Mean lethal dose (LD$_{50}$) and growth reduction (GR$_{50}$) due to gamma radiation in Wilman lovegrass (*Eragrostis superba*)

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Abstract:

Gamma radiation can be utilized as a mutagenic agent for inducing genetic variability in plant species. However, the adequate dose of radiation must be established before starting a mutagenesis-based breeding program. The objective was to determine the mean lethal dose (LD$_{50}$) and the dose for the mean growth reduction (GR$_{50}$) in Wilman lovegrass (*Eragrostis superba* Peyr.). For this purpose, the seeds were radiated with doses of 0 (control treatment), 100, 200, 300, 450, 600, 900,

1400, 2000 and 4000 Gray. The evaluated variables were germination percentage, germination speed index, plumule length, radicle length, forage yield, seed production, leaf-stem ratio and chlorophyll concentration index. The data were analyzed through regression analyses and the Dunnett's mean comparison test. The LD_{50} and GR_{50} generated for each of the evaluated variables were calculated with the resulting regression equation. Based on the values of LD_{50} and GR_{50}, a weighted mean was estimated; the LD_{50} was weighted with 30 % while the GR_{50} with 10%. In general, all the evaluated characteristics were affected by the radiation treatment ($P<0.05$), and their behavior was adjusted to linear square models ($P<0.05$), allowing the determination of the LD_{50} and the GR_{50}. The weighting average of both parameters, estimated at 2486 Gy, is recommended for the genetic breeding of Wilman lovegrass. The results of this study will allow implementing mutagenesis-based plant breeding programs in Wilman lovegrass.

Key words: Cobalt 60, Gamma radiation, Mutations, DL_{50}, RC_{50}.

The median lethal dose (LD_{50}) and the median growth reduction (GR_{50}) are parameters utilized to establish the adequate irradiation dose to induce mutations in plant breeding programs. Mutation induction has the purpose of generate genetic and phenotypic variability, which may allow to select plants with characteristics that are not found in nature. Several researchers agree that the highest probability to generate useful mutations for breeding programs occurs at doses where the 50% of the irradiated individuals die[1-4]. Likewise, other researchers have pointed out that another dose with a high probability of producing effective mutations, besides the LD_{50}, is where the 50% of a growth reduction (GR_{50}) occurs[5,6]. Notably, both parameters (LD_{50} and GR_{50}) are based on the assumption that low doses of irradiation produce minimum impacts on the genome, which rarely generate phenotypic changes; whereas high doses may produce multiple impacts on the genome which consistently produce aberrations or negative changes[7,8]. Therefore, the first step in a mutagenesis-based breeding process is to determine the LD_{50} and the GR_{50}.

In Mexico, most of the arid and semi-arid grasslands are degraded due to the overgrazing and other anthropogenic practices[9]. Such degradation has caused a drop in the vegetation cover, large stretches of bare ground and biodiversity loss, reducing the functionality and productivity of these ecosystems. An alternative to rehabilitate these degraded systems is the revegetation by seeding, for which the use of native vegetation is recommended. Nevertheless, this type of vegetation has a limited establishment ability in areas with a high degree of degradation.

Therefore, the alternative is to use introduced species with a higher rusticity and establishment ability, like the Wilman lovegrass (*Eragrostis superba*). This grass is considered one of the species with the highest potential to be used in grassland rehabilitation programs[10], as it has a higher establishment ability than the native species used in this type of programs, like sideoats grama (*Bouteloua curtipendula*) and blue grama (*Bouteloua gracilis*), and even introduced species like weeping lovegrass (*Eragrostis curvula*)[11]. However, it has a lower nutritional value and forage yield than other introduced grasses, such as buffel grass (*Pennisetum ciliare*), klein grass (*Panicum coloratum*) and even weeping lovegrass[12]. The aforementioned suggests the possibility to subject the Wilman lovegrass to a plant breeding process. Nevertheless, since it is an introduced species, there may be little genetic variability of this species in Mexico. In this sense, mutagenesis may be an alternative for the genetic improvement of Wilman lovegrass, as it has proven to be a viable technique for inducing variability in grasses[13,14,15]. However, the dose with the highest probabilities of producing effective mutations in this species is unknown so far. For this reason, the objective was to determine the LD_{50} and the GR_{50} in Wilman lovegrass in order to establish the optimal dose of gamma radiation for mutagenesis induction into this species.

