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Determination of the method of induction of mutations by *gamma* radiation in soybeans (*Glycine max* L. Merrill) for tolerance to carbonic rot produced by the fungus *Macrophomina phaseolina* (Tassi Goid.) ☆

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ABSTRACT

In recent years, there has been an increase in the appearance of charcoal root rot disease in soybeans crops (*Glycine max* L. Merril). Charcoal rot is caused by the soil-borne fungus *Macrophomina phaseolina*. This disease is typically exacerbated by water deficiency and high temperatures. To evaluate the soybean genotypes' response to this pathogen, novel genotypes developed through gamma irradiation of 150 Gy and 200 Gy were tested under, in field and greenhouse conditions. Additionally, total phenol content was analyzed as a potential indicator of plant tolerance. The results indicate that the incidence of disease in non-irradiated genotypes was 100 %, in genotypes irradiated with a dose of 150 Gy it was 87 %, and those irradiated with a dose of 200 Gy a 100 %. An increase in the level of total phenols was observed in the tolerant genotypes as well as some mutant genotypes with characteristics that show tolerance to the charcoal root rot disease. The results suggest that gamma radiation-induced mutation may be an effective method for breeding disease-resistant soybean varieties.

- This variability method can be applied to any plant species.
- This method can cause mutations in any part of the genome, this allows its application to be unlimited.
- It is a method that can be used in a complementary way to other plant breeding methods.

☆ **Related research article:** None.

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Background

The soybean (*Glycine max*) in Paraguay is one of the agricultural exports that generates large foreign exchange earnings. The environmental conditions of Paraguay, including its climate, soil and photoperiod, have favored the rapid cultivation expansion in its territory's main agriculture agricultural areas the last 20 years [1]. However, these achievements in soybean production can be affected by diseases caused by phytopathogenic fungi. One of these diseases is the charcoal root and stem rot caused by the fungus *Macrophomina phaseolina*, it is present in all soybean-growing areas has begun to cause serious economic damages [2]. The soil fungus *M. phaseolina* is well kown as causal agent of seedlings burning and ash rot of stems and roots in numerous hosts in the world. The disease caused by this fungus has been described in >400 plant species and the wide range of hosts includes economically important crops such as fruit-bearing plants [3].

Most of the desired genetic variation explored in breeding programs has occurred naturally and is conserved in germplasm collections. However, when these collections cannot provide a source for a particular trait, it is necessary to turn to other sources of variation. In such cases, mutation techniques provide tools for the rapid creation of the desired characteristics. In crop breeding programs, it is necessary to have a broad genetic base that guarantees sufficient variability to select the best genotypes. Within breeding programs, the "Improvement by Mutation Induction" consists of three phases: generation of genetic variability by mutation, genotypes selection, and evaluation of selected genotypes with agronomic traits [4]. A narrow genetic base represents difficulties for the different improvement programs; for example, the lack of genetic variability in elite germplasm for resistance genes disease makes it possible that a pathogen devastates the entire production because they are all genetically similar [5]. In the case of soybean showed that genetic variations for productivity and other agronomic traits may be limited by the lack of genetic diversity in crosses between genotypes of elite parents [6].

Mutations are the primary origin of genetic variability. Therefore, some control over their frequency and or spectrum can be considered a valuable tool for improving cultivated plants [7]. The first one arrived in the mid-1930s in Holland regarding the success of induced mutants in plant breeding. It was obtained in tobacco by Tollenar and consisted of a mutant with light green leaves (viridis) of excellent quality that was cultivated in an important cultivation area in Indonesia. In general, in 1980, the number of crop genotypes from mutant breeding programs officially released as cultivars reached 225 [8].

This study evaluates a method of inducing mutations through gamma radiation is to spot with the objective of selecting promising soybean genotypes with tolerance to the carbonic rot disease caused by the fungus *M. phaseolina*.

Method details

Fig. 1 shows a diagram of all the steps that will be described below.

Soybean genetic material

For the different tests, soybean seeds of the NK 3363 Syngenta® variety, supplied by CEMIT/DGICT/UNA, were used. As a part of a soybean classical breeding program, the seeds were induced to mutation by applying *gamma* radiation. The germination capacity, vigor, and health of the seed lot used were also assessed.

