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Lethal dose 50 of NaCl and ethyl methanesulfonate in jalapeño pepper (*Capsicum annuum* L.) seedlings and tolerance to salinity

Dose letal 50 de NaCl e metanossulfonato de etila em mudas de pimenta jalapeño (*Capsicum annuum* L.) e tolerância à salinidade

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ABSTRACT

Soil salinity is a factor affecting crop production and yield. An estimated 74% of agricultural soils are saline, a problem that could be aggravated due to climate change. Our goal was to determine the lethal dose 50 (LD_{50}) of NaCl and ethyl methanesulfonate (EMS) in jalapeño pepper (*Capsicum annuum* L. cv. Jalapeño M.) for selecting putative salt-tolerant mutants. Root length was used as an indicator of the LD_{50} of NaCl in seedlings cultured for 35 days in MS medium containing 0 (without NaCl), 50, 100, 150, 200, or 250 mM NaCl and germination rate as an indicator of the LD_{50} of EMS in seeds exposed to 0% (without EMS), 0.01%, 0.1%, 0.25%, or 0.5% EMS for 3 and 6 h. We selected salt-tolerant seedlings after 35 days in medium with the determined LD_{50} of NaCl. Canonical and probit analyses to the LD_{50} of NaCl and EMS results and multinomial linear regression for germination and survival were applied. LD_{50} of NaCl (150 mM) and EMS (0.3%) were determined. Seed exposure to 0.5% EMS for 6 h reduced germination (67%) and seedling survival (18%). We obtained four putative salt-tolerant mutants by culturing in medium containing the LD_{50} of NaCl, two from seeds exposed for 6 h to 0.01 EMS, and one each from of the seeds treated with 0.1% or 0.5% EMS. The results show that it is possible to select putative salt-tolerant mutants of jalapeño pepper through mutagenesis with EMS and *in vitro* culture in media containing the LD_{50} of NaCl.

Index terms: Mutagenesis; *in vitro*; stress; genetic improvement.

RESUMO

A salinidade do solo é um factor que afeta a produção e o rendimento das culturas. Estima-se que 74% dos solos agrícolas são salinos, um problema que pode ser agravado devido às alterações climáticas. O objetivo deste trabalho foi determinar a dose letal 50 (LD_{50}) de NaCl e metanossulfonato de etila (EMS) em pimenta jalapeño (*Capsicum annuum* L. cv. Jalapeño M.) para a seleção de mutantes putativos tolerantes ao sal. Foi utilizado o comprimento da raiz como indicador do LD_{50} de NaCl em plântulas cultivadas durante 35 dias em meio MS contendo 0 (sem NaCl), 50, 100, 150, 200, ou 250 mM de NaCl e a taxa de germinação como indicador do LD_{50} de EMS em sementes expostas a 0% (sem EMS), 0.01%, 0.1%, 0.25%, ou 0.5% EMS durante 3 e 6 h. Plântulas tolerantes ao sal após 35 dias em meio com o LD_{50} para NaCl foram selecionadas. Foram usadas análises canônicas e probit nos resultados de LD_{50} de NaCl e de EMS e regressão linear multinomial para germinação e sobrevivência. Foram determinados o LD_{50} de NaCl (150 mM) e EMS (0.3%). A exposição de sementes a 0.5% EMS por 6 h reduziu a germinação (67%) e sobrevivência das plântulas (18%). Quatro mutantes putativos tolerantes ao sal através de cultivos em meio contendo o LD_{50} de NaCl, dois de sementes expostas durante 6 h a 0.01 EMS, e um de cada uma das sementes tratadas com 0.1% ou 0.5% de EMS foram obtidos. Os resultados mostram que é possível selecionar mutantes putativos tolerantes de pimenta jalapeño ao sal por meio de mutagênese com EMS e cultura *in vitro* em meios contendo o LD_{50} de NaCl.

Termos para indexação: Mutagênese; *in vitro*; stress; melhoramento genético.

INTRODUCTION

Soil salinity is one of the environmental factors affecting plant growth and development that has negative consequences on crop yields. Recent studies estimated the global surface of saline soils to be over nine hundred million hectares, an extension that is growing (Singh, 2022) and might reduce the availability of agricultural soils by 30% in the next 25 years (Shrivastava; Kumar, 2015) and, due to climate change, by over 50% in the year 2050 (Wang; Vinocur; Altman, 2003).