The experiment was carried out under laboratory and greenhouse conditions. Seeds of Wilman lovegrass var. Palar were radiated. The seeds were imported from the Curtis & Curtis company, located in Clovis, New Mexico, USA. Nine radiation doses and a control were evaluated: 0 (control), 100, 200, 300, 450, 600, 900, 1400, 2000 and 4000 Gray (Gy). The exposure times for the evaluated doses were determined using the Gafchromic dosimetry system and a RADCAL® Accu-dose ion chamber (Serial No. 4094118, USA). The seeds were radiated at the MOSCAFRUT SAGARPA//IICA Complex in Metapa de Domínguez, Chiapas, Mexico, in collaboration with the "Rosario Izapa" Experimental Station of INIFAP in Chiapas. The doses were applied within a Nordion GammaBeam™-127 panoramic Gamma irradiator, with a dry storage source and an activity of 15 000 Curies of Co-60.

The experimental design utilized for this test was completely randomized, with 10 radiation treatments and four repeats per dose, and considering each 90 mm Petri dish with cotton and filter paper and 50 seeds per repeat as the experimental unit. The Petri dishes were moisturized with 25 ml of water at the beginning of the test, and were subsequently sprayed with 2.0 ml every other day during the 15 d of duration of the test. The Petri dishes were then placed in a Precision Scientific 6M incubator at a temperature of 28 ± 2 °C. Those seeds that attained a 0.5 cm plumule or radicle were considered as germinated[16]. The assessed variables were: germination percentage (GP), germination speed index (GSI), plumule length (PL), and radicle length (RL). In order to obtain the RL and the PL, three seedlings per Petri dish were allowed to grow for 7 d after germination. The GSI was estimated using the following equation[16]:

$$GSI = \sum n_i/t$$

Where: GSI = germination speed, n_i = number of seeds germinated per day, t = day of germination.

The greenhouse experiment was assessed using a randomized block experimental design with 10 repeats per treatment, establishing the blocks perpendicularly at the sunlight expose during the day. Twenty (20) seeds were sown in each flowerpot in order to ensure emergence and establishment; however, only one plant was left per pot. The seeds were sown in black polyethylene bags with a height of 26 cm and a diameter of 18 cm, perforated at the bottom. The bags were filled with 23 cm of alluvial loamy sandy soil. They were watered up to the saturation point every two or three days, according to the desiccation of the soil. The test was conducted during 16 wk from April to August. The assessed variables were forage yield (FY), seed production (SP), leaf:stem ratio (LSR), and chlorophyll concentration index (CCI). All the seeds of each plant were collected and put into paper bags in order to calculate the SP. For the FY, the biomass was cut at 5 cm from the ground; the stems and the leaves were then separated and placed in paper bags. The samples of both forage and seeds were dried in an oven at 65 °C during 72 h. Once the samples were dry, they were weighed in a Mettler Viper BC analytical scale. The CCI was measured at the middle part of five leaves selected at random, using an Opti-Sciences CCM-200 meter.

In order to ensure the ranges of temperature (T) and relative humidity (RH) required by Wilman lovegrass, measurements were taken at the nursery with an HMP60 probe (Vaisala, Woburn, MA, USA). These data were registered every second and were averaged every minute in a CR1000 datalogger (Campbell Scientific Inc., Logan, UT, USA). The mean T during the experiment was 26.7 ± 5.6 °C, with a minimum temperature of 17.1 °C and a maximum temperature of 44.7 °C. The mean RH was 52.0 ± 16.8 %.

The data were analyzed with a regression analysis for each variable. The mean lethal dose (LD_{50}) was estimated using the resulting regression equation from the germination percentage. The same procedure was utilized to estimate the mean growth reduction (GR_{50}) of the variables germination speed index, plumule length, radicle length, forage yield, seed weight, leaf:stem ratio, and chlorophyll concentration index. Furthermore, all the data were subjected to a variance analysis and to Dunnett's mean comparison test, where the influence factor was the radiation dose with a significance level of 0.05 ($\alpha=0.05$). The analyses were performed using the REG and GLM procedures of the SAS 9.1.3 statistical package[17].

A weighted mean was estimated based on the results of the LD_{50} and the GR_{50} obtained for the eight variables. The LD_{50} was weighted with 30 %, and the GR_{50}, with 10 % for the remaining variables (GP, GSI, PL, RL, FY, SP, LSR, and CCI). A single value was thus obtained, whereby the optimal dose of gamma radiation for inducing mutagenesis in Wilman lovegrass was established. The germination percentage was the variable to which the highest weighting was assigned, because the death of the individual indicates the maximum damage that radiation can produce.

