilled water, each rinse lasting 15 min.

2.1.3. Experimental Design. A randomized complete block design (RCBD), with three replicates, was used to plant 50 seeds of each variety subjected to a single treatment. The

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Table 1: List of faba bean (*Vicia faba* L.) varieties used: name, registering institution, release year, and key seed characteristics.

Name	Institution	Year of release	Characteristics
Aguadulce superlonga	AGRIN	2003	Large-sized grains with a beige coat and a black hilum
Reina mora	SEMILLAS FITO	2004	Large-sized grains with a purple coat and black hilum
Yasmine	INRA Morocco	2022	Medium- to large-sized grain with a beige coat and black hilum
Zina	INRA Morocco	2018	Small- to medium-sized grain with a beige coat and black hilum
Alfia 05	INRA Morocco	1986	Small rounded grain with a beige coat and black hilum
Alfia 17	INRA Morocco	1986	Small rounded grain with a beige coat and black hilum

seeds were sown in cell trays distributed across three separate benches, with each bench designated for a specific replication. The trial was conducted within a greenhouse environment where the emergence of seedlings and plantlets was counted on a daily basis.

2.2. Measurements and Calculations. Using data from the number of seedlings that emerged, root length, shoot length, and the fresh weight of plantlets, various germination indices have been computed over a 21-day span, marking the end of the experimentation period. This endpoint was determined as new seedlings ceased to emerge, and old ones initiated the display of etiolation.

2.2.1. Germination Percentage (GP). The GP illustrates the number of seeds germinated per 50 seeds sown in each replicate and treatment, which was calculated using the following equation:

$$\mathrm{GP} = \frac{\text{Final number of seedlings emerged}}{\text{Total number of seeds sown}} \times 100. \tag{1}$$

2.2.2. Germination Energy (GE). GE refers to the percentage of seeds that have sprouted within a specific timeframe, typically observed over a span of 7–14 days after seeding (GE7 and GE14, respectively). It serves as a measure of the speed at which germination occurs, providing insights into the vigor and robustness of seedlings. GE was calculated using the following formula [26]:

$$\mathrm{GE} = \frac{\text{Germinated seeds after 7 (or 14 days)}}{\text{Total germinated seeds after 21 days}} \times 100. \tag{2}$$

2.2.3. Germination Rate Index (GRI). The GRI value, which characterizes the percentage of germination on particular days, was computed using the following equation [27]:

$$\mathrm{GRI} = \frac{G1}{1} + \frac{G2}{2} + \frac{G3}{3} + \cdots + \frac{Gx}{x} \times 100, \tag{3}$$

where $G1$ and $G2$ represent the GP on the first and second days after sowing, respectively, and Gx represents the final GP.

2.2.4. Vigor Index (VI). VI was calculated according to Bewley and Black [28] using the following formula:

$$\mathrm{VI} = (\text{Mean shoot length} + \text{Mean root length}) \times \text{Germination percentage}. \tag{4}$$

The average plantlet weight (g) was also recorded.

2.3. Evaluation of Seed Coat Thickness. We conducted an assessment of seed coat thickness across the six different varieties to investigate its potential positive correlation with resistance/sensitivity to EMS toxicity. Employing a micrometer, we measured two distinct areas of the seed coat from three healthy seeds within each variety. Our primary objective was to determine if seed coat thickness demonstrates a positive correlation with parameters assessed under EMS concentrations of 0.5%. This specific concentration was selected due to its previously observed significant induced toxicity across all tested varieties.

2.4. Assay of Antioxidant Activity. We conducted an assessment of antioxidative properties of the six aforementioned varieties. Each variety was subjected to a 0.5% EMS concentration, selected for its ability to induce stress without resulting in a high frequency of lethality, alongside a control group (0% EMS). This investigation utilized the DPPH free radical scavenging assay employing the spectrophotometric method described by Braca et al. [29] with some adjustments. Each treatment comprised 1 g of seed powder from each variety and was replicated two times. To execute the assay, 10 mL of extracts with concentrations of 4, 8, 12, 16, and 20 mg/mL were combined with 3 mL of a methanolic DPPH solution (0.1 mM). The resulting mixture was then incubated in darkness for 30 min before optical density measurements were taken at 517 nm. A blank solution without the extract served as a reference. Antioxidant activity was quantified as the percentage of free radical inhibition, which was calculated using the provided formula.

$$\%\,\text{inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100, \tag{5}$$

where A_{control} represents the absorbance of the DPPH solution without the seed extract, while A_{sample} denotes the absorbance of the DPPH solution with the seed extract.

Low inhibition in a DPPH scavenging assay indicates that the genotype has a weaker ability to neutralize or scavenge the free radicals (DPPH radicals) present in the solution. High inhibition percentages imply strong antioxidant activity, as the genotype is efficient at trapping and neutralizing free radicals [30, 31].

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2.5. Statistical Analyses. To evaluate the sensitivity to EMS treatment, we analyzed seed germination and seedling growth parameters using a two-way analysis of variance (ANOVA). The study focused on two primary comparisons: (1) the interaction between seed size (botanical variety: major vs. minor) and EMS concentration and (2) the interaction between genotype (individual varieties) and EMS concentration. These analyses were consolidated into a single framework, with EMS treatment serving as the common factor. Also, the ANOVA contrast was conducted to compare the antioxidant activity between seeds subjected to EMS and those that were nonstressed for each variety/genotype. In case of significant differences among the genotypes studied, their mean comparison was done via Tukey post hoc test.

To explore relationships between key variables under EMS stress, we constructed a Pearson correlation matrix. This analysis examined associations among germination parameters under 0.5% EMS treatment, seed coat thickness, and antioxidant activity across all varieties. The correlation analysis aimed to uncover potential mechanisms of EMS tolerance by revealing interactions between physical seed characteristics, biochemical responses, and physiological outcomes under mutagenic stress.

All these statistical analyses were performed using SPSS software, while the descriptive data for all calculated parameters was averaged and presented on charts using Excel.

