Phenotypic effects of different doses of physical and chemical mutagens in cotton plants

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ABSTRACT

   Induced mutation techniques have been effectively used to obtain new variability in crop plants. Mutations are the primary source of genetic variability and therefore, some control over their frequency can be considered a valuable tool for plant breeding. Mutagens can be grouped into two broad categories according to their nature: physical and chemical. Doses must be optimized to increase the chances of generating mutants and achieve a population of mutagenized plants that allow a successful selection of mutants of interest. The aim of this study was to test the sensitivity of different mutagenic agents on Guazuncho 3 variety cotton seeds. Four doses of each treatment were evaluated and different phenotypic traits were recorded. The greatest effects on germination percentage, germination velocity index, plant survival and somatic effects were observed in treatments with ethyl methanesulfonate (EMS) as the doses increased. Lower but significant effects were recorded in sodium azide (SA) treatments and no significant differences were observed for X-rays and combined treatments (SA+X-rays). The effects on growth rate were significant in chemical treatments with $R^2 = 0.84$ for SA and $R^2 = 0.97$ for EMS. In addition, it was observed that the EMS and X-rays treatments caused a greater number of off-type plants and chlorotic irregular-shaped spots. The results of this preliminary study determined the mutagens doses to be used on Guazuncho 3 cotton seeds, to generate an M_1 population for developing a mutation breeding program.

   Keywords: cotton, induced mutations, dosimetry.

RESUMEN

   Las técnicas de mutaciones inducidas se han utilizado eficazmente para obtener nueva variabilidad en las plantas de cultivo. Las mutaciones son la principal fuente de variabilidad genética y, por lo tanto, cierto control sobre su frecuencia puede considerarse una herramienta valiosa para el fitomejoramiento. Los mutágenos pueden agruparse en dos grandes categorías según su naturaleza: físicos y químicos. Las dosis deben optimizarse para aumentar las posibilidades de generar mutantes y lograr una población de plantas mutagenizadas que permita una selección exitosa de mutantes de interés. El objetivo de este estudio fue evaluar la sensibilidad de diferentes agentes mutagénicos en semillas de algodón de la variedad Guazuncho 3. Se evaluaron cuatro dosis de cada tratamiento y se registraron diferentes caracteres fenotípicos. Los mayores efectos sobre el porcentaje de germinación, el índice de velocidad de germinación, la supervivencia de las plantas y los efectos somáticos se observaron en los tratamientos con metanosulfonato de etilo (EMS) a medida que aumentaban las dosis. Se registraron efectos menores pero significativos en los tratamientos con azida sódica (SA) y no se observaron diferencias significativas en los tratamientos con rayos X y combinados (SA + rayos X). Los efectos sobre la tasa de crecimiento fueron significativos en los tratamientos químicos con $R^2 = 0,84$ para SA y $R^2 = 0,97$ para EMS. Además, se observó que los tratamientos con EMS y rayos X provocaron un mayor número de plantas fuera de tipo y manchas cloróticas de forma irregular. Los resultados de este estudio preliminar determinaron las dosis de mutágenos a utilizar en semillas de algodón Guazuncho 3, con el fin de generar una población M_1 para desarrollar un programa de mejoramiento de mutaciones.

   Palabras clave: algodón, mutaciones inducidas, dosimetría.

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INTRODUCTION

Cotton is the main source of natural fiber worldwide, used for textile industry and other purposes such as oilseed and animal feed. This crop is cultivated in nearly 100 countries, occupying about 2.5% of the world's arable land, under a great diversity of agroclimatic conditions and widely varied farming practices, being of great importance for the economy of such countries (Boopathi *et al*., 2015; Scarpin *et al*., 2022; Zhang *et al*., 2008). Upland cotton (*Gossypium hirsutum* L.) represents about 95% of the global cotton production, due to its environmental adaptability, high production, and better yield potential (Ijaz *et al*., 2019).

Regarding plant breeding, numerous technologies have emerged over the past decades to join the conventional cross-breeding, such as genomic selection (*i.e.* molecular marker-assisted selection), gene editing and mutation breeding (Jiang, 2016; Suprasanna *et al*., 2015; Voss-Fels *et al*., 2019). Induced mutations have been successfully used in plant breeding programs to artificially generate genetic variation for the development of new varieties with novel traits. Since the 1920s, more than 3,400 mutant varieties have been released worldwide, including cereals, oilseeds, as well as vegetables and industrial crops (https://nucleus.iaea.org/sites/mvd/SitePages/Home.aspx). Specifically, in cotton, the NIAB-78 and NIAB-846 varieties, obtained by irradiation, present heat and bollworm tolerance and improved yield and fiber quality, respectively (Aslam *et al*., 2018; Maluszynski *et al*., 2009). In addition, a cotton variety with tolerance to imidazolinones was recently released, which was obtained by mutagenesis using sodium azide (Tcach *et al*., 2022).

Although mutations occur spontaneously in nature, their frequency is very low, and can be increased by exposing biological material to mutagens (Landau *et al*., 2021; Mba *et al*., 2010; Oladosu *et al*., 2016).

The chemical mutagens most commonly used in plant breeding are: ethyl methanesulfonate (EMS), sodium azide (SA) and N-methyl-N-nitrosourea (MNU) and they mainly produce point mutations. Physical mutagens most commonly used are gamma- or X-rays, which often cause large deletions, leading to loss of several genes, DNA breakage and chromosome aberrations.

The first step of breeding mutation is to optimize the mutagen dose to achieve a high mutation rate while avoiding unintended damage, serious effects on germination and plant development (Ke *et al*., 2019; Mba, 2013; Mba *et al*., 2010; Nielen *et al*., 2018b; Oladosu *et al*., 2016). Initial studies on induced mutations have mainly aimed at finding the optimum combination of mutagen and dose to elicit the best response.

The level of damage induced on the M_1 generation determines the number of seeds that can be used in the M_2 or M_3 populations to select the desired phenotype. Several parameters can be assessed to determine the somatic effects after a mutagenic treatment, including delay in seed germination, level of disturbances in the cell cycle, frequency of chromosomal aberrations in meristematic tissues, reduced seedling emergence, reduced seedling and plant growth, appearance of chlorophyll deficiencies, reduced fertility and plant survival to maturity, and sterility (Maluszynski *et al*., 2009).

In the last decade, some researchers have reported mutagenesis experiments on cotton, which include treatments using seeds or somatic embryos as plant material, and employing EMS, SA, gamma or X-rays. These studies have been used for morpho-physiological feature analyses, selection of lines with enhanced agronomical traits such us fiber quality and yield or stress tolerance among others, which could also serve as a valuable resource for functional genomic research on complex allotetraploid traits (Abid *et al*., 2020; Aslam *et al*., 2016, 2013; Auld *et al*., 2007, 1998; Bechere *et al*., 2010, 2007, 2014; Brown *et al*., 2012; Ganesan *et al*., 2005; Kalwar and Dahot, 2017; Lian *et al*., 2020; Muthusamy and Jayabalan, 2013, 2011; Patel *et al*., 2014; Shim *et al*., 2019; Tcach *et al*., 2022; Witt *et al*., 2018a, 2018b). In those mutagenesis studies, most experiments have followed previously published basic protocols, but few cotton-specific works have assayed dose sensitivity with different type of mutagens in cotton seeds.

In this context, the aim of the present work was to comparatively evaluate the phenotypic effects of EMS, SA, X-rays and a combined treatment of SA+X-rays on M_1 plants coming from seeds of an elite variety of cotton (Guazuncho 3) as a guidance to choose the most adequate doses of each mutagen to generate an M_2 population in order to select mutants of interest for a cotton breeding program.

MATERIALS AND METHODS

Plant material

Experiments were performed using seeds from tetraploid cotton (*G. hirsutum*) of the variety Guazuncho 3, belonging to the germplasm collection of the EEA INTA Saenz Peña, Chaco, Argentina. This line is an intermediate-short cycle crop, with good yield and quality features, high percentage of ginning, intermediate vigor, low number of vegetative branches and low tolerance to abiotic stress (Bonacic Kresic *et al*., 2004).

Mutagenic treatments

The mutagenic treatments were carried out at Instituto de Genética "Ewald A. Favret" (IGEAF) CICVyA-CNIA-INTA. A total of four doses were evaluated for each mutagenic treatment as summarized in table 1, using 100 healthy seeds, coming from the same production lot, for each assay. No special handlings (including delinting) were applied to the seeds before each mutagenic treatment.

For X-rays treatments, dry seeds were irradiated with an X-rays generator Philips MG160 (160 kV max. and 30 mA max.) at the indicated doses at 120 kV and 15 mA. For SA mutagenesis, different concentrations were prepared in phosphate buffer at pH 6.0, and seeds were incubated in each solution for 18 hrs at 18°C and 165 rpm in an orbital shaker. Then, the seeds were thoroughly washed seven times with water and air-dried. Similarly, EMS treatments were performed by incubating the seeds in the corresponding dilution following the same treatment conditions mentioned above for SA. For the combined treatment (SA+X-rays), the SA protocol was carried out first, and then the air-dried seeds were subjected to a 100 Gray (Gy) X-rays dose. Finally, the same number of seeds without mutagenic treatment was used as control.