Plants adapt to soil salinity by three mechanisms: tolerance to osmotic stress, ionic stress, or oxidative stress. Osmotic stress is caused by an instantaneous reduction in the plant's water potential due to increased salt concentration in the soil (Ahmadi; Souri, 2018). Ionic stress is due to the metabolic damage caused by the gradual accumulation of Na^+ and Cl^- in plant tissues caused by Na^+/K^+ and Na^+/Ca^{+2} disequilibrium. Oxidative stress is due to the excessive production of toxic oxygen-receiving substances (ROS) affecting molecules involved in photosynthesis, which, among other effects, causes a reduction in leaf area, chlorophyll content, and somatic conductance (Munns; Testler, 2008; Kamran et al., 2020). However, the negative effects of soil salinity largely depend on the presence of ions from salts other than NaCl (Ahmadi; Souri, 2020).

The cultivation of chili pepper (*Capsicum annuum* L.) in Mexico is highly important in social, economic, and cultural terms, as shown by the crop being a main source of income for rural households and the over 50,000 chili pepper producers in the country, giving employment to over 15 million people. According to (Sánchez-Toledano et al., 2021), in 2021, more than 100 varieties of chili pepper cultivated in Mexico yielded over 3,200,000 t of the crop, of which jalapeño pepper represents 31% of the total. The importance of the cultivation of chili pepper in Mexico is due to its high per capita consumption in the country, which ranges from 8 to 9 kg (Castellón- Martínez et al., 2012), and to the nutraceutical properties of the chemical composition of its fruits, including vitamins (A, C, and D), antioxidants, and capsaicinoids (Bulle; Yarra; Abbagani 2016). However, species in the genus *Capsicum* are highly susceptible to soil salinity stress. For the jalapeño pepper, Wallender and Tanji (2011) reported a reduction of its fruit productivity at a conductivity of 4 dSm^{-1} (approximately 40 mM), because of which there is a need for salinity-tolerant genotypes to ensure its availability to consumers. However, despite the efforts made by researchers to generate tolerance to soil salinity, conventional plant breeding programs have advanced at a slow pace (Rai et al., 2011; Ahmadi; Souri, 2020).

Biotechnological plant breeding methods are an alternative to shorten the period needed by conventional approaches to obtain results, among which alkylating mutagenic agents and *in vitro* tissue culture have shown to be highly efficient for developing abiotic factor stress tolerance (Atak et al., 2004; Yaycili; Alikamanoglu, 2012). Tissue culture is defined as the assortment of techniques allowing the production of disease-free seedlings under controlled aseptic conditions (Oseni; Pande; Nailwal, 2018; Su et al., 2021). Natural mutagenesis – defined as the spontaneous alteration of the genome's nucleotide sequences – occurs at a low frequency (10^{-5}–10^{-8}), but we currently know that the process can be accelerated through physical or chemical procedures (Viana et al., 2019; Aklilu, 2021).

Chemical mutagenesis induction with alkylating agents is a strategy for increasing mutation frequency to induce crop genetic variation (Viana et al., 2019). Ethyl methanesulfonate (EMS) is the most commonly used alkylating agent in plant breeding programs because it allows the evaluation and selection of desirable crop characteristics from large seedling populations (Krupa-Małkiewicz et al., 2017). However, we first need to know the lethal dose 50 (LD_{50}) of EMS for the crop of interest, because its effect depends on the plant species (Arisha et al., 2014) and the LD_{50} of EMS is an indicator of the mutation frequency (Corazón-Guivin et al., 2022). Despite the known advantages of using induced mutagenesis and *in vitro* culture to obtain salt-tolerant mutants, the available research that adopts this approach fails to provide a clear description of the steps followed, particularly regarding jalapeño pepper. Therefore, our goal in the present work was to determine the lethal dose 50 (LD_{50}) of NaCl and ethyl methanesulfonate (EMS) in jalapeño pepper seedlings to evaluate and select putative salt-tolerant mutants of the crop.