Seed irradiation for mutation induction

All irradiations were performed at the Center for Technological Applications and Nuclear Development (CEADEN) in Cuba. The irradiator used was of the MP-γ−30 type, with a capacity of 4 liters and an initial activity of 10.45 kCi. The calibration of the radiation source, exposure duration, and other specific irradiation conditions were managed by CEADEN technicians.

Radiosensitivity evaluation

The first step to induce mutations was determining the optimal radiation dose, as genotypes exhibit varying radiosensitivity, even among the crops of the same species. In radiation experiments, it is crucial to determine the mean lethal dose (LD50), evaluating various doses of radiation to compare survival and growth capacity relative to the control. In the initial phase, seeds were irradiated with doses of: 50, 100, 150, 200, 250 and 300 Gy. This preliminary test aimed to determine the feasibility and the direct effect of radiation. The mean lethal dose (LD50) was estimated based on these results.

In addition, a phenotypic evaluation of the population was conducted at 7, 14, and 21 days post-induction, A total of 600 mutation-induced seeds, along with the control (non-irradiated seeds), were assessed [Fig. 2]. The results of the preliminary test, including germination rates and seedling lengths, guided the selection of the most suitable doses, suggesting irradiation with 150 and 200 Gy.

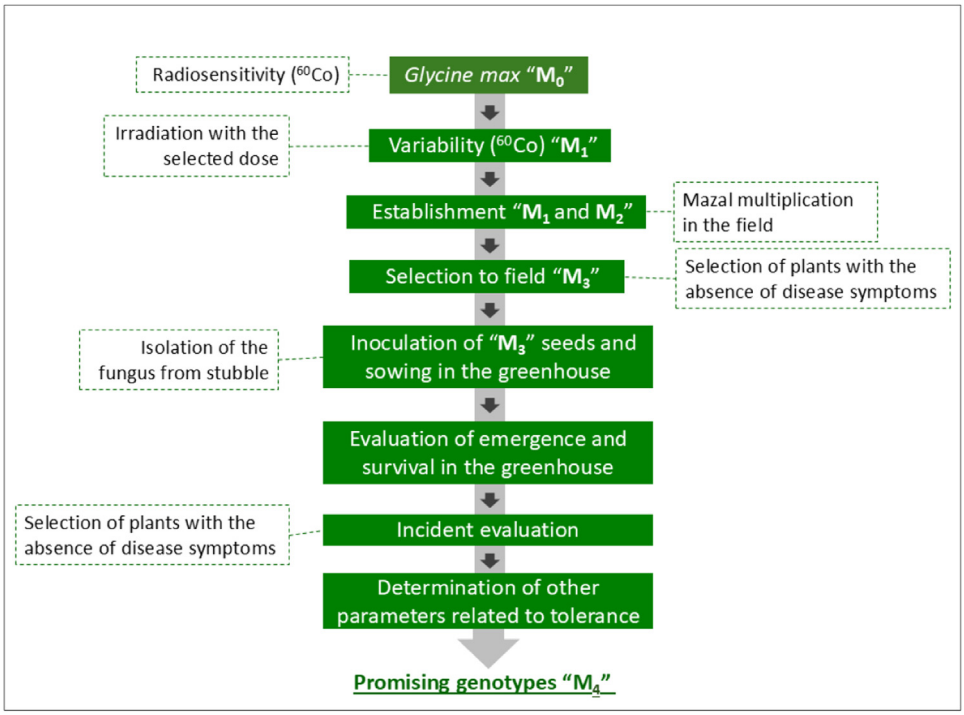

Fig. 1. Stages from starting material to candidate genotypes.