Plant material handling after mutagenic treatments

The treated and control seeds were sown in trays of 0.5 m x 0.35 m, using inert substrate previously sterilized by autoclave. These trays were placed in a growth chamber at 30°C for 15 days to evaluate germination and other parameters described

	X-rays				Sodium azide			
	(Gy)				(mM)			
Dose	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
	200	300	400	500	2	4	8	16
	Ethyl methanesulfonate				Combined (SA+X-rays)			
	(% v/v)				(mM-Gy)			
Dose	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
	0.1	0.2	0.3	0.4	2 – 100	4 – 100	8 – 100	16 – 100

Table 1. Mutagens and doses evaluated in cotton seeds.

below. Then, 15 representative seedlings from each mutagenic treatment and control were randomly selected, transplanted into 5-liter pots (2.5 kg soil/pot) and grown under semi-controlled conditions in a greenhouse for further studies. In this step, only 15 plants of each treatment were selected due to the limited capacity of the greenhouse and personnel to handle them.

Evaluation of different mutagenic treatments in generation 1 (M_1)

For each treatment, the number of emerged seedlings (fully expanded cotyledons) was evaluated 15 days after sowing to determine the germination percentage (GP). The germination velocity index (GVI) was also calculated as described in Meneses *et al*. (2011) and Nakagawa (1994). Finally, survival percentage (Sv) was calculated as the total emerged seedlings divided by the number of plants that emerged and continued their development until the complete expansion of the second true leaf (Spencer Lopes *et al*., 2018). Growth rate (g/days) on vegetative stage was also determined as: (plant weight 40 days after emergence − plant weight 20 days after emergence)/20 days.

Additionally, some morphological features were evaluated during the entire growth period, such as the presence of malformed plants or off-type plants, chlorophyll deficiencies or chimeras. Furthermore, phenological data were recorded from 15 plants selected from each treatment that were transplanted into pots as described below, until maturity.

Post-harvest analysis

At plant maturity, some agronomical and morphological traits were analyzed in the 15 selected plants from each mutagenic treatment: i) plant height, ii) fruit retention percentage, which is the percentage of retained fruits in the plant over the total reproductive positions [(N° retained fruits/N° of reproductive positions)*100], iii) number of fruit abortions, identified by scars instead of developed fruits retained on the plant, iv) number of reproductive positions, v) harvest index (HI), which is the ratio of harvested product (lint and seed) to the above ground plant dry weight or biomass (stems, leaves and fruit), and vi) number of seeds per boll.

Statistical analysis

Statistical analysis was carried out using general linear and mixed models with the statistical program InfoStat (Di Rienzo *et al*., 2011) through its interface with the R program. Fisher's LSD test was used to compare the mean values at a significance value of 5% ($p < 0.05$).

RESULTS

Effects of mutagenic treatments on seed germination, survival and vegetative growth rate

In order to determine the most suitable dose of each mutagen or treatment for cotton and to analyze the differences between them, some important phenotypic characteristics, such as germination percentage (GP), germination velocity index (GVI) and seedling survival were studied. The parameters analyzed showed differences in germination depending on the mutagen employed and dose, as shown in supplemental figure 1. The major effect on GP of M_1 cotton seeds was observed in EMS treatments, where it strongly decreased as the concentration of mutagen increased, with an R^2 of 0.96 for its regression (p-value: 0.003), attaining only 20% of germinated seedlings with 0.4% EMS (figure 1A). Additionally, SA treatments showed significant differences in GP (p-value: 0.029) between doses, presenting an R^2 of 0.84. However, no significant differences were observed in the GP values for the X-rays treatments, as well as in the combined treatment (SA+X-rays) as the mutagen doses increased (p-value: 0.196 and 0.081, respectively) (figure 1A).

Figure 1B shows the GVI obtained in this study. Considering that a low index could be attributed to a low number of germinated seeds and/or a delay in germination, it was considered that GVI should be analyzed in conjunction with GP results. EMS treatments showed a clear decrease in GVI values as the concentration of mutagenic agent increased, resulting in a GVI 10-fold lower at 0.4% EMS, compared to the control (figure 1B). In contrast, X-rays treatments showed non-significant differences for this index among the doses applied.

Interestingly, SA assays exhibited a lower GVI at higher doses, leading to a 2-fold reduction at the highest SA concentration compared to the control (figure 1B). In addition, in combined treatments (SA+X-rays), a similar but less pronounced behavior was observed compared to SA (figure 1B).

Figure 2 shows the survival of the seedlings over the total number of germinated plants for each dose of the mutagenic treatments assayed. The most damaging treatment was the highest concentration of EMS (0.4%), causing more than 60% of plant mortality, while the survival of seedlings was above 66-73%, for SA, X-rays treatments or their combination, even for the highest doses (figure 2).

Figure 3 shows the effect of the different mutagenic treatments and their doses on growth rate of cotton at vegetative stage. Both chemical mutagens (EMS and SA) generated a significant reduction in the growth rate, being more pronounced for EMS, which produced almost no growth per day at the highest dose. On the other hand, with X-rays and the combined (SA+X-rays) treatments the growth rate in the evaluated stage was not significantly affected.