MATERIAL AND METHODS

Seed disinfection

Seeds of Jalapeño pepper (*Capsicum annuum* L. cv. Jalepeño M.) were acquired from a commercial supplier and disinfected by immersion for 10 m in 70% ethanol (Sigma–Aldrich) and 10% commercial chlorine (CLORALEX ®). After immersion in each solution, seeds were rinsed twice in sterile deionized water for 10 m.

Seed germination

Viable seeds were selected by the Emongor, Mathowa, and Kabelo (2004) precipitation-flotation method. The selected seeds were sown in half strength Murashige and Skoog (1962) medium (½MS) supplemented with 30 g L^{-1} sucrose, 0.4 mg L^{-1} thiamine, 100 mg L^{-1} myo-inositol, and 7 g L^{-1} Phyto agar adjusted to pH 5.7 with 1 M NaOH or HCl and sterilized at 121 °C at 15 lb for 15 m. A total of 200 seeds were sown in Petri dishes (6 seeds/dish) that were incubated for 30 days until radicle emergence (germination criterion) in a growth chamber adjusted to a 16 h light photoperiod, 30 µmol m^{-2} s^{-1} light, a temperature of 24±1 °C, and 80% relative humidity. All reagents used were supplied by Sigma–Aldrich.

Treatments and variables assessed for the determination of the lethal dose 50 (LD_{50}) of NaCl

The LD_{50} of NaCl was determined by sowing 20 three-week-old jalapeño pepper seedlings in ½MS medium supplemented with 0 (control), 50, 100, 150, 200, or 250 mM NaCl dissolved in sterile deionized water and incubated in a growth chamber under the same conditions described above. After 35 days of incubation and the appearance of stress symptoms, morphological variables and chlorophyll content were measured as follows: stem diameter (SD) using a digital Vernier caliper, chlorophyll content (CH) using a SPAD 502 plus chlorophyll meter, number of leaves (NL), number of internodes (NI), root length (RL), and number of roots (NR) were measured using ImageJ v.1.32 software. Finally, the results were plotted, and the LD_{50} was determined. The experiment was performed with a completely randomized design considering each seedling as an experimental unit.

Treatments and determination of the lethal dose 50 (LD_{50}) of EMS

Seeds (disinfected as described above) were immersed for 3 and 6 h in 0% (control), 0.01%, 0.1%, 0.25%, or 0.5% EMS dissolved in sterile deionized water. For each treatment and the control, 60 seeds were sown in Petri dishes with ½MS medium in three repetitions, giving a total of 1,800 seeds, and incubated in a growth chamber under the same conditions as described above. Every three days, the number of germinating seeds was recorded until the 30th day, after which the results were plotted and the LD_{50} of EMS was calculated.

The germinated seeds were transferred to 900 mL flasks with ½MS medium for the growth and development of seedlings until two 21-day cycles were completed, after which the survival percentages after the first subculture were calculated for each treatment and the control. Both experiments were performed with a completely randomized 5×2 factorial design, where Factor 1 was the EMS concentration and Factor 2 was the exposure time.

Selection of soil salinity-tolerant (NaCl) seedlings

A representative seedling sample was taken as described by Aguilar-Barojas (2005) using the Equation 1.

$$n = \frac{Z^2 * p * q * N}{e^2 (N-1) + Z^2 * p * q} \quad (1)$$

where: n = sample size; N = population size; p = odds in favor; q = odds against; Z = confidence level; and e = sampling error.

Putative salt-tolerant mutant seedlings were selected by sowing seedlings derived from previously EMS-treated seeds in ½MS medium supplemented with 150 mM NaCl (LD_{50} of NaCl). Of the total mutants obtained, 12 seedlings for each EMS treatment and immersion period (3 and 6 h) were selected based on their vigorous phenotypes. The number of seedlings with a developing root system was counted after 30 days of incubation.

Statistical analyses

For the NaCl results, a canonical discriminant analysis was performed to identify the phenotypic characteristics most affected by salinity to choose a variable for measuring the LD_{50} of NaCl, after which it was determined by a probit analysis.