3. Results and Discussion

3.1. Effect of Seed Size/Botanical Variety on Sensitivity to Different EMS Concentrations. Results of ANOVA indicated very highly significant effect of the EMS treatment on all evaluated traits ($p \leq 0.001$), with the exception of shoot length and root length, which showed significance at the 1% and 5% levels, respectively. However, with the exception of plantlet weight, ANOVA indicated that the seed size did not significantly affect the response to EMS across various parameters ($p > 0.05$), including GP, GE at seven and 14 days, GRI, average stem, and root lengths as well as VI (Table 2). Also, the effect of the interaction between EMS treatment and seed size was not significant on any of the parameters studied, including plantlet weight, which was the only one parameter significantly influenced by seed size. This indicates that different seed sizes reacted similarly to different EMS levels, maintaining their ranking with regard to this trait.

This finding is not in line with previous studies having suggested that the seed size might influence sensitivity to EMS-induced mutations [21, 22, 32]. Nevertheless, it is important to note that the observed differences in those studies were incidental and have not been thoroughly investigated across various varieties to provide a comprehensive understanding. In contrast, the present investigation represents a significant advancement in comprehending the complex relationship between botanical varieties, specifically seed size, and the response to increasing concentrations of EMS. Unlike prior studies that made limited comparisons involving two or three varieties, our study expands the scope by examining six varieties. This broader approach challenges previous assumptions by demonstrating that both very large and very small grains exhibit responses to EMS that do not strictly adhere to seed size proportionality in terms of sensitivity or resistance. This underscores the necessity for a reassessment of our understanding of sensitivity/resistance to EMS toxicity.

3.2. Genotypic Response Variation to Increasing EMS Concentrations. ANOVA revealed a very highly significant effect ($p \leq 0.001$) of the genotype as the main effect on various parameters including GP, GE at seven and 14 days, GRI, root length, VI, and plantlet weight across various EMS concentrations. Furthermore, the effect of the genotype was highly significant ($p \leq 0.001$) on shoot length (Table 2). On the other hand, all the genotypes investigated were comparable for root length. The effect of interaction between EMS treatment and genotype was found to be nonsignificant across all the evaluated parameters. This indicates that regardless of the concentration of EMS applied, each variety consistently exhibited the same response pattern across increasing EMS concentrations. These results underscore the substantial influence of a genotype within the same species on EMS sensitivity as observed by Sharma et al. [33] on urad bean, Ndou et al. [34] and Olaolorun et al. [35] on wheat, and Yadav et al. [36] on rapeseed, highlighting the necessity of testing a broad spectrum of genotypes across different species rather than relying solely on established findings from other varieties or genotypes in the existing literature when determining the DL50. Therefore, conducting comprehensive studies that encompass diverse array of varieties is important to establish more accurate ranges recommended for individual species.

3.3. Germination Percentage. The daily germination patterns observed over a 21-day period (Figure 1) revealed distinct germination dynamics for each genotype in response to ascending EMS concentrations (0.05%, 0.5%, and 1%) compared to an untreated control group. In general, GP decreased across all tested varieties with increasing EMS concentrations. Among the genotypes, large-seeded Reina mora and small seeded Zina consistently exhibited superior responses across all EMS concentrations, including the highest one (1%), compared to the rest of varieties. In contrast, Aguadulce superlonga exhibited lower GPs across most concentrations, ranking particularly low, except under 0.05% where it marginally surpassed Alfia 17, with a 71.3% versus 70.7% germination rate. Similarly, at 1% concentration, Aguadulce superlonga exhibited a comparatively low GP of 22.7%, although it was not the lowest, as Alfia 17 recorded a slightly lower value of 21.3%. Interestingly, Yasmine (faba bean major) and Alfia 5 (faba bean minor) displayed intermediate germination rates across all concentrations, with Yasmine showing improved relative performance at 1% EMS. This variability could be attributed to several factors, including genotype-specific activation thresholds for stress response mechanisms, differential DNA repair efficiencies [37], varied antioxidant responses [38], or

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Table 2: ANOVA analysis: Influence of faba bean genotypes (varieties) and faba bean seed size (botanical variety) on EMS response in 10 parameters (mean square and level of different significance).

	Df[1]	GP%[2]	GE7%[3]	GE14%[4]	GRI[5]	Root length	Shoot length	Vigor index	Plantlet weight
EMS treatment	3	9194.74	7051.04	8762.22	5974.31	19.59	190.60	13,799,098.10	52.07
p value		**0.000**	**0.000**	**0.000**	**0.000**	**0.045**	**0.001**	**0.000**	**0.000**
Genotype	5	370.89	602.00	459.47	466.93	12.94	147.12	1,331,200.86	37.47
p value		**0.000**	**0.000**	**0.000**	**0.000**	0.111	**0.001**	**0.000**	**0.000**
Seed size	1	8.00	2.00	32.00	11.05	14.69	1.24	169,563.82	48.84
p value		0.723	0.900	0.514	0.700	0.174	0.852	0.461	**0.015**
EMS treatment ∗ genotype	15	49.85	90.86	50.67	37.68	8.29	19.26	166,592.18	6.19
p value		0.189	0.341	0.296	0.545	0.290	0.782	0.780	0.379
EMS treatment ∗ seed size	3	93.78	51.33	89.04	40.69	0.99	33.29	289,094.27	1.94
p value		0.226	0.746	0.319	0.649	0.943	0.426	0.427	0.861

Note: Bold p values indicate statistical significance at $p < 0.05$.
[1]degrees of freedom.
[2]germination percentage
[3]germination energy at 7 days.
[4]germination energy at 14 days.
[5]germination rate index.