Overall, for the germination and seedling stage parameters determined in this study, the EMS treatments produced greater effects on the cotton variety used (Guazuncho 3) than the other mutagens tested, even in the SA+X-rays combination. Finally, the X-ray treatments showed small changes in the survival of seedlings, but no differences were observed on GP or GVI, while their combination with SA exhibited a distinctive behavior, probably due to SA.
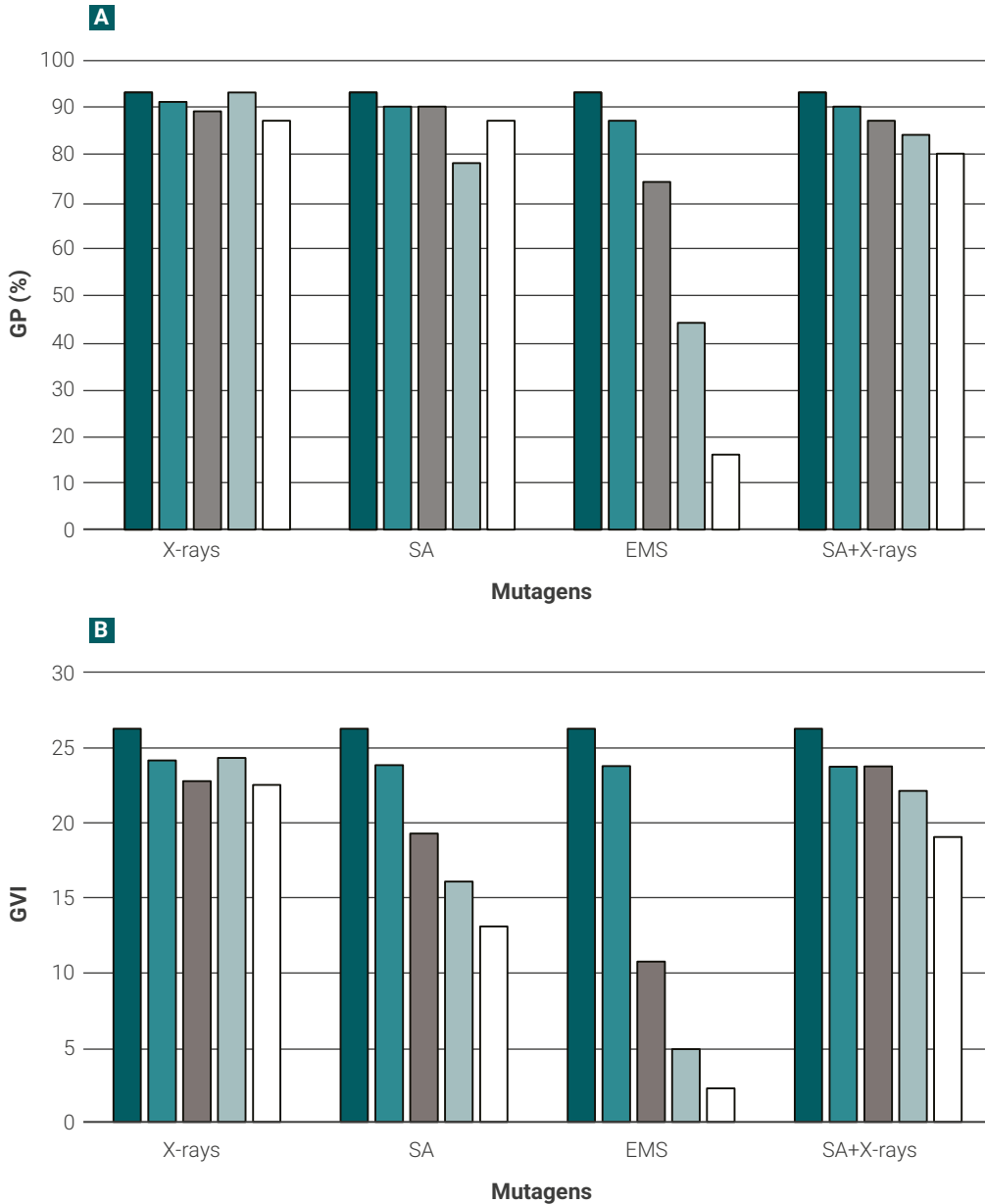
Figure 1. Germination percentage (A) and germination velocity index (B) of Guazuncho 3 cotton seeds under mutagenic treatments. Results obtained for the untreated control plants (dark blue bars), dose 1 (blue bars), dose 2 (gray bars), dose 3 (light blue bars) and dose 4 (white bars), for each mutagenic treatment. EMS: ethyl methanesulfonate, SA: sodium azide.
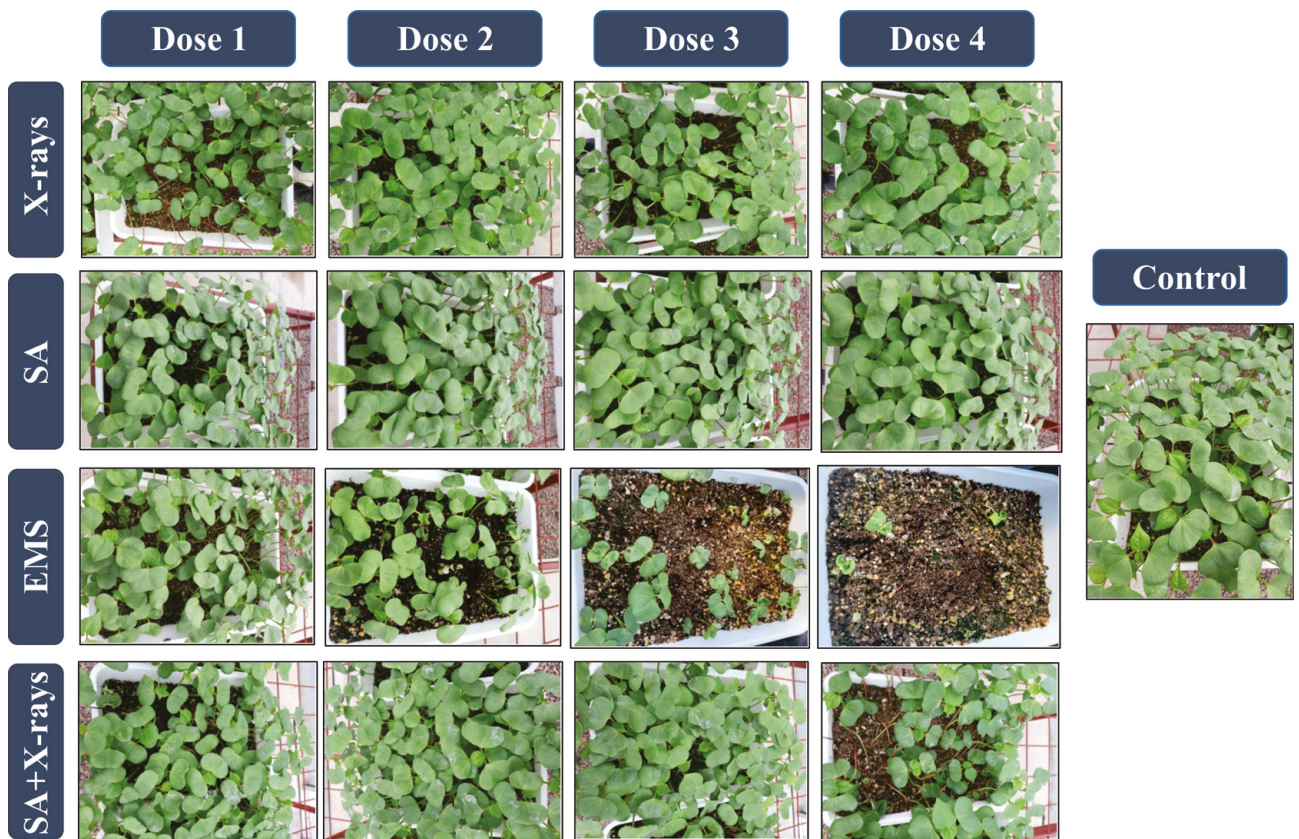
Supplemental figure 1. Cotton seeds germination. The figure shows the obtained seedlings with the different mutagenic treatments and doses whose quantification is presented on figure 1.
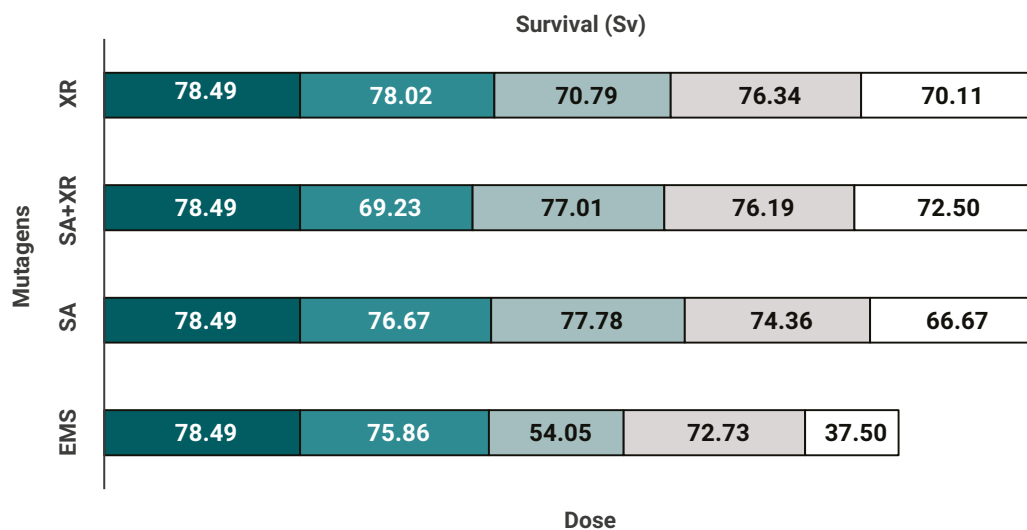

Figure 2. Seedling survival percentage. Percentage of seedlings that survived until the second leaf was fully expanded, over the total number of germinated seeds, under the different mutagenic treatments and doses. Dark blue bars (control), blue (dose 1), light blue (dose 2), gray (dose 3), white (dose 4). Survival percentage was determined as described in Materials and methods. EMS: ethyl methanesulfonate, SA: sodium azide.
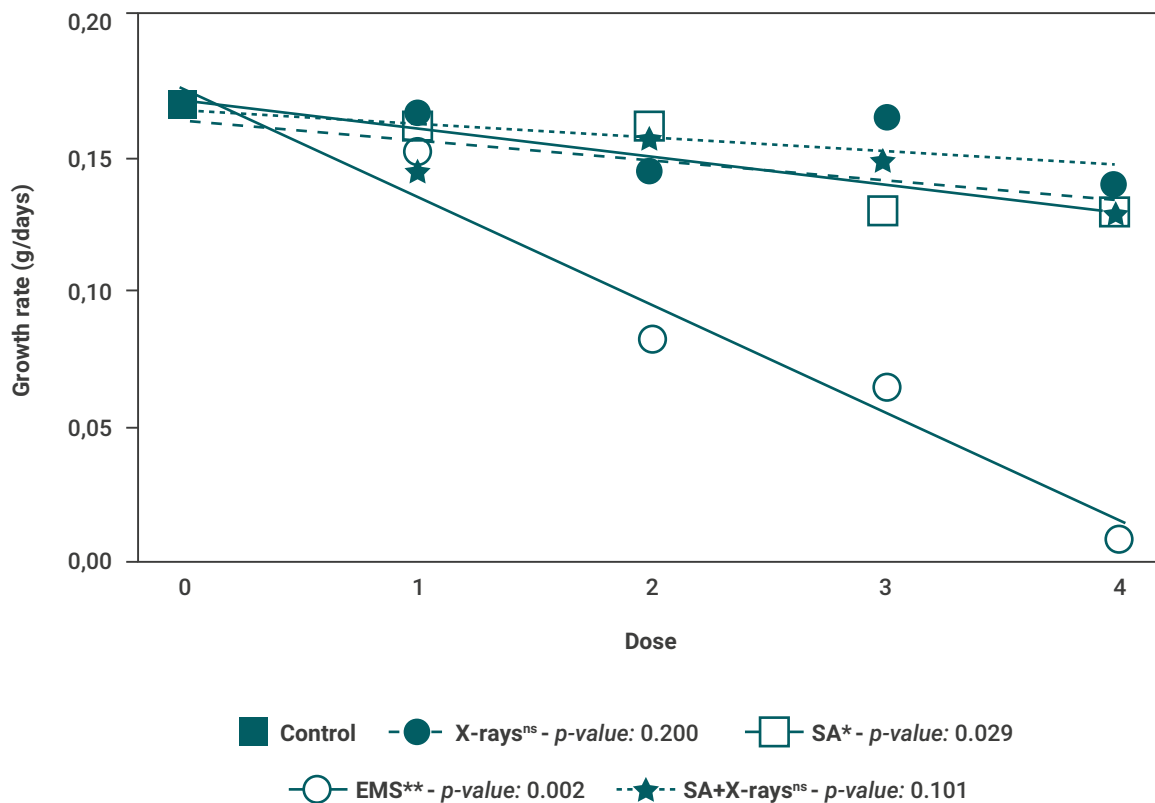
Figure 3. Growth rate vs. mutagen dose determined in vegetative stage. The growth rate was calculated as described in Materials and methods. EMS: ethyl methanesulfonate, SA: sodium azide.