To analyze the EMS results, multinomial logistic regression was performed with the germination and survival data. The LD_{50} of EMS was calculated from the germination data using probit analysis. Data were analyzed in SAS v. 9.0 software.

RESULTS AND DISCUSSION

Effects of NaCl on the assessed morphological variables of seedlings

The results of the discriminant canonical analysis for the phenotypic characteristics we assessed (SD, CH, NL, NI, RL, and NR) showed that while the concentration of NaCl in the medium had effects on all the variables, the first statistically significant ($p \leq 0.05$) correlation corresponded to root length (RL), explaining 91% of the negative effect of salinity, and the second correlation corresponded to stem diameter (SD), explaining only 4% of the variation (Figures 1 and 2). Based on these results – and because roots are in charge of water and nutrient intake by the plant, therefore strictly determining the remaining assessed variables (Ji et al., 2013; Uga, 2021)– we chose root length as the selection criterion for salinity stress tolerance.

Determination of the lethal dose 50 (LD_{50}) of NaCl based on root length

As described in the previous section, we observed a statistically significant inhibitory effect ($p \leq 0.05$) on root length of the increased NaCl concentration of the culture medium. The addition of 50, 100, 150, 200, and 250 mM NaCl caused 4.6%, 34.6%, 50.0%, 56.2%, and 100% root length inhibition, respectively. A 50% inhibition of root growth (LD_{50}) occurred after 30 days of culture in a medium containing 150 mM NaCl (Figure 3).

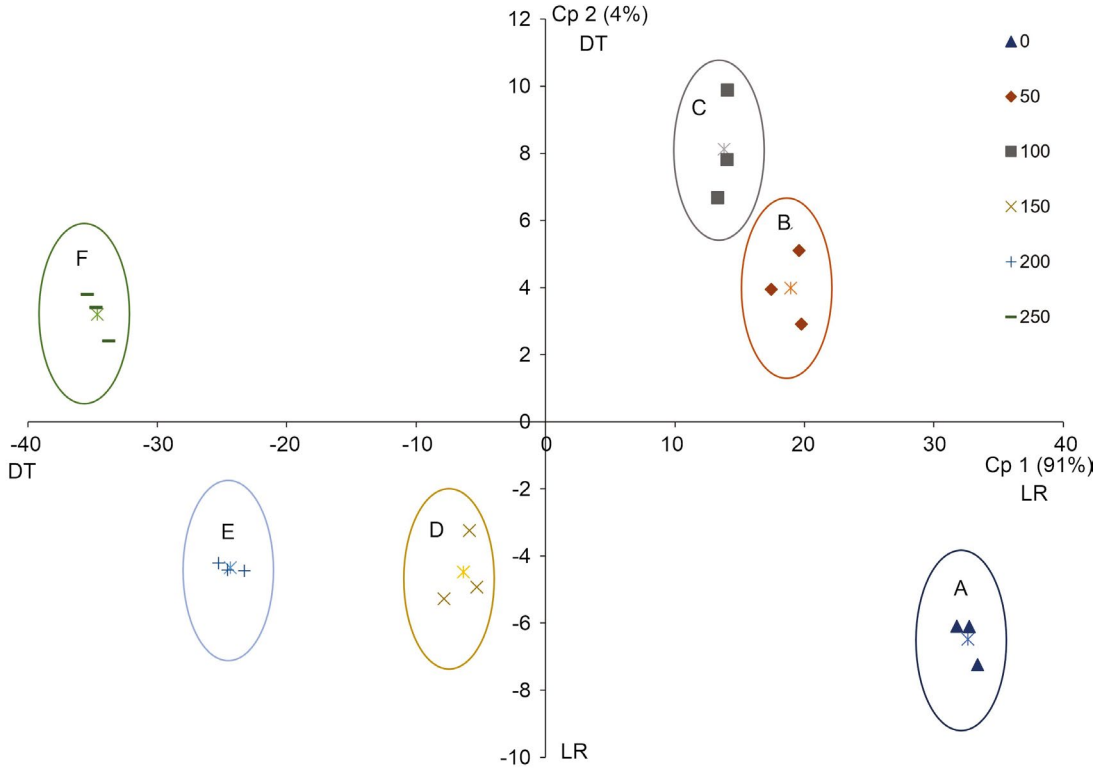

Figure 1: Canonical dispersion of the assessed variables of jalapeño pepper seedlings exposed to different NaCl concentrations. (A) Control (0 mM), (B) 50 mM, (C) 100 mM, (D) 150 mM, (E) 200 mM, and (F) 250 mM. RL= root length; SD= stem diameter.