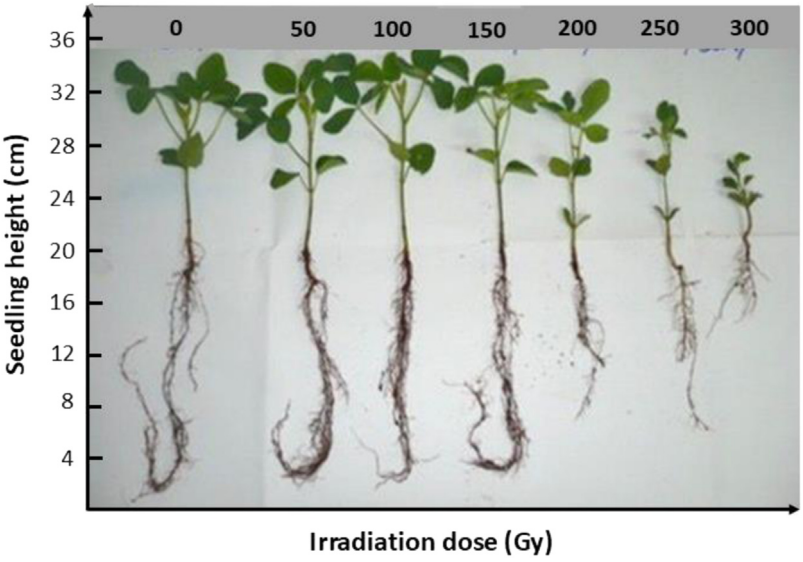

Fig. 2. Dosimetry: Evaluation of the effect of different radiation doses (50, 100, 150, 200, 250 and 300 Gy.) observed in soybean seedlings in the trial to determine the irradiation dose of the seed lot.

Irradiation of the seed lot

Doses of 150 and 200 Gy were selected, and these doses affected height by around 40 %. The nomenclature used for this technique suggests: M_0: Seeds before irradiation; M_1: Seeds after irradiation; M_2: Seeds product of the Multiplication to the field of M_1; M_3: Seeds product of field multiplication of M_2.

After irradiation of the seeds (M_1), these materials were sown for massive multiplication in the field, with the crop requirements for proper growth, which produced the next generation of seeds (M_2 and M_3).

Fig. 3

Fig. 3. Establishment M_1 and M_2: Multiplication in the field in generations of M_1 and M_2 with the conditions required by the crop.

First field selection (hot spot) of genotypes induced to mutation (M_3)

In M_3, of the 1000 seeds irradiated with a dose of 150 Gy, and that were multiplied in the field, 450 produced viable seeds, and of the seeds irradiated with a dose of 200 Gy, only 150 produced viable seeds.

For the selection of soybean genotypes with possible tolerance to *M. phaseolina*, the M_3 150 and M_3 200 seeds were sown in a field with natural infestation of *M. phaseolina* located In Experimental Field of the Faculty of Agrarian Sciences FCA/UNA located in the City of San Lorenzo, Central Department.

The presence of the pathogen in the soil was corroborated by isolating the fungus from soil samples and stubble from plants with fungus signs. Sowing was done manually, sowing 600 seeds in total, 450 $M_3$150 seeds and 150 M_3 200 seeds. The distance used was 12 cm between plants and 50 cm between rows. The general cultural care recommended for said crop was conducted until the moment of collecting the seeds. No chemical control was applied.

To identify promising genotypes for tolerance to this pathogen, the progress of the disease in the crop was evaluated [Fig. 4], using a severity scale [Table 1] [9]. Seeds were then collected from the selected plants (which showed no symptoms of the pathogen) and were stored at 4 °C until the next greenhouse test.