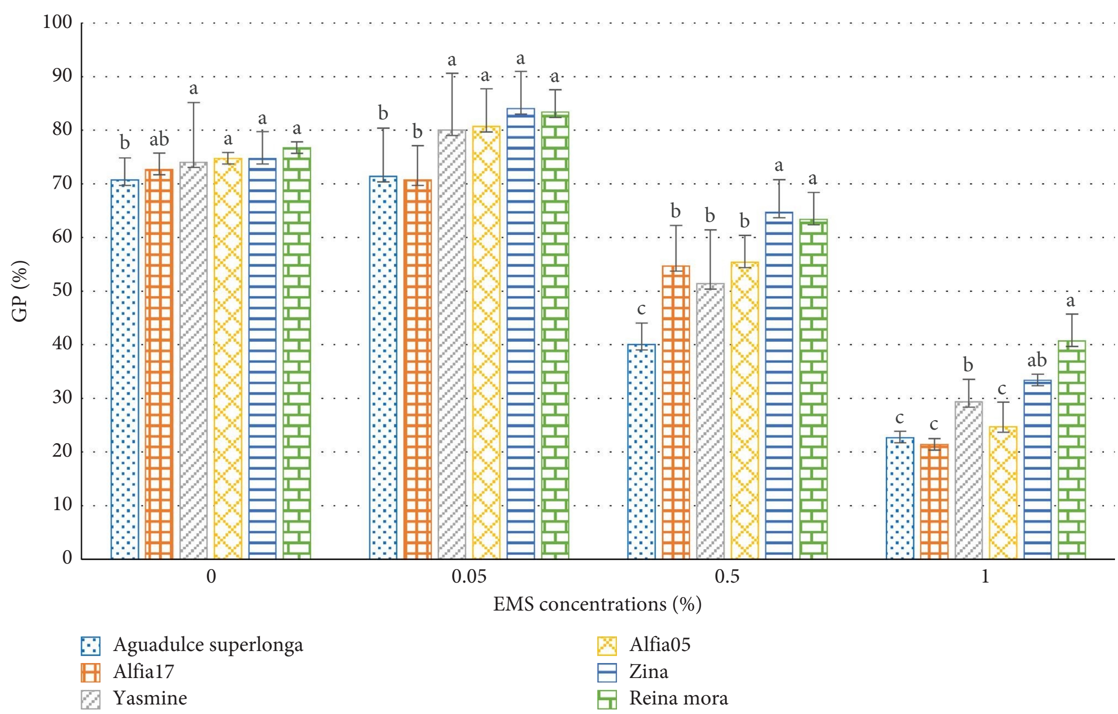

Figure 1: Germination percentage across six faba bean varieties under increasing EMS concentrations (0.05%, 0.5%, and 1%) and control. The different letters in the exposure groups represent statistically significant differences, as determined by the Tukey post hoc test.

epigenetic factors influencing gene expression patterns under stress [39]. All tested varieties, except for Reina mora (with a GP of 40.7%), exhibited lower GPs compared to the findings reported by Elmeer et al. [17], who observed a GP of 40% under 1% EMS. However, under 0.5% EMS, our tested varieties surpassed all the 50% GPs reported by Elmeer et al. [17], except for Aguadulce superlonga, which registered a GP of 40%.

A notable finding emerged: The 0.05% concentration led to higher germination rates in all tested varieties, except for Alfia 17, compared to the untreated control seeds. A recent study has reported that a low EMS concentration improves the GP in sunflower [40]. In a papaya study investigating germination methods, low EMS concentrations outperformed the untreated control, boosting germination. This might be due to a potential hormetic effect, stimulating beneficial seed germination [41]. Hormesis theory suggests that low doses of stressors can trigger positive biological responses, potentially improving seed germination. Another factor could involve the activation of specific stress-response pathways within the seeds, which could enhance their resilience and subsequently improve germination rates.

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Further exploration of these aspects could reveal the underlying mechanisms, providing valuable insights into improving germination under challenging or stressful conditions. Examining EMS concentrations below 0.05% on Alfia 17 and similar varieties might identify optimal levels for enhancing germination in each genotype, potentially leading to more effective seed treatments.

Reina mora and Zina, which demonstrate superior tolerance to EMS, represent promising candidates for mutagenesis breeding as a starting material. These varieties can be used to develop a large population of mutants, allowing the exploration and identification of agronomically valuable traits.

3.4. Germination Kinetics. During the initial 7-day period postsowing, the application of EMS treatment at a concentration of 0.05% not only improved GPs but also accelerated the process, as demonstrated by the increased GE across all varieties except for Yasmine, which showed a GE of 61.3% under the 0.05% EMS treatment compared to the control's 62% (Figures 2 and 3). At the 7-day mark, significant improvements in GE were observed: Aguadulce superlonga exhibited 65.3% under the 0.05% treatment compared to 36% in the control, Alfia 17 showed 59.3% against the control's 52%, Zina demonstrated 66.7% versus the control's 64.7%, and Reina mora displayed 72.7% compared to 67.3% in the control. The ranking of varieties based on GE at the seventh day remained consistent between the 0.05% treatment and the control, except for Aguadulce superlonga, which improved from the last place in the control group to second-to-last under the 0.05% EMS treatment. Consequently, Reina mora emerged as the variety with the fastest germination rate under the 0.05% treatment.

At Day 14, the 0.05% EMS treatment continued to accelerate the germination of Zina (GE of 82% compared to 74.7% in the control), Reina mora (GE of 81% compared to 76%), Yasmine (GE of 80% compared to 72.7%), and Alfia 05 (79.3% compared to 70% in the control). However, it appeared to impede the germination of Alfia 17, as indicated by a GE at Day 14 of 67.3% compared to 72.7% in the control. In addition, Aguadulce superlonga displayed a GE at 14 days of 63.3% under the 0.05% EMS treatment compared to 65.3% in the control, suggesting a slower germination rate under this treatment, thus ranking it as the slowest among the varieties.

At a higher EMS concentration (0.5%), exceeding the commonly utilized ranges in the literature (Abo-Hegazi [11], Khursheed et al. [13, 14], and Nurmansyah et al. [15]) and subjecting seeds to significant EMS-induced stress, the 7 day postsowing period revealed Reina mora exhibiting the highest GE at 39.3%, closely followed by Zina with a GE of 38%. Subsequently, Yasmine displayed a GE of 35.3%, Alfia 17 showed a GE of 27.3%, and Alfia 05 had a GE of 26.7%. Once again, Aguadulce superlonga ranked last with a GE of 23.3%.