Figure 2: Effect of NaCl concentration on the morphology of jalapeño pepper seedlings. (A) Control (0 mM), (B) 50 mM, (C) 100 mM, (D) 150 mM, (E) 200 mM, (F) 250 mM (scale bar: 1 cm).

Our determination of the LD_{50} of NaCl agrees with the results of Bojórquez-Quintal et al. (2015), who evaluated the behavior of two varieties of chile habanero (*C. chinense* Jacq.) – the salinity-stress tolerant variety Rex and the salinity-stress sensitive variety Chichén-Itzá– after seven days of hydroponic culture under salinity stress conditions (150 mM NaCl). The authors found changes in the dry and fresh weight of seedlings and toxicity symptoms. In a study of the response to NaCl concentration of five Tunisian varieties of *C. annuum* (Alaya, Skhira, Sgay, Maghraoua, and Farch) cultured in the greenhouse for 21 days, Gammoudi et al. (2016) observed physiological

and biochemical changes and a reduction in root surface area at concentrations ranging from 120 to 170 mM NaCl. While the latter NaCl concentration range included the LD_{50} value we determined as inhibitory of root length in jalapeño pepper, the authors of the study used different experimental conditions and exposure times and did not clarify the strategy they followed for choosing the NaCl dose that they applied. Although salinity had a negative effect on all the morphological variables of jalapeño pepper seedlings, we corroborated that statistically and visually, the root is the organ most affected by salinity (Figures 1 and 2). Finding the most affected variable provided the strategy for determining the DL_{50} to NaCl (Figure 3).

EMS mutagenesis in seeds

According to the results of our multinomial regression analysis, seed germination was significantly ($p \leq 0.05$) affected by EMS; however, the exposure time showed no interaction with germination. The highest germination percent we observed was 85.0% for the control, decreasing to 5.11% at 0.5% NaCl for 3 h and 28.3% at 0.5% for 6h (Figure 4). The reduction in germination percentage might have been caused by EMS exposure resulting in random point mutations in coding or noncoding genomic DNA regions, specifically in embryos, which provides a viable strategy for inducing high mutagenesis rates for the selection of desired characteristics (Serrat et al., 2014).

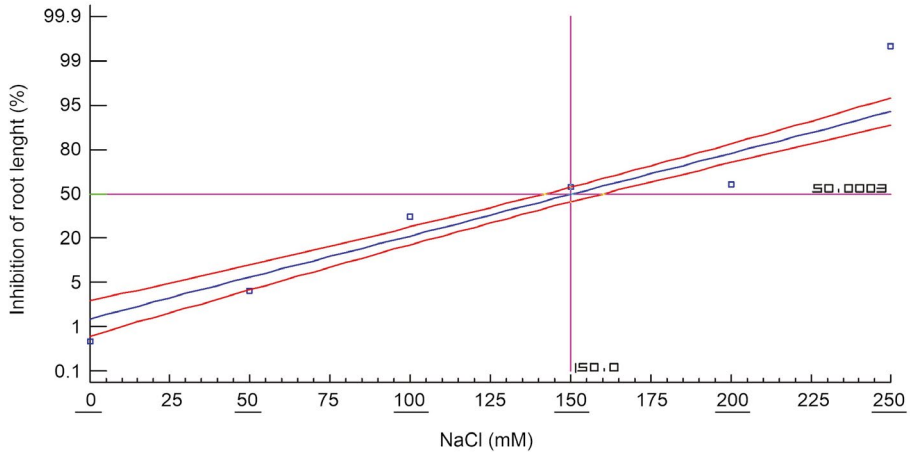

Figure 3: Lethal dose 50 (DL_{50}) of NaCl in jalapeño pepper seedlings after 30 days of culture.