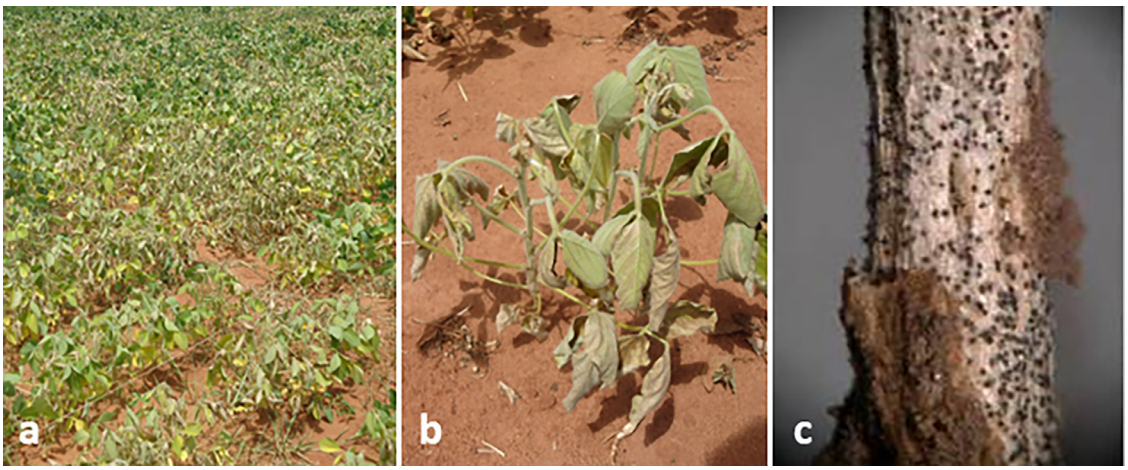

Fig. 4. Selection of genotypes in the field in M_3: Grown in plots with natural infestation *of M. phaseolina* in the soil. a: normal plants; b: plants with symptoms of charcoal rot., c: presence of microsclerotia of the fungus *M. phaseolina*.

Table 1
Charcoal rot severity scale due to *M. phaseolina* in soybean plants [9].

Lesion	Description of symptoms
0	No symptoms were observed at the time of inoculation.
1	Mycelial growth is observed. Lesions 0.5 to 1 cm long at the point of inoculation.
2	Lesions with brown-reddish edges 1 to 2 cm long at the point of infection.
3	Necrotic lesions that cover half of the neck of the plant. Slight chlorosis of the foliage.
4	Necrotic lesions with the presence of microsclerotia that completely cover the surface of the plant neck.
5	Death of the plant.

Second greenhouse selection of genotypes induced to the mutation (M_4) with seeds inoculated with the fungus M. phaseolina

A of *M. phaseolina* (strain: MPMA-18) obtained from soybean plants with symptoms of carbonaceous stem rot kept was selected for inoculation test. The strain is maintained at Centro Multidisciplinario de Investigaciones Tecnológicas. The isolate was cultured on potato dextrose agar (PDA) medium, from Sigma-Aldrich®, and this was used for subsequent tests.

The seeds were inoculated as described by [9]. To quantify the density of microsclerotia, five 100-µl aliquots were counted using a stereoscopic magnifying glass [10]. The seeds were placed in expanded polystyrene trays with 2 cm cells, in which each tray received 1 mL of fungal suspension. Soybeans seeds were left to rest for 10 min then dried on filter paper for two hours at room temperature.

Then, seeds were sown in 50 × 50 cm plastic pots, containing sterilized substrate (proportional mixture 3:3:2 of red soil, washed sand and earthworm humus, respectively). The experimental unit consisted of five soybean seeds arranged in a pot, in three replicated plots per genotype, all from a genotype, M_4 150 and M_4 200. The design used for planting was completely random, and there was also a control with the same treatment with non-irradiated seeds. Plants were subjected to an irrigation scheme that allowed maintaining the substrate at a field capacity of 70 % from day 41 of emergence. To provide the conditions that favors disease development. The substrate humidity was registered in each pot daily using a soil tensiometer.

Emergence and survival of seedlings in pots

Seedling emergence was evaluated at 7 days after sowing in all treatments. The percentage of emerged seedlings was determined for each genotype Seedling survival was evaluated at 14 days after sowing in all treatments. The percentage of seedlings that survived for each genotype evaluated was determined.

Incidence of charcoal rot disease

From 41 days (phenological stage V_6) after sowing, until the end of the crop cycle (ripe grains, R_8), the plants with signs of the disease were collected [Fig. 5]. The presence of microsclerotia of *M. phaseolina* at the base level of the stem and in the roots of the plant was determined by direct observation with a stereoscopic lens. Root sowing was carried out in PDA medium, to confirm or not the presence of the pathogen. (At the end of the cycle, the roots of all the plants were sown in PDA culture medium, to confirm or rule out the presence of the disease). At the end of the crop cycle (ripe grains, R_8), infection of plants with no symptom's disease was determined, following the same methodology of incidence due to charcoal rot already described, by sowing root and stem sections.

Fig. 5. Selection of genotypes under controlled conditions in M_3: Grown in pots infested with a known strain of *M. phaseolina*. a: normal plants; b: plants with symptoms of charcoal rot. c: pure culture of plant isolates with disease symptoms. d: presence of microsclerotia of *M. phaseolina*.

Charcoal rot severity assessment

For both the field evaluations in the M_3 greenhouse and the M_4 greenhouse, the severity of the disease was evaluated, observationally, using the charcoal rot severity scale of *M. phaseolina* [9], the which is presented in Table 1.