After 14 days, Reina mora and Zina maintained their positions as the fastest germinating varieties, achieving rates of 62.7% and 62% GE, respectively. Aguadulce superlonga retained its lowest rank, exhibiting a GE of 37.3%, while the three intermediate varieties showed a slight overlap. Among these, Alfia 05 displayed the fastest germination with a GE of 54.7%, closely followed by Alfia 17 at 53.3%, and then Yasmine, exhibiting the slowest germination rate at 49.3% GE.

At the highest EMS concentration (1%), inducing severe stress across all tested varieties, the GE after 7 days postsowing displayed the minimal variation (ranging from 20.7% to 24.7%) and did not maintain the same overall rankings observed at lower concentrations. Yasmine exhibited the quickest initiation of germination with a GE of 24.7%, followed by Reina mora (23.3%), Aguadulce superlonga (22%), Zina (21.3%), Alfia 05 (20.7%), and finally Alfia 17 (20%). However, at 14 days postsowing, the varieties that had previously shown better responses to EMS resumed their usual rankings and accelerated in germination. Reina mora emerged as the fastest at this stage with a GE of 37.3%, followed by Zina (30.7%), Yasmine (28%), Alfia 05 (24.7%), Aguadulce superlonga (22.7%), and Alfia 17 (20.7%).

3.5. Germination Rate Index. Without EMS treatment, the varieties exhibited an overall GRI from lowest to highest: Aguadulce superlonga (41.2%), Alfia 17 (51.1%), Yasmine (58.2%), Zina (60.8%), Alfia 05 (61.3%), and Reina mora (62.8%). This sequence indicated inherent differences in their germination capabilities under normal conditions (Figure 4).

The application of a 0.05% EMS treatment notably enhanced the overall GRI across all varieties, resulting in a reordering of their rankings over time, with Aguadulce superlonga displaying the lowest GRI (44.2%), followed by Alfia 17 (51.3%), Alfia 05 (59.3%), and Zina (62.7%). Meanwhile, Reina mora (65.3%) and Yasmine (66%) exhibited the highest GRIs. These findings indicate that the 0.05% EMS treatment positively influenced most varieties, accelerating their germination rates and altering their original ranking.

At a stronger EMS concentration of 0.5%, the varieties displayed an almost similar ranking to the control, exhibiting GRIs as follows: Aguadulce superlonga (24.5%), Alfia 17 (33.9%), Yasmine (34.4%), Alfia 05 (34.9%), Zina (40.4%), and Reina mora (41.6%).

At the highest EMS concentration (1%), the varieties demonstrated distinct GRIs. Alfia 17 recorded the lowest GRI (16%), followed by Aguadulce superlonga (17.2%), Alfia 05 (18%), Yasmine (20.7%), Zina (21.4%), and Reina mora (25.5%).

Throughout all EMS concentrations, the extreme ranges, both the lowest and highest levels of GRI, were consistently maintained by the same varieties, preserving their respective positions. This pattern suggests a consistent genotypic resilience or sensitivity within these varieties across varying EMS treatments. However, although GRI rankings generally correlated with GP rankings across the different EMS levels, notable deviations were observed. Specifically, Yasmine and Alfia 05 exchanged their relative positions in GRI at 0% and 0.05% EMS, with Alfia,05 exhibiting a higher GRI despite Yasmine's superior GP. Similarly, at 0.5% and 1% EMS, Zina outperformed Reina mora in GRI, despite having lower GPs at certain concentrations. These deviations highlight that GP and GRI offer complementary insights, GP reflects overall

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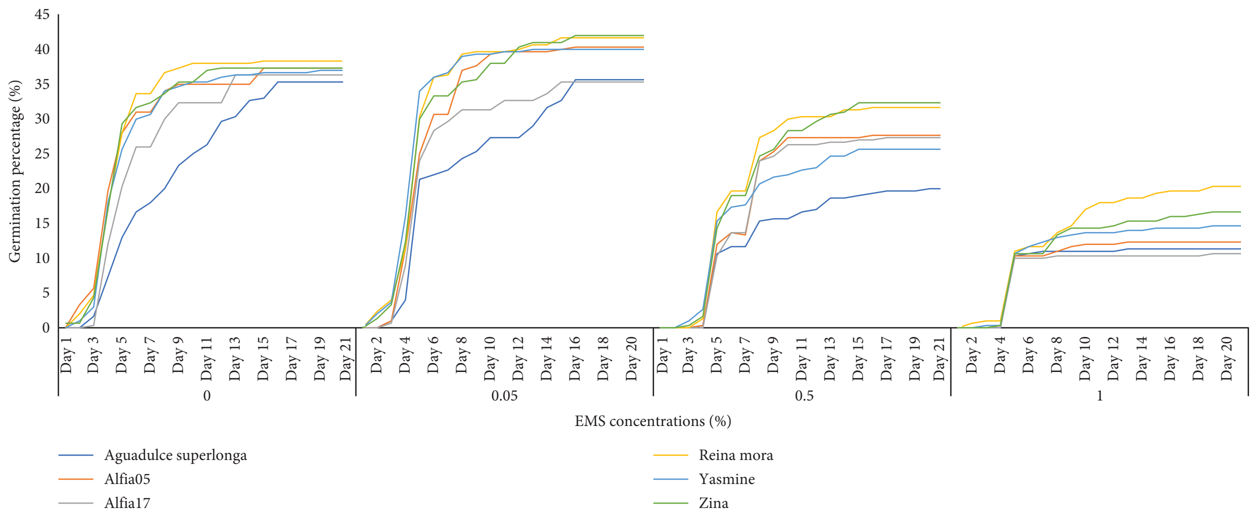

Figure 2: Germination kinetics over 21 days across six faba bean varieties under gradually increasing EMS concentrations (0.05%, 0.5%, and 1%) and control.