Phenological and phenotypic alterations in M_1 plants

Cotton (Guazuncho 3) showed a significant delay in the development of the vegetative stage when treated with X-rays or EMS, as presented in Figure 4. The time from seedling emergence to squaring for the X-rays treatments was of 54.3 and 53.8 days for 300 Gy (dose 2) and 500 Gy (dose 4) respectively, compared to 48 days in control plants (figure 4). Similarly, the plants from trials with 0.3% and 0.4% EMS exhibited the longest emergence-squaring time, 60.9 and 57 days, respectively, while for SA and SA+X-rays treatments no significant differences were found at this stage regardless of the dose used (figure 4). Although all doses tested for EMS generated a shorter time to reach maturity (from the first open boll to the harvest time) compared to the control, the shortest time (25.2 days) was obtained in 0.3% EMS-treated plants, while 34.4 days were recorded for the control plants (figure 4). Finally, no significant differences were found in SA or SA+X-rays mutagenic treatments regardless of the doses tested.

Phenotypic alterations were also observed in some treated plants (supplemental figure 2). Phenotypic effects were classified into two groups: i) Malformed or off-type plants, and ii) Plants with chlorophyll deficient irregular-shaped spots as indicators of chimeras. Regardless of the applied dose of the mutagen, phenotypic variations were grouped and analyzed according to the type of mutagen (figure 5). Results indicated that more than 50% of the total plants with malformations

came from EMS treatments, followed by X-rays (around 30%), while the SA treatments and the combination SA+X-rays resulted in less than 10% of malformed or off-type plants (figure 5A). Similarly, X-rays and EMS mutagenic treatments showed the highest proportions of plants with chlorophyll deficient irregular-shaped spots accounting for 50% and 44%, respectively. Whilst only 5% corresponded to the combined mutagens (SA+X-rays) and 2% of plants treated with SA presented this type of phenotypic effects (figure 5B), this chlorotic effect is even less marked for the latter compared with X-rays or EMS (supplemental figure 2 H-I).

Morphological and agronomic traits in selected plants

At maturity or harvest time, some morphological and agronomic characteristics were determined in 15 selected plants from each mutagenic treatment. Both EMS and SA showed differences in plant height among the doses used (supplemental table 1). Plants coming from SA treatments exhibited a slight increase in height at lower doses compared to the control, while this effect reverted with the higher concentration of SA. Plant height from EMS treatments decreased as the dose increased, reaching only 16.27 cm at 0.4%, compared with the control plants (supplemental table 1).

Other agronomic traits, such as the percentage of retained fruits, the number of reproductive positions and the harvest

index (HI), were affected in plants subjected to mutagenic treatments. A reduction of 2.7- and 1.8-fold was determined for retention percentage and number of reproductive positions, respectively, with 500 Gy (dose 4) of X-rays. However, no significant differences were found for the number of abortions and the number of seeds per boll in plants that were treated with X-rays (supplemental table 1). Likewise, the EMS mutagen triggered similar effects on these evaluated agronomic traits, reducing fruit retention and the number of reproductive positions by 4.4- and 4.0-fold, respectively, at the highest dose (0.4%). Nevertheless, the plants treated with EMS conserved their few reproductive positions, since they presented fewer abortions when the dose increased, showing a mean of 3.50 and 1.60 abortions with 0.3% and 0.4% EMS, respectively (supplemental table 1). In addition, no significant differences were obtained for the HI or number of seeds per boll. The trial with SA did not show differences in the agronomic characteristics studied, nor did the combination of SA+X-rays.

DISCUSSION

The dosimetry of any mutagen is a crucial aspect for a mutation breeding program. In cotton, mutants for diverse characters have been obtained using different mutagenic agents at distinct doses (The Joint FAO/IAEA Mutant Variety Database (MDV) https://nucleus.iaea.org/sites/mvd/SitePages/Home.aspx). Tcach et al. (2022) isolated a mutant tolerant to imidazolinones in advanced cotton lines using 1 mM sodium azide pH 3 or 6. However, Ul-Allah et al. (2019) concluded that sodium azide doses of 15 and 25 mM induced genetic variation in cotton germplasm suitable for use in a breeding program in nine different genotypes. In addition, Auld et al. (1998) generated variability in fiber quality parameters in the upland cotton variety HS 2000 using 3% v/v EMS. Similar studies were described by Brown et al. (2015). Comparable doses of EMS (2.45%) were used by Cutts (2013) in different germplasm that exhibited herbicide tolerance. Conversely, Deho et al. (2020) observed differences in morphological and agronomic parameters in eight mutant lines with 0.03% EMS. Regarding cotton treatments with ionizing radiation, there are several mutants obtained mostly with gamma rays annotated in the IAEA MDV using different doses (https://nucleus.iaea.org/sites/mvd/SitePages/Home.aspx).

So, there are many differences in the range of doses between varieties of the same crop, making it difficult to establish a correlation between them. This might happen because the dose and type of mutagen can vary between genotypes and it is therefore advisable to perform a dosimetry in each case before the final treatment.
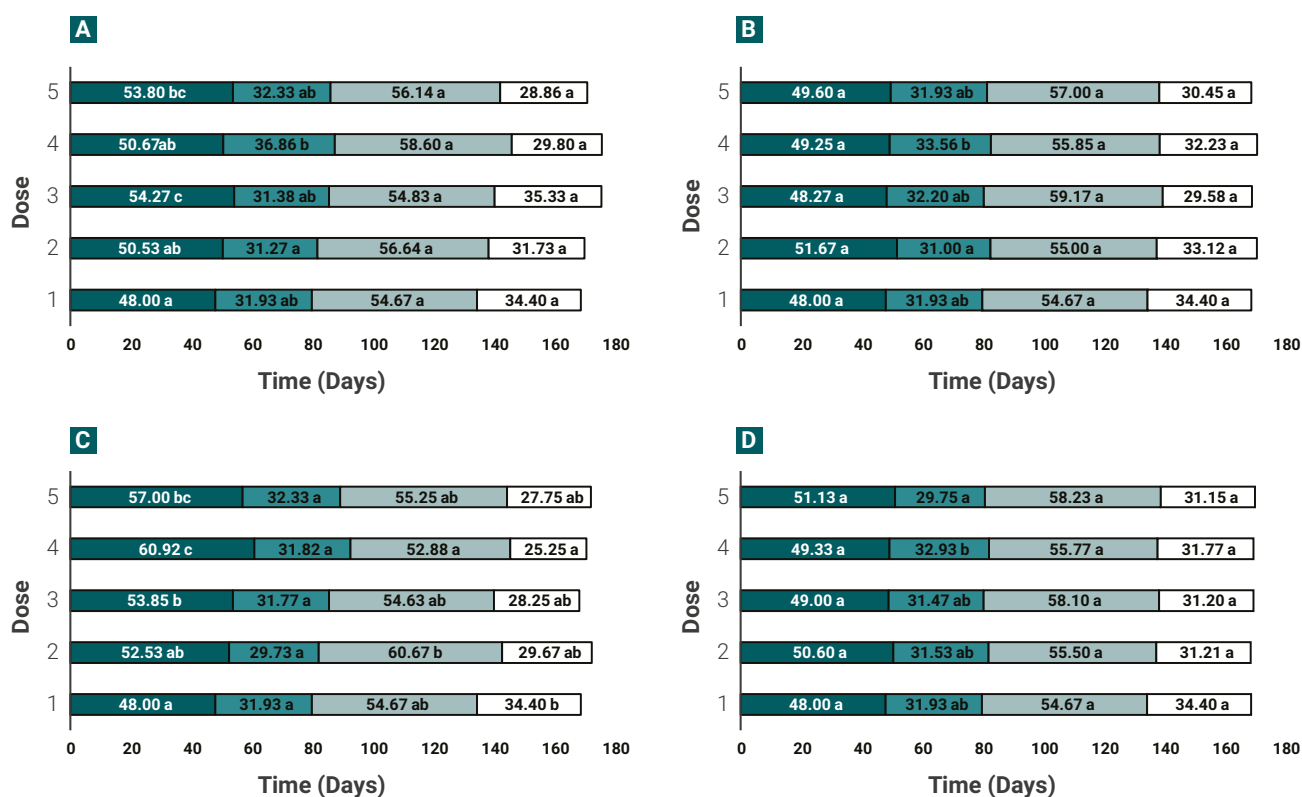

Figure 4. Phenological development of cotton crop under different mutagenic treatments. (A) X-rays, (B) SA, (C) EMS and (D) SA+X-rays in different doses. Phenology was divided in: the number of days from seedling emergence to squaring (dark blue; vegetative stage), number of days from first square to first flower (blue), days from first flower to first open boll (light blue) and days from first open boll to harvest time (white). EMS: ethyl methanesulphonate, SA: sodium azide. *<0.05, **<0.01, ***<0.001, ns: no significance.