Quantification of total phenols in soybeans leaves in response to M. phaseolina

The concentration of toral phenols in soybean plant leaves, with and without signs of diseases caused by *M. phaseolina*, were determined. The trifoliolate leaves were collected in the V_6 stage in the greenhouse test plants, maintaining the previously described experimental unit. Leaves were ground with liquid nitrogen to obtain a fine powder. A 100 mg of the fine powder was weighed and extracted with 10 mL of 70 % acetone in water for 30 min. The supernatant was collected by filtration through filter paper. The extracts were used to quantify the total phenols usin the Folin-Ciocalteu method [11] (Folin-Ciocalteu 2 N, p.a. grade - Brand: Biopack - Origin Argentina) using the gallic acid standard (Gallic Acid (Anhydrous) p.a. grade - Brand: Biopack - Origin Argentina).

The total phenolic content was determined in three phases: T1 (no water stress), T2 (24 h after being subjected to water stress), and T3 (48 h after suffering water stress).

Data analysis

The results were subjected to an analysis of variance. The means were compared with each other using the Tukey test with a significance level of 5 %.

Method validation

Field selection (M_3)

without symptoms at the R7 stage were selected, including 250 healthy plants (223 from the 150 Gy treatment and 27 from the 200 Gy treatment). Fifteen seeds were collected from each, these were kept individualized, totaling 3750 seeds of the M_4 generation.

Stage 1: Emergence and survival of seedlings in the vegetation house

Table 2 shows the results of the emergence percentage of the different genotypes evaluated. The control "T_1" (without irradiation, without inoculation) had an emergence percentage of 96 %, the control "T_2" (without irradiation, inoculated) had an emergence of 70 %, with a difference of 28 % between these treatments.

Table 2
Emergence and survival (%) of soybean seedlings sown in pots with *M. phaseolina* infested soil. Greenhouse evaluation M_3.

Treatment	Genotype	Emergence (%)	Survival (%)
Not irradiated (control) N:1 R:50	1	96	100
Not irradiated (infested soil) N:1 R:50	1	70	90
Irradiated 150 Gy (i) N: 223 R:3.345	5	0	–
	2	12	82
	2	18	98
	11	20	78
	4	22	96
	4	40	98
	1	52	100
	10	60	98
	6	74	100
	10	78	100
	8	84	90
	40	90	92
	120	100	96
Media	–	83,7	94
Irradiated 200 Gy (i) N: 27 R:405	2	0	–
	3	60	60
	3	68	100
	6	76	98
	5	86	100
	8	100	92
Media	–	76	90

Table 3
Evaluation of the Incidence of charcoal rot disease (M_3 150 Gy and M_3 200 Gy) in a greenhouse in infected soil.

Treatment	Incidence		
	Injuri	Root infection	Total
Nod irradiated (ni)	0 % a	2 % a	2 % a
Nod irradiated (i)	90 % c	10 % b	100 % c
Irradiated 150 Gy (i)	65 % b	22 % c	87 % b
Irradiated 200 Gy (i)	100 % d	–	100 % c

Lesion 0 according to the severity scale of charcoal rot caused by *M. phaseolina* in soybean plants, adapted from Ramírez and Orrego (2008). (nor): not inoculated. (i): inoculated. Plant symptoms: visual evaluation of plants. Root infection: planting roots in PDA and appearance of the pathogen. Gy: Grey. TT: Tukey's Test: Averages followed by the same letter do not differ from each other, Averages followed by a different letter differ from each other, at a 5 % level of significance.

Among the genotypes irradiated with a 150 Gy dose evaluated in the M_4 generation and inoculated with *M. phaseolina*, a variable emergence percentage was obtained, ranging from five genotypes with 0 % emergence to 120 genotypes with 100 % emergence. In total, 223 genotypes were evaluated.

The genotypes irradiated with a dose of 200 Gy evaluated in the M_4 generation and inoculated with *M. phaseolina* had an emergence percentage, ranging from 2 genotypes with 0 % emergence to eight genotypes with 100 %. In total, 23 genotypes were evaluated.

Considering only the treatments, (T_1: non-irradiated plants, not inoculated with M. *phaseolina*; T_2: non-irradiated plants, inoculated with *M. phaseolina*; T_3: M_4 plants irradiated with 150 Gy inoculated with *M. phaseolina*; T_4: M_4 irradiated plants with 150 Gy inoculated with *M. phaseolina*) we have that T_1 had 96 % emergence, T_2 had 70 % emergence, T_3 had 83.7 % and T_4 had 76 %.