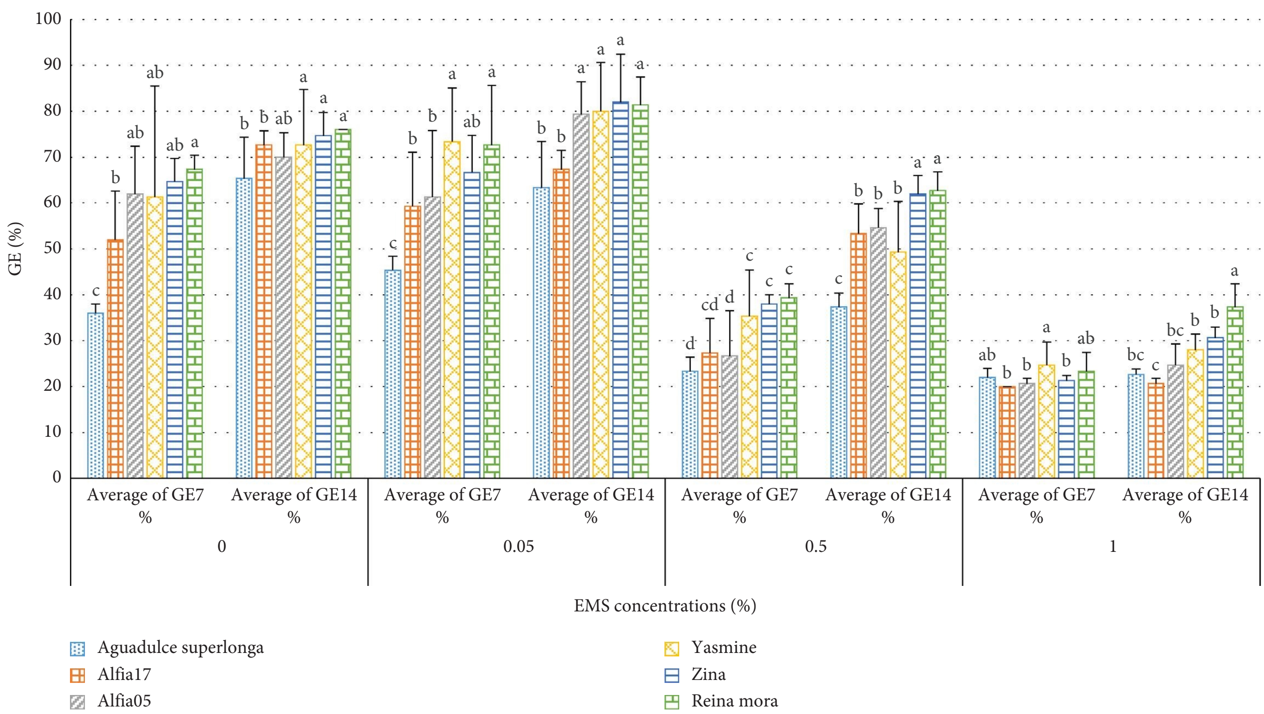

Figure 3: Comparison of germination energy across six faba bean varieties under increasing EMS concentrations (0.05%, 0.5%, and 1%) and control on days 7 and 14. The different letters in the exposure groups represent statistically significant differences, as determined by the Tukey post hoc test.

seed viability under mutagenic stress, while GRI captures the speed and uniformity of germination. The discrepancies reveal genotype-specific responses to EMS, where some varieties exhibit faster or more uniform germination despite lower overall success rates.

3.6. Vigor Index. The VI varied across different EMS concentrations and varieties. In the control group (0% EMS concentration), the VI ranged from 2127.6 in Alfia 17 to 2925.7 in Yasmine (Figure 5). At 0.05% concentration, there was an overall improvement in VI across all varieties except for Aguadulce superlonga which remained the lowest (1846.1), followed by Alfia 17 (2524.5), Reina mora (2679.6), Yasmine (2801), Zina (2972.8), and then Alfia 05 (3079.3). Under 0.5% concentration, Aguadulce superlonga consistently exhibited the lowest VI (734.6), while Reina mora showed the highest one (1721.5). At 1%, the ranking remained similar to that at 0.5%, except for Alfia 05 and Alfia 17 switching places. Again, Aguadulce superlonga showed

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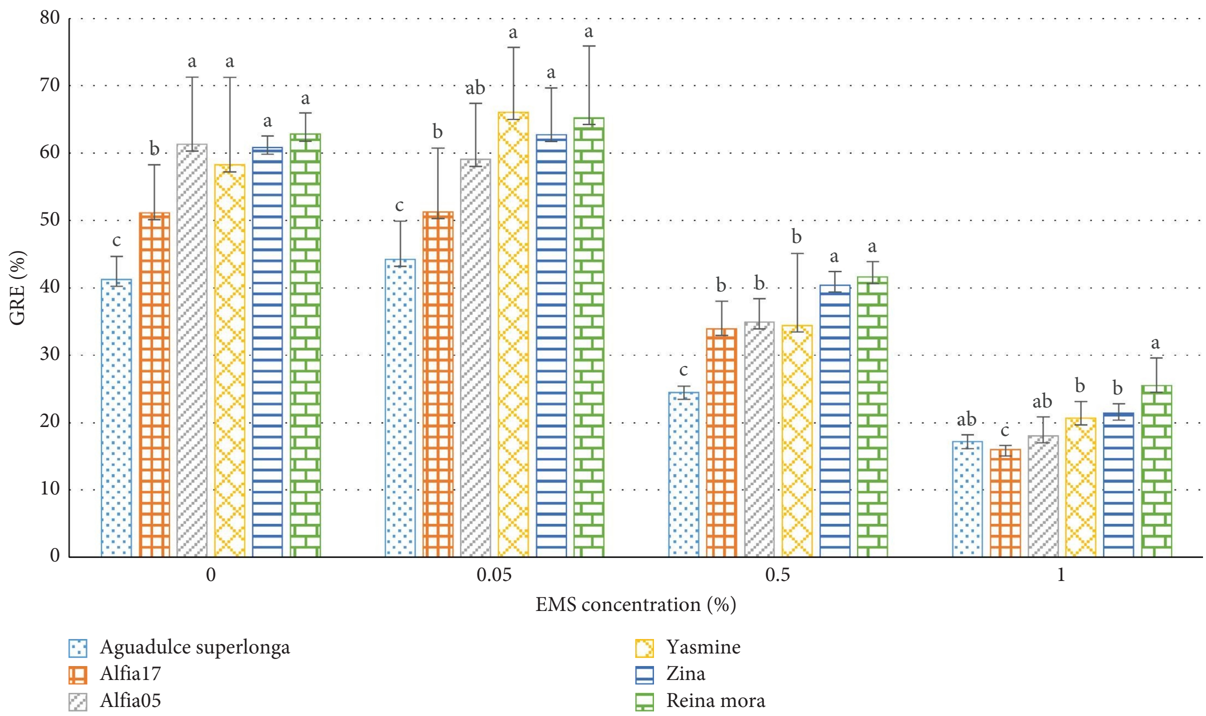