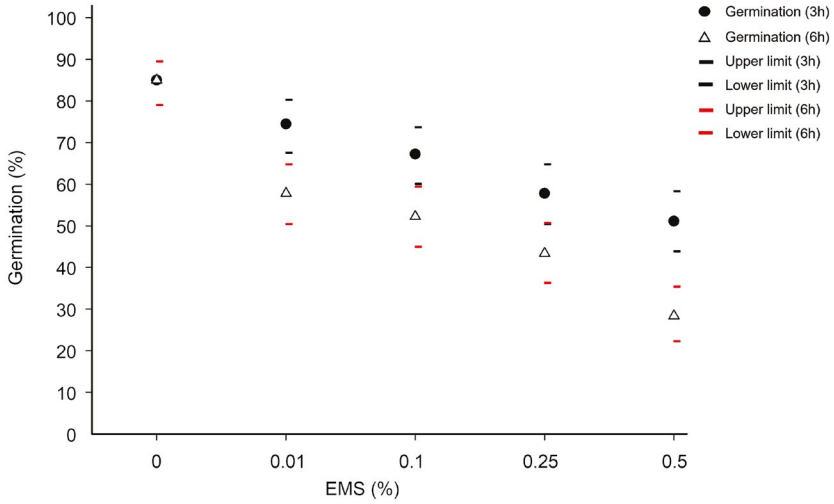

Figure 4: Effect of 3 and 6 h of exposure to EMS on the germination percentage of jalapeño pepper seeds 15 days after sowing.

Kumar et al. (2013) attributed the reduction in germination percent caused by EMS to possible damage to cellular components, such as alteration of enzymatic activity, or to the delay or inhibition of physiological and biological processes as a consequence of mutations in genomic DNA. In a study using *C. annuum* cv. B12 seeds, Arisha et al. (2014) reported 50% germination inhibition in the first generation (M1) after a 12 h exposure to 0.6% EMS, and in a second study (Arisha et al., 2015), the authors observed germination percentages between 41% and 60% in the M2 generation. Pharmawati et al. (2018) exposed *C. annuum* cv. Hot Pepper Smart seeds to 0.5% EMS for 6 h, observing a 21% reduction in germination percent in the M1 generation relative to control seeds, the authors suggested that the high concentration of EMS inhibited the germination physiological process. Other studies by Jabeen and Mirza (2004) using *C. annuum* and by Viana et al. (2019) using rice show that the germination percent after exposing seeds to EMS varies according to the plant species, cultivar, and experimental conditions.

Determination of the lethal dose 50 (LD_{50}) of EMS

Based on our experimental data of seed germination and probit analysis, we determined that treatment of jalapeño pepper seeds with 0.3% EMS inhibited germination by 50% and by 24%, 36%, 47%, and 70% at 0.01%, 0.1%, 0.25%, and 0.5% EMS concentrations, respectively (Figure 5). The reduction in germination percent that we observed could have been due to the inhibition of physiological and biological processes, enzymatic activities necessary for germination (Devi; Mullainathan, 2011), and mitosis, or by EMS causing hormonal disequilibrium (Kumar; Gupta, 2009).

Although the mutagenic agent EMS might have variable effects on genomes, the response also depends on the time of exposure, the concentration of the mutagen, and the type of plant material being used. Our results of germination of jalapeño pepper seeds differ from those reported by Corazón-Guivin et al. (2022), who treated inchi (*Plukenetia volubilis* L.) seeds with 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, or 3.0% EMS for 30 h and found that a 3.0% concentration reduced germination by 50%. In another study, Arisha et al. (2014) evaluated the lethality of exposure to 0%, 0.25%, 0.5%, 0.75%, 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, 2.25%, and 2.5% EMS for 6 h and 12 h in *Capsicum annuum* seeds, reporting an LD_{50} of EMS of 1.0%. The latter authors mention that it is important to consider the time of exposure to the mutagenic agent; however, in our study, the time of exposure to EMS was not a determining factor, at least for the stage of germination (Figures 4 and 5).