The behavior of the GP exhibited a quadratic tendency ($P=0.008$). Furthermore, there were differences ($P<0.05$) between all the radiation doses and the control treatment (T-0). In general,

the GP tended to diminish as the radiation dose increased (Figure 1a). The regression equation indicates that the LD_{50} in Wilman lovegrass occurs at 1,529.1 Gy. Likewise, the GSI exhibited a quadratic tendency (P=0.001), and there were differences (P<0.05) between all the radiation treatments and the T-0. The GSI also diminished as the radiation dose increased (Figure 1b). According to the regression equation, for this variable, the GR_{50} occurred at 805.3 Gy.

Figure 1: Effect of different doses of radiation with Co-60 on the germination percentage (a), the germination speed index (b), the plumule length (c) and the radicle length (d) of Wilman lovegrass (*Eragrostis superba*)
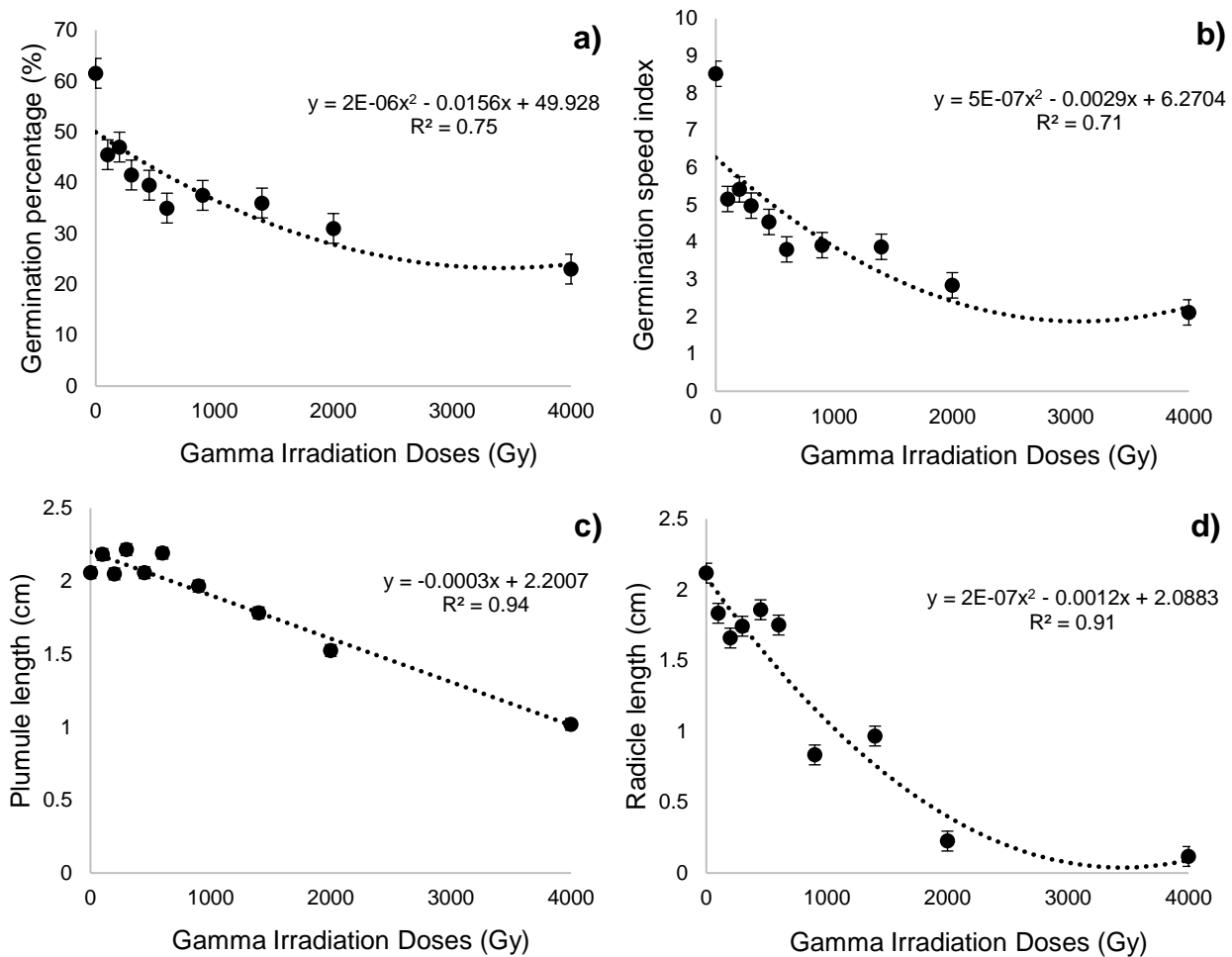