Incidence of M. phaseolina*: Plants with disease symptoms and root infection*

In Table 3 can be seen that with the genotype not irradiated and not inoculated with M. *phaseolina*, the 100 % of the plants did not show symptoms of the disease in the greenhouse, however, in the laboratory tests (roots sowed in PDA medium) showed 5 % infection by this fungus.

Emergence and survival of seedlings in the vegetation house

In a study on root and stem diseases in soybean plants, observed that initial infections of *M. phaseolina* in soybean seeds remained latent until the plants reached advanced stages of growth [12], where most of the infected plants germinated and emerged developing the disease later.

In preliminary germination power tests of the seeds, germination of seeds inoculated with *M. phaseolina* and natural infection by *M. phaseolina* in seeds collected in the field, "M_4 generation", in a sampling 1200 seeds were taken randomly and planted in *blotter test*. This test showed a germination power of 92 %, germination percentage of seeds inoculated with *M. phaseolina* higher than 90 %, and only 2 % of natural infection by the fungus *M. phaseolina*. These values would suggest that fungal infection does not prevent seed germination and subsequent seedling emergence.

Regarding the survival evaluated 14 days after planting, in all treatments, T_1 had 100 % survival, T_2 had 90 % survival, T_3 had 94 % survival, and T_4 90 % survival.

It should be considered that between the treatments irradiated with 150 and 200 Gy and inoculated with the fungus *M. phaseolina* (T_3 and T_4), there are 223 and 27 genotypes, respectively and they are considered different.

Incidence of M. phaseolina*: Plants with disease symptoms and root infection*

According to some research, referring to the symptoms of root and stem rot caused by *M. phaseolina*, they mention that those symptoms manifest when the crop is under hydric stress, with a dry soil profile and high temperatures [2]. In the present research, to promote the appearance of symptoms of this disease, field capacity of the substrate was maintained at around 70 % of the field capacity. The typical symptoms, which show black to gray lesions on stems, loss of leaflets, vigor reduction, furthermore, the climate (warm and dry), as a factor that predisposes the germination of sclerotia, thus infecting the seedlings developing the typical symptoms of the disease [12].

In the inoculated genotype and non-irradiated "Control", it was observed a 90 % infection of plants in the greenhouse and a 10 % infection of roots taken from plants that did not show symptoms in the greenhouse were observed, totaling a 100 % infection by the fungus.

In a selection of crops, 50 soybean cultivars (commercial seeds) were evaluated, which showed variable results in the percentage of infection, in which the cultivar that presented the highest tolerance was with a 32.73 % of infection, while the most susceptible

cultivar presented 87, 73 % of infection of M. *phaseolina* [2]. In the investigation they stated that none of the cultivars evaluated showed tolerance or resistance to this pathogen, since the percentage of diseased plants was greater than 30 %.

In the genotypes inoculated with *M. phaseolina*, it is observed that those irradiated with a dose of 150 Gy had an incidence of 65 % of plants with symptoms and plants without symptoms, whose roots were planted in plates containing PDA medium 22 % of infection caused by this fungus, totaling 87 % of infection in these genotypes.

In the genotypes from seeds irradiated with 200 Gy, all were infected by the fungus. These results suggest that none of these mutant genotypes (M_4) from seeds irradiated with 200 Gy have any tolerance to the fungus.

In an evaluation of 16 soybean mutant genotypes have evaluated (mutation induced by *gamma* irradiation) under greenhouse conditions to study tolerance to charcoal rot disease caused by *M. phaseolina* and have obtained a variation in the behavior of these genotypes, observing highly susceptible mutant genotypes, moderately susceptible mutants, which reduced survival, and disease-tolerant genotypes [13]. These variations between mutants in the reaction against the pathogen may be due to changes in the genetic composition of the mutants because of *gamma* irradiation [14].

In general, some mutation-induced genotypes tolerant to charcoal rot disease were obtained that should be subject to further evaluation to confirm tolerance. These results are in agreement with those reported data [15,16], who indicate that treated seeds of locally adapted varieties of some crops with *gamma* irradiation produce some promising.