Survival of seedlings produced by seeds treated with EMS

The results of our multinomial analysis showed statistically significant ($p \leq 0.05$) differences in the survival of seedlings after 30 days of incubation in each treatment and exposure period. The interaction between the EMS concentration and the exposure time was also statistically significant ($p \leq 0.05$), which means that the effect of the EMS concentrations depended on the period of exposure. We observed that as the concentration of EMS and the exposure time increased, the survival percentages were lower relative to the control (93.1%). The treatments with 0.01% and 0.5% EMS for 3 h resulted in the lowest survival percentages (75.7% and 78.2%, respectively; Figure 6). These results suggest that the latter concentrations were lethal for the seedlings, possibly due to damage at the cellular and even chromosomal level (Dhamayanthi; Reddy, 2000; Bhat; Ansari; Aslam 2012).

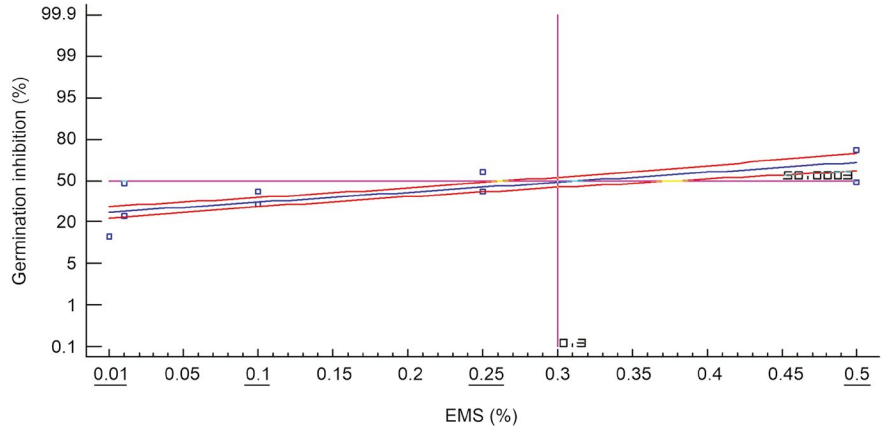

Figure 5: Lethal dose 50 (LD_{50}) of EMS for jalapeño pepper seed germination 15 days after sowing.

In the case of the treatments with 0.01%, 0.1%, 0.25%, or 0.5% EMS for 6 h, the survival percentages were higher (89.4%, 90.4%, 98.7%, and 85.0%, respectively) than the treatments in which the exposure time was 3 h (Figure 6). The latter result might have been due to the seeds treated with EMS during the longest period (6h) having more favorable random mutations allowing survival than seeds treated during a 3 h period (Figures 4 and 6) (Krupa-Małkiewicz et al., 2017).

The variation in the survival of seedlings from seeds exposed to different concentrations of EMS that we observed was also reported by Sonavane (2017), who assessed the effect on bean (*Phaseolus vulgaris*) seedlings of several concentrations of EMS (0.05%, 0.10%, or 0.15%) and found the highest survival (96.4%) at the lowest concentration (0.05%), while the higher concentrations reduced the survival to 82.3% and 81.9%, respectively, attributing that effect to chromosomal damage. Dhamayanthi and Reddy (2000) observed that the M2 of *C. annuum* seeds treated with 0.8 and 1.0% EMS for 12 h had a lower survival than that of seedlings from untreated seeds, suggesting that this could have been due to irregular meiosis causing chromosomal changes that affected the seedlings' vigor.

Selection of putative NaCl-tolerant mutants

We evaluated 20% of the total surviving seedlings in the selection experiment and only observed salt-tolerant mutants in seedlings produced by seeds treated with 0.01%, 0.1%, or 0.5% EMS. After 35 days of culture, we observed two mutant seedlings produced by seeds treated with 0.01% EMS for 6 h, both of which displayed tolerance to NaCl by forming roots, while seedlings from seeds treated with 0.1% and 0.5% EMS for 6 h showed one tolerant mutant each, together representing 3% of the sample we evaluated at a 90% confidence level (Figure 7). In contrast, the remaining seedlings originating from seeds exposed to mutagenesis by EMS failed to survive culture in medium containing the LD_{50} of NaCl that we established experimentally (150 mM), most of them lacking roots, showing necrosis at the stem base and leaf margins, chlorosis, and defoliation.