Supplemental figure 2. Representative pictures from M_1 mutagenized cotton seedlings and plants with observed abnormal phenotypes. A: control plant, EMS-treated seedlings/plants with malformations (B-C-D), chlorotic regions and malformed branches (E-F), malformed EMS plant at maturity (G), chlorotic effect with SA (H) and with X-rays (I).
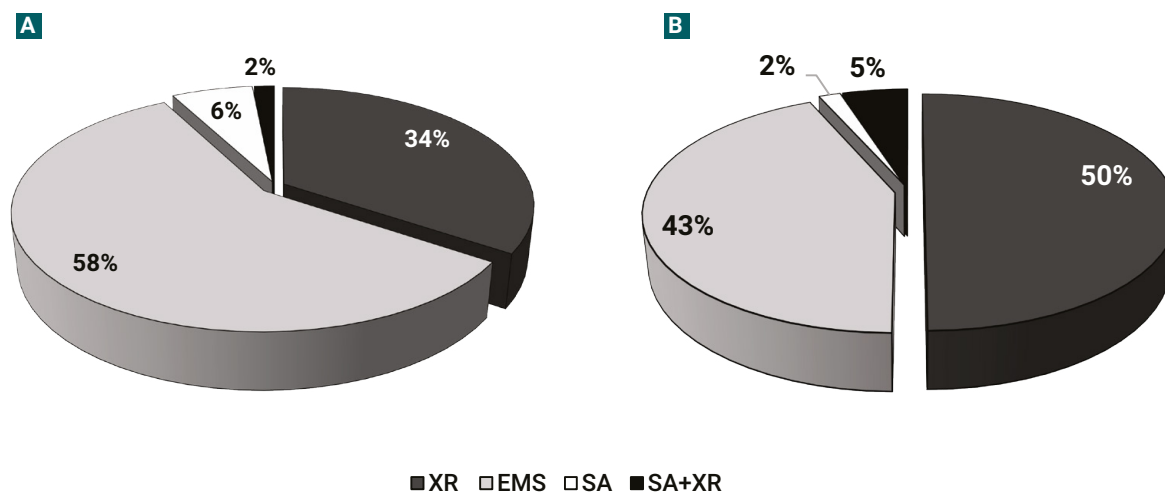
Figure 5. Phenotypic effects of mutagenic treatments on cotton seedlings and plants. (A) Percentages of malformed or off-type plants. (B) Plants with chlorophyll deficient irregular-shaped spots. EMS: ethyl methanesulfonate, SA: sodium azide.

Germination Percentage

Both chemical mutagens, SA and EMS, significantly reduced the GP compared to the controls. It is well known that the SA and EMS have an effect over seed germination, delaying or decreasing it (Espina *et al*., 2018; Gruszka *et al*., 2012).

The greatest effect was observed in the EMS treatment, which produced an abrupt reduction in the number of germinated seeds as the dose was increased. High doses of radiation or chemical agents produce physiological and biochemical alterations, *i.e.* they can alter hormones such as auxins, produce changes in the content of ascorbic acid, generate free radicals and increase the α-amylase activity, among others. Besides, some authors suggest that this reduction in seed germination could be due to an effect of mutagens in the meristematic tissue of the embryo. Together, these alterations inhibit vital functions that can result in the death of certain cells and even the embryo (Kurtar *et al*., 2017; Lagoda, 2012).

Although there were significant differences in GP for SA treatments, an increase in germinated seeds was observed with the highest dose evaluated. Similar results were obtained for other cotton germplasm by Baimuhametova *et al*., (2017), who even recommended 1 to 5 mM SA for cottonseed treatment. These authors suggested that SA has high mutagenicity and at certain concentrations this chemical could have a stimulating effect on germination. Additional studies showed a stimulating effect of SA on germination of pure lines in wild oats (*Avena fatua* L.) (Upadhyaya *et al*., 1982) due to an alternative respiration mechanism or pathway, since a stimulation in oxygen uptake was observed in the presence of SA (Upadhyaya *et al*., 1983). Nevertheless, more studies are needed to confirm the relationship between the alternative respiration by SA and the stimulation of germination.

On the other hand, no effects on germination of cotton seeds were observed with X-rays regardless of the dose used. These results are in agreement with studies that suggest that ionizing radiation only affects cell division and not the elongation of predetermined cells, because large damage to DNA will be evidenced at replication at cell division, thus not affecting germination (Prina, 1989). However, other studies showed a decrease in GP as the ionizing radiation dose increased in different crops, such as soybean (Mudibu *et al*., 2012), linen (Bhat *et al*., 2017), wheat (Ahumada-Flores *et al*., 2021), sorghum (Wanga *et al*., 2020). Results from Aslam *et al*. (2013) showed more than 50% decrease in GP at 300 Gy of gamma rays treatment in cotton, in contrast to what was observed in this work.

It is important to consider that the level of the effects observed from irradiation treatments in the different germplasm can be affected by different biological, environmental and chemical factors, such as, the water content of the seeds, the atmosphere, temperature, storage after irradiation, genetic differences, cell cycle phases, etc. (Spencer Lopes *et al*., 2018). Thus, we could assume that our results are due to the type of seed, its hardness, as well as the absence of delinting and hydration of the seeds.

Germination Velocity Index

Mutagens have been reported to cause a delay in germination time, determined by the germination velocity index (GVI) (Adeosun *et al*., 2020; Khan *et al*., 2009; Nakagawa, 1994). EMS, SA and combined (SA+X-rays) treatments showed significant differences in GVI. EMS exhibited a clear tendency to decrease the GVI as the dose increased, mainly influenced by the number of germinated seeds than by the germination time. In contrast, our results showed that SA treatment did not affect the number of germinated plants, thus the decrease observed in GVI as the dose of SA increased was the result of a delay in seed germination. Finally, a similar but milder behavior compared to SA was observed in GVI for combined treatments. This lower phenotypic effect of the combined treatments could be attributed to a protection effect exerted by sodium azide against physiological damage, chromosomal damage and lethality caused by radiation, as it was previously suggested (Prina and Favret, 1983).

Treatment	Dose	Plant height (cm)		Retention (%)		N° Abortions		N° reproductive positions		HI		N° seeds.boll^{-1}
Control	0	62.47	a	30.09	a	5.93	ab	8.47	a	0.35	a	17.34
X-rays	1	61.93		31.25 a		5.73		8.40 a		0.33 ab		17.96
	2	59.40	62.15 a	16.59 b	20.05 bc	4.87	4.78 b	6.33 ab	6.55 b	0.30 ab	0.28 b	19.38
	3	63.60		21.14 ab		4.87		6.80 ab		0.23 c		17.13
	4	63.67		11.23 b		3.67		4.67 c		0.26 bc		17.66
	Signif.	ns		***		ns		*		*		ns
SA	1	63.13 abc		21.04		7.47		9.40		0.29		20.46
	2	66.73 a	63.56 a	25.08	24.19 ab	7.53	7.16 a	10.00	9.38 a	0.29	0.31 ab	18.45
	3	64.19 ab		26.30		6.88		9.19		0.31		19.38
	4	60.13 c		24.21		6.80		8.93		0.35		21.99
	Signif.	*		ns		ns		ns		ns		ns
EMS	1	60.40 a		22.65 a		4.20 ab		6.00 ab		0.32		18.98
	2	56.21 ab	44.66 b	21.99 a	18.52 c	5.00 ab	3.55 c	6.79 ab	4.98 c	0.32	0.33 a	19.98
	3	46.64 b		23.14 a		3.50 bc		5.14 b		0.33		15.71
	4	16.27 c		6.85 b		1.60 c		2.13 c		0.40		17.67
	Signif.	***		**		***		***		ns		ns
SA+ X-rays	1	60.07		25.01		6.20		8.20		0.31		19.19
	2	66.27	63.20 a	26.61	26.86 a	6.67	6.43 a	9.07	8.75 a	0.33	0.32 a	18.07
	3	63.73		28.26		6.27		8.73		0.34		20.52
	4	62.75		27.51		6.56		9.00		0.31		17.56
	Signif.	ns		ns		ns		ns		ns		ns
Signif.		***		**		***		***		*		ns

Supplemental table 1. Morphological and agronomic traits analyzed in different treatments and doses. Parameters were evaluated at maturity. Retention: percentage of retained fruits on the plant, N° abortions: number of bolls that do not develop fully, HI: harvest index. Signif.: statistical significance, ns: no significance, *<0.05, **<0.01, ***<0.001.

The delay in seed germination is a common effect in chemical mutagenic treatments such as SA. This can be attributed to the inhibition of necessary physiological and biological processes, including enzyme activities alteration, hormonal imbalance, and inhibition of mitotic processes. The metabolite formed between azide and alanine, L-azidoalanine, causes mutations by interacting with enzymes and DNA in the cell, being a strong inhibitor of cytochrome oxidase, consequently inhibiting the oxidative phosphorylation process. Furthermore, azide acts as a potent inhibitor of the proton pump, altering mitochondrial membrane potential. Together, these SA-induced effects cause an obstruction in the main ATP biosynthesis pathway, which can directly influence the slowing of the germination rate (Gruszka *et al*., 2012; Khan *et al*., 2009; Leitao, 2012).

Survival

Previous studies have reported that the mutagens affect not only germination but also seedling survival (Sv), at least until second leaf is fully expanded, in different plant species (Ahumada-Flores *et al*., 2021; Aslam *et al*., 2013; Espina *et al*.,

2018; Ingelbrecht *et al*., 2018; Kodym and Afza, 2003; Nielen *et al*., 2018a; Spencer Lopes *et al*., 2018). Plant survival was also affected by the mutagenic treatments. The highest percentage of seedling mortality was obtained with 0.4% EMS, being 3-fold higher compared to the control. While, for SA, X-rays and the combined treatment of both, a survival of approximately 70% was observed at their respective higher doses. Therefore, the results revealed that EMS caused more seedling lethality than X-rays and SA, and this reduction in survival rate could be associated with the toxic effect of EMS (Bhat *et al*., 2017). Besides, Espina *et al*. (2018) suggested that the effect of EMS on germinated soybean seedlings survival could be reduced due to cell damage and poor root and shoot development. This result has also been observed in pepper (Arisha *et al*., 2015) and cucumber (Shah *et al*., 2015). Finally, for SA a significant reduction in survival was also observed for the highest dose. Similar results were observed by Khan *et al*. (2009), who attributed this effect to physiological alterations and imbalance between promoters and inhibitors of growth regulators, which led to seedling death.