Figure 4: Variation in the germination rate index among six across six faba bean varieties under increasing EMS concentrations (0.05%, 0.5%, and 1%) and control. The different letters in the exposure groups represent statistically significant differences, as determined by the Tukey post hoc test.

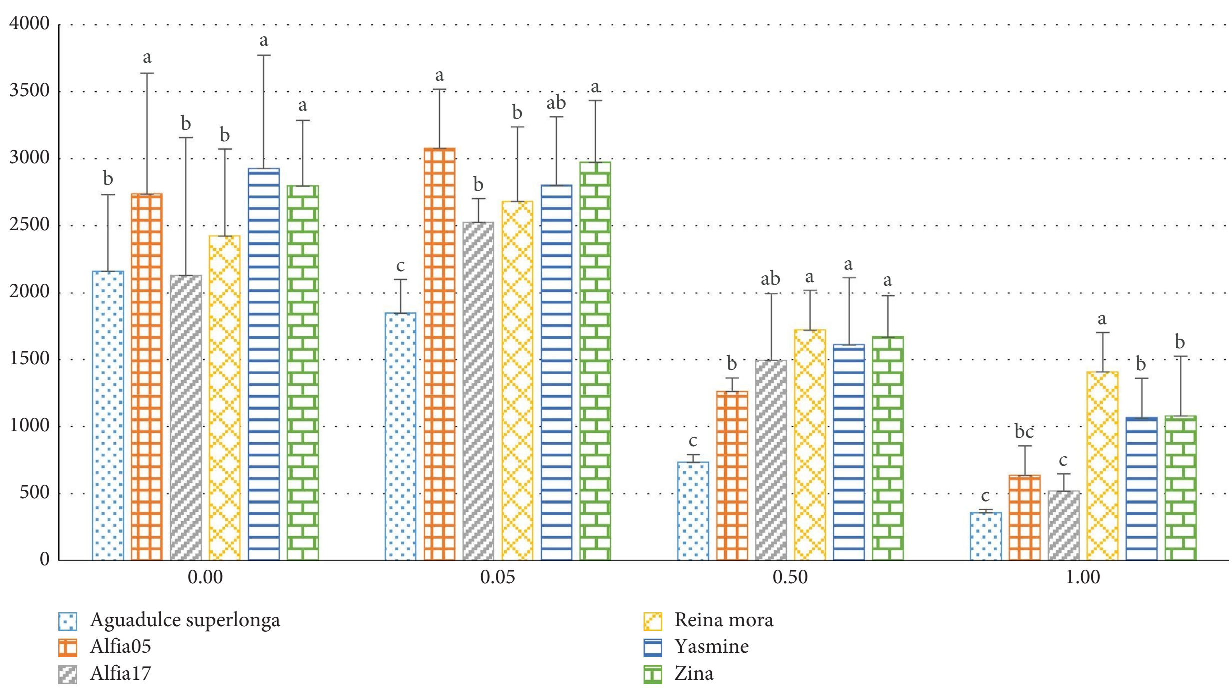

Figure 5: Variation in the vigor index among six faba bean varieties under increasing EMS concentrations (0.05%, 0.5%, and 1%) and control. The different letters in the exposure groups represent statistically significant differences, as determined by the Tukey post hoc test.

the lowest VI (358.1), whereas Reina mora exhibited the highest (1407.8). At 0.5% EMS, Alfia 05 exhibited good germination (55.33%) but showed a significant decline in vigor, ranking second to last, suggesting that energy expended during germination under stress may hinder subsequent growth. At 1%, the ranking remained similar to

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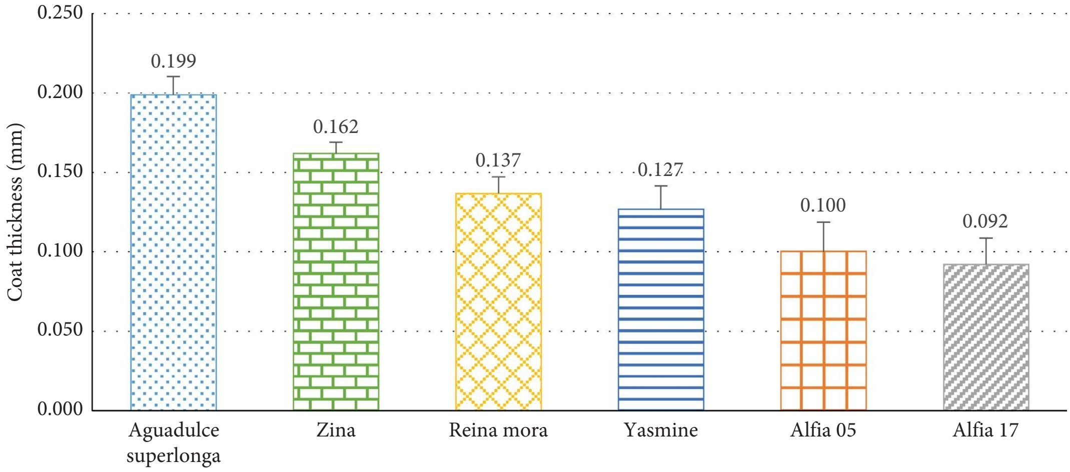

Figure 6: Variation in coat thickness among the six faba bean varieties studied.

that at 0.5%, except for Alfia 05 and Alfia 17 switching places. Again, Aguadulce superlonga showed the lowest VI (358.1), whereas Reina mora exhibited the highest (1407.8).