The latter results are similar to the reports of Jabeen and Mirza (2004), Yaycili and Alikamanoğlu (2012), Espina et al. (2018), and Lethin et al. (2020). The latter authors suggested that variations in the concentration of EMS and exposure period act upon the presence or absence of salinity-tolerant mutants and that such treatments might cause mutations within nuclear regulatory DNA regions –both in coding or noncoding regions of the nuclear genome– that, in turn, positively or negatively regulate the expression of genes involved in the tolerance to soil salinity.

Salinity stress slows the division and elongation rate of root epidermal cells, therefore reducing primary root growth and inhibiting the initiation of lateral roots (Jung; McCouch, 2013). Salinity-tolerant plants develop characteristic mechanisms allowing them to grow in saline environments, including the exclusion of Na^+ and Cl^- ions from roots and their compartmentalization in vacuoles and the synthesis of organic molecules (compatible solutes) such as proline and glycinebetaine, allowing them to maintain cell turgor and eliminate free radicals during salinity stress periods, which favors photosynthesis and the growth of roots and shoots (Roy; Negrão; Tester, 2014).

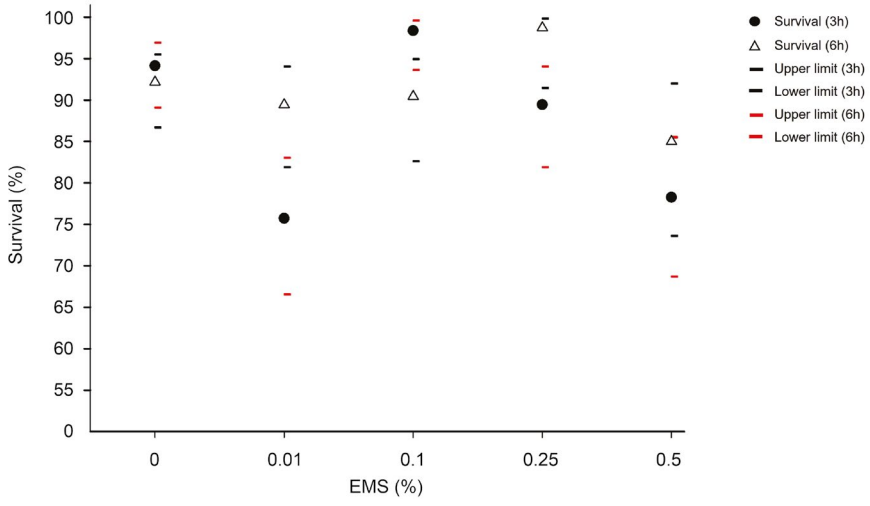

Figure 6: Survival percentages of jalapeño pepper seedlings produced by seeds treated with different concentrations of EMS and exposure periods.

Figure 7: Putative mutants tolerant to the LD_{50} of NaCl produced by seeds treated with 0.5% EMS for 6 h (A), 0.01% EMS for 6 h (B and C), and 0.1% EMS for 6 h (D) (scale bar: 2 cm).

CONCLUSIONS

LD_{50} of NaCl and EMS (150 mM and 0.3%, respectively) were determined for the jalapeño pepper. These values might be used for future mutant selection. Exposure of seeds to 0.01%, 0.1%, or 0.5% EMS for 6 h allows putative salinity-tolerant mutants to be obtained by selection in substrates containing the LD_{50} of NaCl. The combination of *in vitro* culture and mutagenesis with EMS is a viable tool for inducing saline tolerance in jalapeño peppers.

AUTHOR CONTRIBUTION

Conceptual Idea: Poot-Poot, W.A.; Methodology design: Poot-Poot, W.A.; Urías-Salazar, A. A; and Ayi-Gutiérrez, B.A.; Data collection: Urías-Salazar, A.A.; Delgado-Martínez, R.; and Ayi-Gutiérrez, B.A. ; Data analysis and interpretation: Silva-Espinosa, J.H.T. ; Segura-Martínez, M.T.J. ; Delgado-Martínez, R.; and Poot-Poot, WA., and Writing and editing: Urías-Salazar, A.A. ; Delgado-Martínez, R.; Poot-Poot, W.A.; Silva-Espinosa, J.H.T. ; and Segura Martínez, M.T.J.

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