The PL exhibited a negative linear tendency (P<0.0001). However, only the doses of 2,000 and 4,000 Gy exhibited a lower PL (P<0.05) than the T-0 (Figure 1c). According to the regression equation, in this variable the GR_{50} occurred at 3,905.3 Gy. Likewise, the RL exhibited a quadratic tendency (P<0.0001). For this variable, only the 900, 1400, 2000 and 4000 Gy treatments had a

lower RL ($P<0.05$) than the T-0 (Figure 1d). The equation showed that, for this variable, the GR_{50} occurred at 1 037.8 Gy.

The behavior of the FY exhibited a quadratic tendency ($P=0.0009$). There was an increase in this variable ($P<0.05$) with the doses of 450 and 600 Gy. It subsequently tended to diminish until it reached 4000 Gy, with which dose all the plants died before reaching 21 days of age (Figure 2a). The regression equation for this variable indicates that the GR_{50} occurred at 2,262.8 Gy.

Figure 2: Effect of different doses of radiation with Co-60 on the forage yield (a), seed production (b), leaf:stem ratio (c), and chlorophyll concentration index (d) of Wilman lovegrass (*Eragrostis superba*)
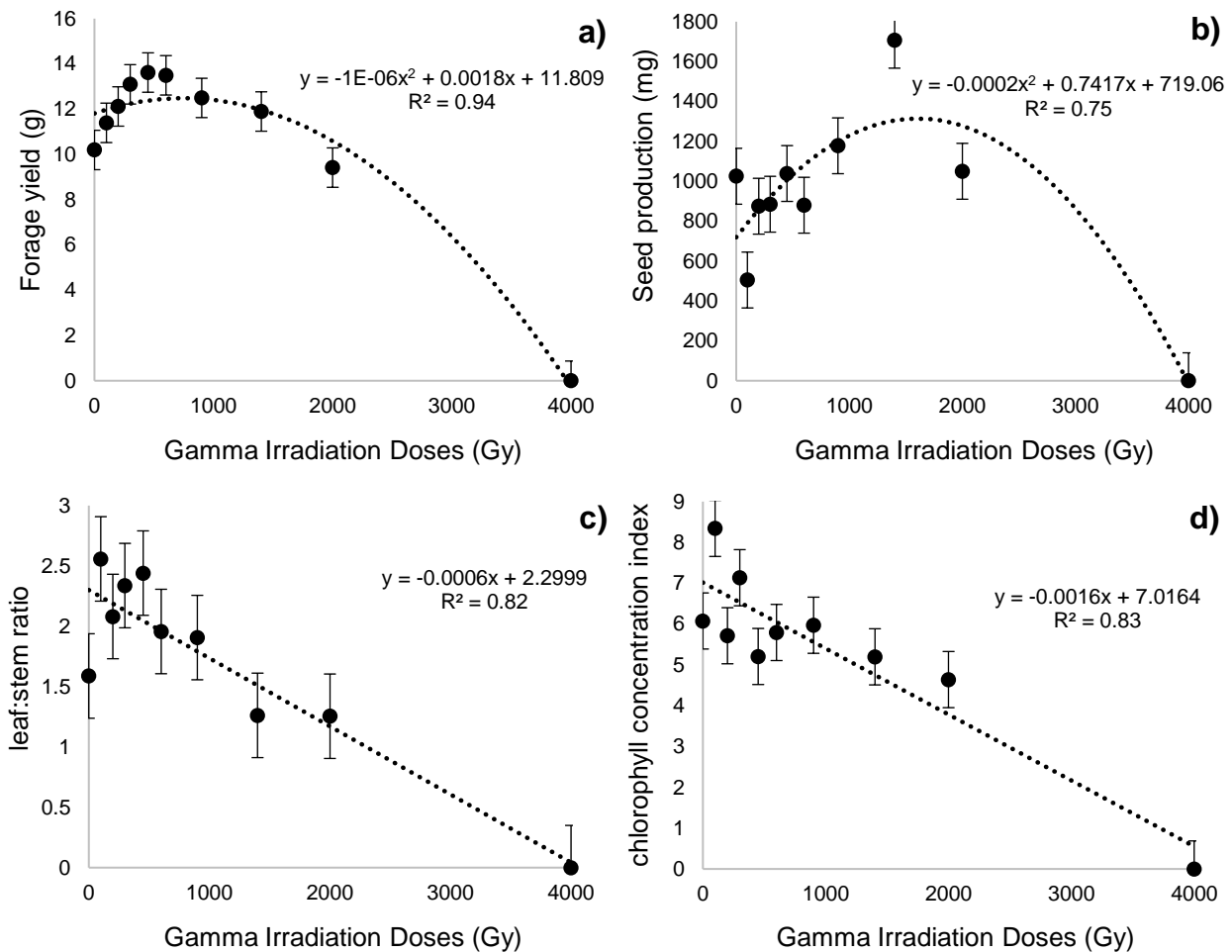
The SP exhibited a quadratic tendency ($P<0.05$). However, the only treatments with differences ($P<0.05$) in relation to the control were 1,400 and 4,000 Gy. There was an increase in the SP with the 1,400 Gy dose ($P<0.05$; Figure 2b). According to the regression equation, the GR_{50} on the SP occurred at 3,968.7 Gy. The LSR showed a linear tendency ($P<0.0001$). The only treatment that