Growth rate and height

A reduction in seedling or plant growth in different crops after mutagenic treatments have been reported before (Ingelbrecht *et al*., 2018; Joshi *et al*., 2011; Spencer Lopes *et al*., 2018; Suprasanna *et al*., 2012). An increase in the concentration of the mutagen has often shown more physiological damage and direct DNA damage, biochemical disturbances, auxin destruction and changes in ascorbic acid content (Joshi *et al*., 2011). Together, these damages are phenotypically reflected in plant growth (Kodym *et al*., 2012).

Our results showed a significant reduction in the growth rate of cotton plants as the chemical treatment dose increased; EMS caused more reduction in plant growth than SA. Also, a significant effect on plant height was observed in both chemical treatments, obtaining a linear reduction in height in EMS treated plants as the dose increased, similarly to previous studies in cotton (Aslam *et al*., 2013).

Interestingly, a low dose of SA recorded taller plants compared to the control. This effect was also previously observed in cotton, *Calendula officinalis* L., *Salvia splendens*, *Amaranthus*, onion (Baimuhametova *et al*., 2017; El-Nashar and Asrar, 2016; Joshi *et al*., 2011). These results could be explained by "hormesis" effect, which has been defined as a favorable response to exposures at low levels of adverse conditions (Jalal *et al*., 2021). Hypothetically, this mild stimulation of plant height would be related to an increase in the rate of cell division, as well as by an activation of growth hormones such as auxins (Joshi *et al*., 2011). Although no significant differences were obtained in growth or height for X-rays treatment in our study, the hormesis effect has been mainly observed in ionizing radiation treatments (Nielen *et al*., 2018b).

Phenology

EMS and X-rays treatments produce chromosomal aberrations that can negatively affect cell division (Hadebe *et al*., 2018). In this context, these damages could be responsible for the observed significant delay in the vegetative stage of cotton plants treated with these mutagens. On the other hand, SA+X-rays combination did not show significance compared with X-rays treatment. As it was discussed previously, the differences between these treatments could be attributed to a protection effect exerted by SA against the damages caused by radiation (Prina and Favret, 1983). Additionally, a reduction in maturity time was observed at the end of the plant cycle (crop maturity) for EMS treatment, in higher doses compared with the control.

Somatic mutations

Morphological changes such as variations in leaf shape, dwarfism, branching pattern, physical lesions and chimerism were observed in our study. These phenotypic characteristics have been commonly observed in different crops after mutagenic treatments since they are easily measurable in M_1 generation and are good indicators of mutagenic efficacy (Abid *et al*., 2020; Aslam *et al*., 2013; El-Nashar and Asrar, 2016; Espina *et al*., 2018; Favret, 1960; Landau *et al*., 2021; Lian *et al*., 2020; Prina *et al*., 2012). Our results showed that EMS and X-rays treatments originated the highest number of malformed plants and irregular-shaped spots with chlorophyll deficiencies.

Chimerism of M_1 can be observed as irregular spots in dicotyledonous plants. The sectors with notable changes in the amount of chlorophyll, called chlorophyll-deficient sectors, are easily seen and are usually counted to estimate the frequencies of induced somatic mutations (Prina, 1989).

Prina (1989) pointed out a relationship between somatic mutations and mutations in germ lines in barley after treatments with different mutagens. A ratio of M_2 mutations to M_1 mutated sectors was established, which was approximately 0.4 for X-rays, 2.5 for EMS, and 6 in SA treatments. The author assumed that the frequency of somatic mutations in M_1 can serve as an indicator of the frequency of mutations to be expected in M_2, the generation where a selection process can begin (Prina, 1989). However, these values or relationships between somatic mutations (M_1) and germinal mutations (M_2) are specific to each crop and species. Although this ratio has not been established for cotton yet, the somatic mutations observed and recorded in the present work are good parameters to indicate the level of damage per dose for each mutagenic agent.

Fertility or sterility

M_1 plants typically show reduced fertility, mainly caused by chromosomal rearrangements and genomic mutations during meiosis (Lagoda, 2012). Reduced fertility is also considered as a reduction in reproductive capacity, including a severe growth retardation or growth inhibition that prevents flowering, formed flowers lacking reproductive structures, abortion of pollen or ovules, abortion of fertilized embryos before maturity and formed seeds that do not germinate properly or die after germination (Nielen *et al*., 2018b). Therefore, fertility or sterility could be assessed as the number of flowers, fruits and seeds produced at crop maturity.

In our work, fertility was considered as the number of seeds produced per boll that reached physiological maturity. No significant differences were found between treatments or between doses. All the plants that reached the reproductive stage produced seeds, considering then that there were no mutagenic effects on meiosis.

On the other hand, significant differences were observed in the number of reproductive positions, abortions and fruit retention percentage. The greatest effects were recorded in EMS and X-rays treatments, assuming that the effects on these variables could be mainly due to physiological effects (Nielen *et al*., 2018b).

CONCLUSIONS

The treatments with EMS were the most aggressive for cotton seeds, since they showed the greatest number of plants with altered phenotypic effects (PG, height, survival, etc.), followed by X-rays and then SA treatments. Finally, the combined treatment SA+X-rays showed the least efficacy for the evaluated traits. According to our results, the intermediate doses (300 Gy, 4 mM, 0.2%, for X-rays, SA and EMS, respectively) would be the most recommended for cotton (Guazuncho 3 elite variety). In summary, this study provided an approximation of the most appropriate doses of each mutagen to use to generate the M_1 population. The frequencies of mutations generated by each of these doses can only be evaluated in the M_2 generation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

ABID, M.A.; WANG, P.; ZHU, T.; LIANG, C.; MENG, Z.; MALIK, W.; GUO, S.; ZHANG, R. 2020. Construction of Gossypium barbadense Mutant Library Provides Genetic Resources for Cotton Germplasm Improvement. Int. J. Mol. Sci. Artic. 21, 6505. https://doi.org/10.3390/ijms21186505

ADEOSUN, C.A.; ELEM, K.A.; EZE, C.D. 2020. Mutagenic effects of sodium azide on the survival and morphological characters of tomato varieties. Niger. J. Biotechnol. 37, 55-62. https://doi.org/10.4314/njb.v37i1.6

AHUMADA-FLORES, S.; GÓMEZ PANDO, L.R.; PARRA COTA, F.I.; DE LA CRUZ TORRES, E.; SARSU, F.; DE LOS SANTOS VILLALOBOS, S. 2021. Technical note: Gamma irradiation induces changes of phenotypic and agronomic traits in wheat (Triticum turgidum ssp. durum). Appl. Radiat. Isot. 167, 109490-109490. https://doi.org/10.1016/j.apradiso.2020.109490

ARISHA, M.H.; SHAH, S.N.M.; GONG, Z.H.; JING, H.; LI, C.; ZHANG, H.X. 2015. Ethyl methane sulfonate induced mutations in M2 generation and physiological variations in M1 generation of peppers (Capsicum annuum L.). Front. Plant Sci. 6, 399. https://doi.org/10.3389/fpls.2015.00399

ASLAM, U.; CHEEMA, H.M.N.; AHMAD, S.; KHAN, I.A.; MALIK, W.; KHAN, A.A. 2016. COTIP: Cotton TILLING platform, a resource for plant improvement and reverse genetic studies. Front. Plant Sci. 7, 1863. https://doi.org/10.3389/fpls.2016.01863

ASLAM, M.; HAQ, M.A.; BANDESHA, A.A.; HAIDAR, S. 2018. NIAB-846: High yielding and better quality cotton mutant developed through pollen irradiation technique. Pakistan J. Agric. Sci. 55, 767-776. https://doi.org/10.21162/PAKJAS/18.5133

ASLAM, U.; KHAN, A.A.; CHEEMA, H.M.N.; IMTIAZ, F.; MALIK, W. 2013. Kill curve analysis and response of ethyl methanesulfonate and γ-rays in diploid and tetraploid cotton. Int. J. Agric. Biol. 15, 11-18.

AULD, D.L.; BECHERE, E.; KRIFA, M.; KEBEDE, H.; HEQUET, E.; WRIGHT, R.; MISRA, S. 2007. Registration of 'Raider 276', a High-Yielding, Improved-Quality Upland Mutant Cotton Cultivar. J. Plant Regist. 1, 115-116. https://doi.org/10.3198/JPR2007.01.0059CRC

AULD, D.L.; ETHRIDGE, M.D.; DEVER, J.K.; DOTRAY, P.D. 1998. Chemical mutagenesis as a tool in cotton improvement. https://doi.org/10.2/JQUERY.MIN.JS

BAIMUHAMETOVA, E.A.; LASHTABOVA, S.V.; GOLOVINA, V.Y.; KIMSANBAEV, O.H.; KULUEV, B.R. 2017. Application of induced mutagenesis for increasing the genetic polymorphism of cotton. BIOMICS 9, 370-379.