Reina mora demonstrated higher VI under elevated EMS concentrations (0.5% and 1%) compared to the other varieties, revealing its highest resistance to EMS toxicity as well as enhanced recovery capabilities.

These findings hold substantial implications for mutagenesis breeding of faba beans. While a high GP alone does not guarantee a high survival rate, as shown in several studies [16, 42, 43], a good VI might imply better recovery and survival rates. A high number of surviving mutants after sowing is crucial, as it broadens genetic diversity and provides a more extensive pool of mutants for exploration and characterization. This expanded diversity is essential for identifying novel traits and advancing the development of improved cultivars.

3.7. Evaluation of Seed Coat Thickness. Figure 6 shows the coat thickness values recorded for the six varieties investigated. Contrary to expectations, our findings suggest that seed coat thickness may not directly account for the tolerance or sensitivity to EMS. The variety demonstrating the highest sensitivity to EMS treatment, Aguadulce superlonga, paradoxically exhibited the thickest seed coat, measuring 0.199 mm (Figure 6). Further analysis through the correlation matrix (Tables 3 and 4) supported this observation, revealing no statistically significant correlation between seed coat thickness and the parameters associated with EMS sensitivity neither understressed nor nonstressed conditions. This finding refutes the hypothesis that varieties with thicker seed coats might absorb EMS differently from those with thinner coats, suggesting that thick coat varieties absorb less EMS than thin coat varieties, thus impacting their sensitivity to it.

3.8. Antioxidant Activity. The evaluation of antioxidant activity on nonstressed seeds revealed distinct groupings. Yasmine exhibited the highest level of free radicals' inhibition (57.4%–68.4%), with both Aguadulce superlonga (46.9%–59.1%) and Alfia 05 (48.2%–50.7%) closely following, displaying similar higher inhibition percentages across various concentrations. In contrast, Reina mora, Zina, and Alfia 17 expressed notably lower levels of free radicals' inhibition in nonstressed seeds, ranging from 28.1% to 36.1%, 30% to 33.4%, and 26.8% to 34.3%, respectively (Figure 7).

However, when subjected to 0.5% EMS concentration, an intriguing shift in expression among these groups emerged. Alfia 17 (51%–78.9%), Zina (56.3%–73.8%), and Reina mora (45.2%–69.8%) showed a significant increase in trapping free radicals ($p \leq 0.05$) across concentrations.

This adaptive response likely involves the enhancement of antioxidant defenses to withstand stress-induced oxidative damage and maintain cellular integrity for correct germination [44, 45], a characteristic that was associated with their enhanced resistance to EMS toxicity across all evaluated germination parameters, especially Reina mora and Zina. This defense mechanism is mediated through the upregulation of enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and others involved in neutralizing free radicals [46].

On the other hand, Aguadulce superlonga (36.4%–53.1%), Alfia 05 (35.9%–54.1%), and Yasmine (40.1%–54.8%) (Figure 8) significantly reduced their antioxidant activity ($p \leq 0.05$), compared to their nonstressed state (Table 5). An overproduction of reactive oxygen species (ROS) under severe stress could overwhelm the plant's defense mechanisms [47–49], resulting in a decrease in inhibition percentage as ROS levels exceed the genotype's antioxidant capacity. In addition, these genotypes may prioritize alternative stress response pathways over antioxidant defense mechanisms when subjected to stress [50].

Various environmental stressors have been demonstrated to disrupt the balance of redox reactions, suppress antioxidant mechanisms, and elevate the production of ROS within peroxisomes [51–54]. For instance, in tomato plants, salinity has been observed to diminish the levels of ascorbic acid (AsA) and glutathione (GSH), while also triggering lipid peroxidation within peroxisomes [52]. This may result in

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Table 3: Pearson correlation coefficients among germination traits, coat thickness, and antioxidant activity in nonstressed faba bean seeds of six varieties.

	GP[1]	GE7[2]	GE14[3]	GRI[4]	Vigor index	Plantlet weight	Coat thickness	Antioxidant activity
GP	1	0.786**	0.760**	0.694**	0.575**	−0.195	−0.114	−0.068
GE7		1	0.815**	0.966**	0.585**	−0.370*	−0.184	−0.107
GE14			1	0.755**	0.354	−0.293	−0.003	−0.262
GRI				1	0.499**	−0.379*	−0.220	−0.085
Vigor index					1	−0.068	−0.206	0.308
Plantlet weight						1	−0.061	0.281
Coat thickness							1	−0.254
Antioxidant activity								1

[1]Germination percentage.
[2]Germination energy at 7 days.
[3]Germination energy at 14 days.
[4]Germination rate index.
*,**Significant differences at the probability level of 5% and 1%, respectively.

Table 4: Pearson correlation coefficients among germination traits, coat thickness, and antioxidant activity of faba bean seeds under 0.5% EMS concentration.

	GP[1]	GE7[2]	GE14[3]	GRI[4]	Vigor index	Plantlet weight	Coat thickness	Antioxidant activity
GP	1	0.683**	0.980**	0.946**	0.790**	0.024	−0.058	0.425*
GE7		1	0.700**	0.826**	0.801**	0.468**	−0.162	0.374*
GE14			1	0.966**	0.825**	0.072	−0.061	0.437*
GRI				1	0.880**	0.248	−0.099	0.402*
Vigor index					1	0.390*	−0.015	0.366*
Plantlet weight						1	−0.334	−0.176
Coat thickness							1	0.335
Antioxidant activity								1

[1]Germination percentage.
[2]Germination energy at 7 days.
[3]Germination energy at 14 days.
[4]Germination rate index.
*, **Significant differences at the probability level of 5% and 1%, respectively.