differed ($P<0.05$) from the control was 4,000 Gy radiation, because all the plants with this treatment died, and therefore the LSR was equal to 0. The GR_{50} for this variable occurred at 2,509.9 Gy. The CCI exhibited a linear behavior ($P<0.0001$). However, as the former variable, the only treatment that was different ($P<0.05$) from the control was 4,000 Gy. According to the regression equation, the GR_{50} occurred at 2,486.6 Gy. The weighted mean of the LD_{50} and GR_{50} resulting from the eight variables was 2 159.2 Gy.

Wilman lovegrass germination decreased as the radiation dose increased. This result agrees with those of several authors, who found that the germination of various plant species tends to diminish with increasing radiation doses[18-21]. The reason for this may be that high doses of radiation can inhibit functions that are vital to the cells, which may result in the death of certain cells, and even of the embryo in the seed. This phenomenon tends to increase with the radiation dose, and it is the cause of reduced germination[20]. The radiation may damage compounds related to the plant metabolism —such as auxins, ascorbic acid, chlorophyll, and proteins—, potentially inhibiting the growth of the seedlings[22,23]. It has been observed that, due to these disorders, seeds with high radiation doses cannot germinate, or their seedlings cannot survive beyond a few days[24], as was the case with the 4,000 Gy dose in this study. Furthermore, germination reduction has been ascribed to the fact that the frequency in chromosomal damage augments with increasing doses[22]. This may change the expression of proteins and influence the functioning of the cells, potentially affecting the development of the seedlings and preventing germination[25].

One of the grasses whose radiosensitivity has been most extensively studied is rice (*Oryza sativa*). A study of this species exposed 13 varieties to gamma radiation, and found that the LD_{50} ranged, according to the variety, between 345 and 423 Gy[26]. Another study carried out in two varieties of rice established the LD_{50} at 288 and 354 Gy[27], while other researchers established it at 229 Gy[28]. In other grasses, such as *Pennisetum glaucum* and *Pennisetum typhoides*, the LD_{50} has been determined at 669.3 and 200 Gy, respectively[28,29]. As for forage grasses, the LD_{50} determined for different varieties of sudan grass (*Sorghum sudanense*) ranged between 307 and 342 Gy[3]. Notably, the same agent was utilized in the above studies as in the present one. However, the results are lower than the LD_{50} obtained in this study for Wilman lovegrass. This result may be due, among other factors, to the variations in the moisture content of the seed between species, since the ionizing radiation may break the covalent bonds and cause the breakdown of water molecules, forming free radicals that may indirectly damage the various organelles of the cell and even the DNA molecules within the cell[30].

Like the GP, the GSI of Wilman lovegrass tended to diminish with increasing radiation doses. This result agrees with the findings reported for peanut (*Arachis hypogaea*), where the germination speed diminished with the increased dose of radiation[31]. The delay in germination may be due to the fact that high radiation doses may inhibit processes that are vital for the cell, such as protein synthesis and enzyme activity, which can delay cell division and, therefore, germination[20,32].