BECHERE, E.; AULD, D.L.; CANTRELL, R.G.; HEQUET, E.; KRIFA, M.; MISRA, S.; WAYNE, C.; BECHERE, S.E. 2007. Registration of TTU 0774-3-3 and TTU 0808-1-6-1 Upland Cotton Germplasm Lines with Improved Fiber Length and Strength. J. Plant Regist. 1, 58-59. https://doi.org/10.3198/JPR2006.09.0587CRG

BECHERE, E.; AULD, D.L.; DOTRAY, P; KEBEDE, H. 2010. Registration of Four Upland Cotton (Gossypium hirsutum L.) Genetic Stock Mutants with Tolerance to Imazamox. J. Plant Regist. 4, 155-158. https://doi.org/10.3198/JPR2009.08.0446CRGS

BECHERE, E.; AULD, D.L. 2014. Registration of a Tufted-Naked Seed Upland Cotton Germplasm, 9023n4t. J. Plant Regist. 8, 63-67. https://doi.org/10.3198/JPR2013.06.0025CRG

BHAT, I.A.; PANDIT, U.J.; SHEIKH, I.A.; HASSAN, Z.U. 2017. Physical and Chemical Mutagenesis in Linum usitatissimum L. to Induce Variability in Seed Germination, Survival and Growth rate traits. Curr. Bot. 7, 28. https://doi.org/10.19071/cb.2016.v7.3054

BONACIC KRESIC, I.; IBALO, S.; ERAZZU, L.E.; ROYO, O.M.; FARIÑA NUÑEZ, J.R.; ORTIZ, R.; POISSON, J.A.; MONTENEGRO, A. 2004. Guazuncho 3. INTA.

BOOPATHI, N.M.; SATHISH, S.; KAVITHA, P.; DACHINAMOORTHY, P.; RAVIKESAVAN, R. 2015. Molecular breeding for genetic improvement of cotton (Gossypium spp.), in: Advances in Plant Breeding Strategies: Breeding, Biotechnology and Molecular Tools. Springer International Publishing, 613-645 pp. https://doi.org/10.1007/978-3-319-22521-0_21

BROWN, I.N.; SMITH, C.W.; AULD, D.; HAGUE, S.; HEQUET, E.F.; JONES, D. 2012. Registration of TAM 94L-25-M24, TAM 94L-25-M25, and TAM 94L-25-M30 Mutant Upland Cotton Germplasm with Improved Fiber Length and Strength. J. Plant Regist. 6, 195-199. https://doi.org/10.3198/JPR2011.05.0245CRG

BROWN, N.; SMITH, C.W.; HAGUE, S.; AULD, D.; HEQUET, E.; JOY, K.; JONES, D. 2015. Within Boll Yield Characteristics and Their Correlation with Fiber Quality Parameters following Mutagenesis of Upland Cotton, TAM 94L 25. Crop Science, 55(4), 1513-1523.

CUTTS, G.S. 2013. Genetic Analysis, Inheritance and Stability of Mutation-based Herbicide Tolerance in Cotton (Gossypium hirsutum L.) (Doctoral dissertation).

DEHO, Z.A.; ABRO, S.; RIZWAN, M. 2020. Improvement of cotton yield and fiber quality parameters in upland cotton (gossypium hirsutum l.) Genotypes through chemical mutagen: Nuclear Institute of Agriculture, Tandojam, (NIA), Pakistan. Pakistan Journal of Agriculture, Agricultural Engineering and Veterinary Sciences, 36(1), 8-12.

DI RIENZO, J.A.; CASANOVES, F.; BALZARINI, M.G.; GONZALEZ, L.; TABLADA, M.; ROBLEDO, C.W.; 2011. InfoStat.

EL-NASHAR, Y.I.; ASRAR, A.A. 2016. Phenotypic and biochemical profile changes in calendula (Calendula officinalis L.) plants treated with two chemical mutagenesis. Genet. Mol. Res. 15. https://doi.org/10.4238/gmr.15028071

ESPINA, M.J.; AHMED, C.M.S.; BERNARDINI, A.; ADELEKE, E.; YADEGARI, Z.; ARELLI, P.; PANTALONE, V.; TAHERI, A. 2018. Development and phenotypic screening of an ethyl methane sulfonate mutant population in Soybean. Front. Plant Sci. 9, 394. https://doi.org/10.3389/fpls.2018.00394

FAVRET, E.A. 1960. Spontaneous and induced mutations of Barley for the reaction to mildew. Hereditas 46.

GANESAN, M.; BHANUMATHI, P.; JAYABALAN, N. 2005. Mutagenic effect of sodium azide on somatic embryo regeneration and root growth of cotton (Gossypium hirsutum L. CV. SVPR2). J. Agric. Technol. 1, 365-380.

GRUSZKA, D.; SZAREJKO, I.; MALUSZYNSKI, M. 2012. Sodium Azide as a mutagen, in: Shu, Q.Y., Forster, B.P., Nakagawa, J. (Eds.), Plant Mutation Breeding and Biotechnology. 159-166 pp.

HADEBE, S.T.; MODI, A.T.; ODINDO, A.O.; SHIMELIS, H.A. 2018. Inducing genetic mutation on selected vernonia accessions using predetermined ethylmethanesulfonate dosage, temperature regime and exposure duration conditions. South African J. Bot. 119, 301-306. https://doi.org/10.1016/j.sajb.2018.09.032

IJAZ, B.; ZHAO, N.; KONG, J.; HUA, J. 2019. Fiber Quality Improvement in Upland Cotton (Gossypium hirsutum L.): Quantitative Trait Loci Mapping and Marker Assisted Selection Application. Front. Plant Sci. https://doi.org/10.3389/fpls.2019.01585

INGELBRECHT, I.; JANKOWICZ-CIESLAK, J.; SZURMAN, M.; TILL, B.J.; SZAREJKO, I.; 2018. Chemical mutagenesis. In: SPENCER-LOPES, M.M.; FORSTER, B.P.; JANKULOSKI, L. (Eds.). Manual on Mutation Breeding. 51-82 pp.

JALAL, A.; DE OLIVEIRA JUNIOR, J.C.; RIBEIRO, J.S.; FERNANDES, G.C.; MARIANO, G.G.; TRINDADE, V.D.R.; DOS REIS; A.R. 2021. Hormesis in plants: Physiological and biochemical responses. Ecotoxicol. Environ. Saf. https://doi.org/10.1016/j.ecoenv.2020.111225

JIANG, G.L. 2016. Molecular marker-assisted breeding: A plant breeder's review, in: Advances in Plant Breeding Strategies: Breeding, Biotechnology and Molecular Tools. Springer International Publishing, 431-472 pp. https://doi.org/10.1007/978-3-319-22521-0_15

JOSHI, N.; RAVINDRAN, A.; MAHAJAN, V. 2011. Investigations on chemical Mutagen Sensitivity in onion (Allium cepa L.). Int. J. Bot. 7, 243-248. https://doi.org/10.3923/ijb.2011.243.248

KALWAR, K.; DAHOT, M.U. 2017. Effect of induced mutation by UV radiation on cotton growth, seeds and protease activity. Pakistan J. Biotechnol. 14, 105-107.

KE, C.; GUAN, W.; BU, S.; LI, X.; DENG, Y.; WEI, Z.; WU, W.; ZHENG, Y. 2019. Determination of absorption dose in chemical mutagenesis in plants. PLoS One 14. https://doi.org/10.1371/journal.pone.0210596

KHAN, S.; AL-QURAINY, F.; ANWAR, F. 2009. Sodium azide: A chemical mutagen for enhancement of agronomic traits of crop plants. Environ. We an Int. J. Sci. Technol. 04, 1-21. https://doi.org/10.4172/2155-9597.c1.014

KODYM, A.; AFZA, R. 2003. Physical and chemical mutagenesis. Methods Mol. Biol. 236, 189-204. https://doi.org/10.1385/1-59259-413-1:189

KODYM, A.; AFZA, R.; FORSTER, B.P; UKAI, Y.; NAKAGAWA, H.; MBA, C. 2012. Methodology for physical and chemical mutagenic treatments. In: SHU, Q.Y.; FORSTER, B.P.; NAKAGAWA, J. (Eds.). Plant Mutation Breeding and Biotechnology. 169-180 pp.

KURTAR, E.; BALKAYA, A.; KANDEMIR, D. 2017. Determination of semi-lethal (LD50) doses for mutation breeding of winter squash (Cucurbita maxima duch.) and pumpkin (Cucurbita moschata duch.). Fresenius Environ. Bull. 26, 3209-3216.

LAGODA, P.J. 2012. Effects of radiation on living cells and plants. In: SHU, Y. (Ed.). Plant Mutation Breeding and Biotechnology. 123-134 pp.

LANDAU, A.; BRIZUELA, V.; LENCINA, F.; MARTÍNEZ, A.; TCACH, M.; DÍAZ, D.; PACHECO, M.G.; PRINA, A.R. 2021. Induced mutations in crop plants at Instituto de Genética "Ewald A. Favret": mutants of scientific and/or agronomic interest. ACI Av. en Ciencias e Ing. 12, 14. https://doi.org/10.18272/aci.v12i3.1928

LEITAO, J.M. 2012. Chemical mutagenesis. In: SHU, Q.Y.; FORSTER, B.P.; NAKAGAWA, H. (Eds.). Plant Mutation Breeding and Biotechnology. 135-158 pp.

LIAN, X.; LIU, Y.; GUO, H.; FAN, Y.; WU, J.; GUO, H.; JIAO, C.; TANG, Z.; ZHANG, L.; FAN, Y.; GOU, Z.; ZHANG, C.; LI, T.; ZENG, F. 2020. Ethyl methanesulfonate mutant library construction in Gossypium hirsutum L. for allotetraploid functional genomics and germplasm innovation. Plant J. 103, 858-868. https://doi.org/10.1111/tpj.14755

MALUSZYNSKI, M.; SZAREJKO, I.; BHATIA, C.R.; NICHTER-LEIN, K.; LAGODA, P.J.L. 2009. Methodologies for generating variability. Part 4: Mutation techniques. Plant Breed. farmer Particip. 159-194.