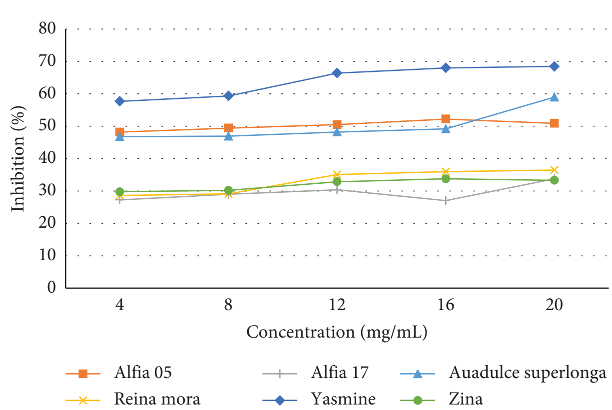

Figure 7: Comparison of antioxidant activity measured by the DPPH method in nonstressed faba bean seeds from six varieties.

a shift of nutrient distribution toward alternate tissues, at the expense of antioxidant enzyme activity, leading to reduced scavenging of free radicals.

3.9. Correlation Among the Parameters Studied. The association between antioxidant activity and sensitivity to EMS, measured through germination parameters, was verified by

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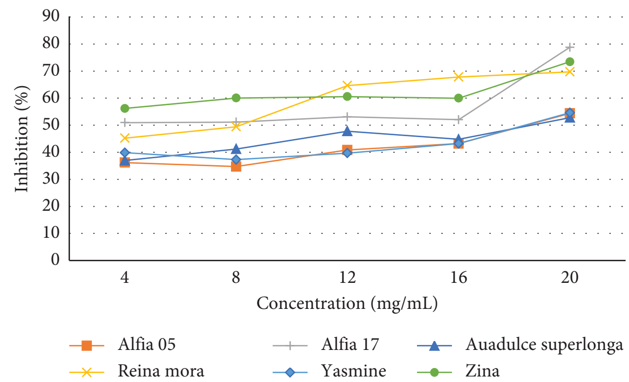

Figure 8: Comparison of antioxidant activity measured by the DPPH method in 0.5% EMS mutagenized faba bean seeds from six varieties.

Table 5: ANOVA one-way with contrast analysis: Comparison of antioxidant activity (DPPH radical scavenging) in six faba bean varieties under control and EMS-induced stress conditions.

Contrast (stressed–nonstressed)	Value of contrast	Std. error	t^1	df^2	Sig. (2-tailed)[3]
Alfia 05	8.366	2.371	3.528	9.701	0.006
Alfia 17	−27.705	3.703	−7.482	9.981	0.000
Aguadulce superlonga	5.300	2.372	2.234	17.515	0.039
Reina mora	−26.316	3.546	−7.421	11.110	0.000
Yasmine	21.037	2.552	8.243	16.576	0.000
Zina	−30.066	2.050	−14.665	10.431	0.000

[1]Standard t-test.
[2]Degrees of freedom.
[3]p value for two-tailed tests.

significant correlations between antioxidant activity and all germination parameters under a 0.5% EMS treatment, with the exception of plantlet weight. However, these correlations were not evident in healthy untreated seeds, as antioxidant activity did not exhibit any significant correlation with the evaluated parameters before inducing stress (Tables 3 and 4). In addition, no correlation was found between coat thickness and free radical inhibition under any condition. Thus, genotypes with efficient mechanisms for scavenging free radicals may play a crucial role in mitigating the negative effects of environmental stressors, beyond just chemical mutagens such as EMS. Therefore, identifying and selecting genotypes with robust antioxidant mechanisms could be a valuable strategy for breeding programs aimed at developing crops with enhanced resilience to a wide range of adverse environmental conditions.

4. Conclusion

In conclusion, this study reveals complex genotype-specific responses of faba bean varieties to EMS mutagenesis. Key findings indicate that genetic makeup, rather than seed size, primarily determines EMS tolerance, while seed coat thickness does not correlate with EMS sensitivity. Antioxidant responses play a crucial role in EMS stress mitigation. Notably, Reina mora and Zina exhibited superior tolerance across EMS concentrations, while Aguadulce superlonga showed consistently lower germination rates. Interestingly, low EMS concentrations (0.05%) improved germination in most varieties, possibly due to hormetic effects. VI analyses further supported these findings, with Reina mora demonstrating the highest resistance and recovery capabilities at elevated EMS concentrations. These results have significant implications for mutagenesis breeding programs, suggesting that genotype-specific responses should be considered when applying EMS treatments. Future research directions could include detailed examination of enzymatic and nonenzymatic antioxidant systems, gene expression analysis of key antioxidant genes, investigation of hormetic effects at low EMS concentrations, and exploration of other factors influencing EMS sensitivity, such as seed dormancy or metabolic activity. This study provides a foundation for optimizing mutagenesis protocols in faba bean breeding, potentially leading to more efficient development of improved cultivars.

Nomenclature

DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EMS	Ethyl methanesulfonate
RCBD	Randomized complete block design
GE7	Germination energy at the seventh day
GE14	Germination energy at the 14th day
GP	Germination percentage
GRI	Germination rate index
VI	Vigor index

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Data Availability Statement

The data will be made available on request.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Oumaima Chetto: conceptualization, data curation, formal analysis, funding acquisition, investigation, and writing – original draft.

Loubna Belqadi: conceptualization, funding acquisition, and supervision.

Mohamed Kouighat: formal analysis, original draft, and writing – review and editing.

Etienne Bucher: funding acquisition and writing – review and editing.

Mohamed El Fechtali: data curation and investigation.

Rokhaya Imelda Ndiaye: data curation and investigation.

Abdelghani Nabloussi: conceptualization, funding acquisition, supervision, methodology, validation, writing – review and editing.

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