The PL and the RL were observed to diminish with increased radiation doses. This result was also found for *Eleusine coracana*, in which the PL and RL were affected by the increase in the radiation dose, and the GR_{50} occurred at 500 Gy for both variables[33]. The same effect was found in rice, where the GR_{50} occurred at 250 and 450 Gy, respectively, for the two variables[34].

Reduction of the plumule and radicle lengths may be due to the fact that ionizing radiation usually affects plant metabolism and cell division, inhibiting or delaying the plant's growth[20,31]. In this regard, Kiong *et al*[22] found that gamma radiation can diminish the amount of proteins and chlorophyll compounds, affecting the plant metabolism. On the other hand, Preuss and Britt[35] found that the gamma radiation may inhibit plant growth because plants have a signal transduction mechanism that monitors cellular damage. This mechanism stops cell division whenever the cell structure is damaged.

The FY and SP of Wilman lovegrass presented an increase with low radiation doses. This may be due to the fact that gamma radiation can increase the amount of photosynthetic pigments like a- and b- chlorophylls and carotenoids[22]. These pigments capture the sunlight photons for the plant to convert into energy compounds through photosynthesis[36]. Therefore, an increase in the concentration of these pigments may increase the photosynthetic rates; this may have been the cause of the increase in forage yield and seed production in this study. The increase in FY and SP may also be due to the fact that gamma radiation sometimes increases the activity of antioxidant enzymes such as the superoxide dismutase and glutathione reductase, reducing the oxidative stress in the plant. This may help the plant deal more easily with the stress factors it faces on a daily basis, such as the fluctuations of temperature and sunlight that it receives, benefitting its growth[37,38]. Nevertheless, after the increase in FY and SP, there was a decrease. This is due to the fact that radiation can also cause negative effects, as the exposure to gamma radiations frequently produces free radicals during the water radiolysis process. These free radicals are unstable chemical species that may damage cell organelles. In an experiment conducted by Wi *et al.*[39], gamma radiation was observed to produce important modifications in the structure of certain cell organelles of *Arabidopsis thaliana*. In this experiment, the tilacoids within the chloroplasts exhibited dilation and damages; the mitochondria displayed distorted forms, and the endoplasmic reticulum exhibited distended membranes. In addition, as it was mentioned above, radiation may cause the destruction of auxins, ascorbic acid, chlorophyll, and proteins, affecting the metabolism of the plants[22,23]. It is unlikely that any of these phenomena may occur with low radiation doses; however, as the radiation dose augments, the probability of their occurrence increases[22]. This may be why, after an increase in forage yield and seed production, there was a reduction of both variables.

The LD_{50} and the GR_{50} are parameters utilized for determining the optimal radiation dose to induce effective mutations in genetic improvement programs. This is because the impact on the genome has been observed to be minimal and difficult to reflect, while with high doses, the genome may suffer multiple impacts that consistently produce anomalies or negative changes[7,8]. Furthermore, low radiation doses may induce an increase in the cell division, the enzyme activity or the amount

of photosynthetic pigments[31,34]. In contrast, high radiation doses may also produce physical damage to the organelles or chemical damages in the cells, due to the destruction of enzymes and hormones, among others. However, because these changes were not produced through genetic modifications, they will not pass on to the next generations. In this sense, low and high radiation doses may generate in plants phenotypic changes that may be mistaken for genetic changes. Therefore, the optimal radiation dose must be established before starting a mutagenesis-based genetic improvement program; this will increase the possibility of finding effective mutations. The optimal radiation dose is established through the mean lethal dose or the mean growth reduction dose[6,7]. In this study, the reduction that occurred in the values of all the variables due to radiation made it possible to determine the LD_{50} and GR_{50}. However, the value of both parameters in each variable ranged between 805 and 3,905 Gy. Because of the amplitude of this range, a weighed mean was estimated in order to establish the optimal dose of the gamma radiation in the seed. The variable to which the highest weighting was assigned was the germination percentage, as the death of the individual indicates the maximum damage that radiation can produce.

Low gamma radiation doses can produce an increase in certain agronomic attributes in Wilman lovegrass. However, in general all the assessed physical parameters were affected by high doses of gamma radiations, which made it possible to determine the mean lethal dose and the mean growth reduction. In order to attain the genetic improvement of Wilman lovegrass, it recommends using the optimal radiation dose obtained through the weighting of both parameters (2,486 Gy). This will contribute to the obtainment of new genetic material that can be utilized for the rehabilitation of grasslands.

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