MBA, C.; AFZA, R.; BADO, S.; MOHAN JAIN, S. 2010. Induced Mutagenesis in Plants Using Physical and Chemical Agents. In: WILEY, J.; CHICHESTER, S. Plant Cell Culture. Ltd, Chichester, UK, 111-130 pp. https://doi.org/10.1002/9780470686522.ch7

MBA, C. 2013. Induced Mutations Unleash the Potentials of Plant Genetic Resources for Food and Agriculture. Agronomy 3, 200-231. https://doi.org/10.3390/agronomy3010200

MENESES, C.H.S.G.; BRUNO, R. DE L.A.; FERNANDES, P.D.; PEREIRA, W.E.; LIMA, L.H.G.; DE ANDRADE LIMA, M.M.; VIDAL, M.S. 2011. Germination of cotton cultivar seeds under water stress induced by polyethyleneglycol-6000. Sci. Agric. 68, 131-138. https://doi.org/10.1590/S0103-90162011000200001

MUDIBU, J.; NKONGOLO, K.C.; KALONJI-MBUYI, A.; KIZUNGU, R.V. 2012. Effect of Gamma Irradiation on Morpho-Agronomic Characteristics of Soybeans (Glycine max L.). Am. J. Plant Sci. 03, 331−337. https://doi.org/10.4236/ajps.2012.33039

MUTHUSAMY, A.; JAYABALAN, N. 2013. Variations in seed protein content of cotton (Gossypium hirsutum L.) mutant lines by in vivo and in vitro mutagenesis. J. Environ. Biol. 34, 11-16.

MUTHUSAMY, A.; JAYABALAN, N. 2011. In vitro induction of mutation in cotton (Gossypium hirsutum L.) and isolation of mutants with improved yield and fiber characters. Acta Physiol. Plant. 33, 1793-1801. https://doi.org/10.1007/s11738-011-0718-8

NAKAGAWA, J. 1994. Seed vigour tests based on seedling characteristics. In: VIEIRA, R.D.; CARVALHO, N.M. (Eds.). Vigor Test on Seeds. 49-85 pp.

NIELEN, S.; FORSTER, B.P.; BADIGANNAVAR, A. 2018a. Types of mutation. In: SPENCER-LOPES, M.M.; FORSTER, B.P.; JANKULOSKI, L. (Eds.). Manual on Mutation Breeding. 83-101 pp.

NIELEN, S.; FORSTER, B.P.; HESLOP-HARRISON, J.S. (Pat). 2018b. Mutagen effects in the first generation after seed treatment: biological effects on mutation treatments. In: SPENCER-LOPES, M.M.; FORSTER, B.P.; Jankuloski, L. (Eds.). Manual on Mutation Breeding. 105-115 pp.

OLADOSU, Y.; RAFII, M.Y.; ABDULLAH, N.; HUSSIN, G.; RAMLI, A.; RAHIM, H.A.; MIAH, G.; USMAN, M. 2016. Principle and application of plant mutagenesis in crop improvement: a review. Biotechnol. Biotechnol. Equip. 30, 1-16. https://doi.org/10.1080/13102818.2015.1087333

PATEL, J.D.; WRIGHT, R.J.; AULD, D.; CHANDNANI, R.; GOFF, V.H.; INGLES, J.; PIERCE, G.J.; TORRES, M.J.; PATERSON, A.H.

2014. Alleles conferring improved fiber quality from EMS mutagenesis of elite cotton genotypes. Theor. Appl. Genet. 127, 821-830. https://doi.org/10.1007/S00122-013-2259-6/TABLES/5

PRINA, A.; FAVRET, E.A. 1983. Parabolic effect in sodium azide mutagenesis in barley. Hereditas 98, 89-94. https://doi.org/10.1111/j.1601-5223.1983.tb00583.x

PRINA, A.R. 1989. Consideraciones sobre la aplicación eficiente de la mutagénesis inducida en fitomejoramiento. Mendeliana 9, 5-49.

PRINA, A.R.; LANDAU, A.M.; PACHECO, M. 2012. Chimeras and Mutant Gene Transmission. In: SHU, Q.Y.; FORSTER, B.P.; NAKAGAWA, H. (Eds.). Plant Mutation Breeding and Biotechnology.

SCARPIN, G.J.; DILEO, P.N.; WINKLER, H.M.; CEREIJO, A.E.; LORENZINI, F.G.; ROESCHLIN, R.A.; MUCHUT, R.J.; ACUÑA, C.; PAYTAS, M. 2022. Genetic progress in cotton lint and yield components in Argentina. F. Crop. Res. 275. https://doi.org/10.1016/j.fcr.2021.108322

SHAH, S.; GONG, Z.H.; ARISHA, M.H.; KHAN, A.; TIAN, S.L. 2015. Effect of ethyl methyl sulfonate concentration and different treatment conditions on germination and seedling growth of the cucumber cultivar Chinese long (9930). Genet. Mol. Res. 14, 2440-2449. https://doi.org/10.4238/2015.March.30.2

SHIM, J.; GANNABAN, R.B.; DE LOS REYES, B.G.; ANGELES-SHIM, R.B. 2019. Identification of novel sources of genetic variation for the improvement of cold germination ability in upland cotton (Gossypium hirsutum). Euphytica 215, 1-16. https://doi.org/10.1007/S10681-019-2510-6/FIGURES/2

SPENCER LOPES, M.M.; JANKULOSKI, L.; GHANIM, M.A.; MATIJEVIC, M.; KODYM, A.; 2018. Physical Mutagenesis, in: Manual on Mutation Breeding. 5-49 pp.

SUPRASANNA, P.; JAIN, S.M.; OCHATT, S.J.; KULKARNI, V.; PRENDIERI, S. 2012. Applications of In Vitro techniques in mutations breeding of vegetatively propagated crops. In: SHU, Q.Y.; FORSTER, B.P.; NAKAGAWA, J. (Eds.). Plant Mutation Breeding and Biotechnology.

SUPRASANNA, P.; MIRAJKAR, S.J.; BHAGWAT, S.G. 2015. Induced mutations and crop improvement. In: BAHADUR, B.; VENKAT RAJAM, M.M.; SAHIJRAM, L. KRISHNAMURTHY, K.V. (ed.). Plant Biology and Biotechnology: Plant Diversity, Organization, Function and Improvement. Springer India. 593-617

pp. https://doi.org/10.1007/978-81-322-2286-6_23

TCACH, M.A.; LANDAU, A.M.; MONTENEGRO, A.; DÍAZ, D.; ACUÑA, C.; PRINA, A.R.; 2022. Isolation and characterization of a new imidazolinone-tolerant mutant in cotton. Crop Sci. 62, 2222-2232. https://doi.org/10.1002/CSC2.20814

THE JOINT FAO/IAEA MUTANT VARIETY DATABASE (MDV). https://nucleus.iaea.org/sites/mvd/SitePages/Home.aspx

UL-ALLAH, S.; AHMAD, S.; IQBAL, M.; NAEEM, M.; IJAZ, M.; AHMAD, M.Q.; NABI, H.G. 2019. Creation of new genetic diversity in cotton germplasm through chemically induced mutation. Int J Agric Biol, 22, 51-56.

UPADHYAYA, M.K.; NAYLOR, J.M.; SIMPSON, G.M. 1982. The physiological basis of seed dormancy in Avena fatua L. I. Action of the respiratory inhibitors sodium azide and salicylhydroxamic acid. Physiol. Plant. 54, 419-424. https://doi.org/10.1111/j.1399-3054.1982.tb00702.x

UPADHYAYA, M.K.; NAYLOR, J.M.; SIMPSON, G.M. 1983. The physiological basis of seed dormancy in Avena fatua. II. On the involvement of alternative respiration in the stimulation of germination by sodium azide. Physiol. Plant. 58, 119-123. https://doi.org/10.1111/j.1399-3054.1983.tb04153.x

VOSS-FELS, K.P.; STAHL, A.; HICKEY, L.T. 2019. Q&A: modern crop breeding for future food security. BMC Biol. 17. https://doi.org/10.1186/s12915-019-0638-4

WANGA, M.A.; SHIMELIS, H.; HORN, L.; SARSU, F. 2020. The Effect of Single and Combined Use of Gamma Radiation and Ethylmethane Sulfonate on Early Growth Parameters in Sorghum. Plants 9, 827.

WITT, T.W.; ULLOA, M.; PELLETIER, M.G.; MENDU, V.; RITCHIE, G.L. 2018a. Irrigation's effect and applied selection on the fiber quality of Ethyl MethaneSulfonate (EMS) treated upland cotton (Gossypium hirsutum L.). J. Cott. Res. 1, 1-11. https://doi.org/10.1186/S42397-018-0016-8/TABLES/6

WITT, T.W.; ULLOA, M.; PELLETIER, M.G.; MENDU, V.; RITCHIE, G.L. 2018b. Exploring ethyl methanesulfonate (EMS) treated cotton (Gossypium hirsutum L.) to improve drought tolerance. Euphytica 214, 1-12. https://doi.org/10.1007/S10681-018-2206-3/FIGURES/3

ZHANG, H.B.; LI, Y.; WANG, B.; CHEE, P.W. 2008. Recent advances in cotton genomics. Int. J. Plant Genomics 2008. https://doi.org/10.1155/2008/742304