Stage 2: Determination of the variation of total phenols in the reaction of soybean genotypes induced by mutation to M. phaseolina

The concentration of total phenols in the first sample (T_1) remained constant in almost all the genotypes, both irradiated-inoculated and irradiated non-inoculated and inoculated did not have large variations. However, as the disease progresses (2 and T_3), it can be observed that the level of total phenols in the genotypes induced by the mutation (M_3) increases.

Changes in total phenol content associated with infection and increased tolerance to M. phaseolina

The determination of total phenols content was carried out in all the available samples, in three times (T_1, T_2 and T_3) to establish if an accumulation of these occurs as a consequence because of the infection with the fungus and if this accumulation is related to tolerance to disease.

An interesting response has been found regarding the accumulation of phenols (Fig. 6). It has been seen that the non-inoculated plants remained relatively constant throughout the test, around 3 ppm per gram of sample; the opposite occurred with the inoculated genotypes that showed a marked trend, given the significant increase in the content of total phenols. This increase can be attributed to the stress produced by the infection by the fungus, however, of the genotypes inoculated with the fungus, the increase was greater in the genotypes from seeds induced to mutation.

Table 4 shows the averages of the phenol content in the last measurement, at this stage where almost all the genotypes evaluated in the trial were infected and withered by the fungus infection, some that had no symptoms and ended normally were observed its cycle, among which the genotype M_{45}, M_{19}, M_{13}, and M_{35} stands out, which were reported as tolerant.

Regarding the phenol content, it is observed that these genotypes (M_{45}, M_{19}, M_{13}, and M_{35}) recorded the highest values in phenol content, between 12.03 and 11.27 mg/g, which may indicate a relationship with tolerance to the fungus. However, as already mentioned, this dynamic is difficult to interpret, because while we observe genotypes with phenol content values very close to the

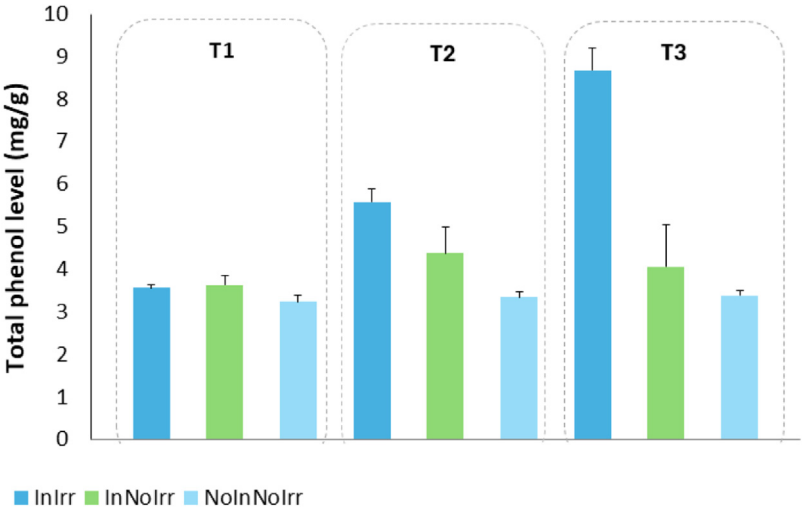

Fig. 6. Total Phenol content level in the three treatments in three measurement times: T_1: 0 h of stress. T_2: 24 h of stress. T_3: 48 h of stress.

Table 4
Averages in the content of total phenols in genotypes induced to mutation and infected by the fungus *M. phaseolina* (3rd sampling).

Averages in the content of Total Phenols (TF) in the different genotypes													
Gen.	mg/g	TT											
M_{45}**	12,03	a											
M_{76}*	11,67	a	b										
M_{19}**	11,43	a	b	c									
M_{13}**	11,38	a	b	c									
M_{35}**	11,27	a	b	c	d								
M_{89}S	10,87	a	b	c	d	e							
M_{12}S	10,85	a	b	c	d	e							
M_{102}*	10,28	a	b	c	d	e	f						
M_{125}**	10,11	a	b	c	d	e	f						
M_{127}S	9,96	a	b	c	d	e	f						
M_{79}S	9,48		b	c	d	e	f						
M_{48}*	9,34		b	c	d	e	f						
M_{30}*	9,25			c	d	e	f						
M_{112}S	9,03				d	e	f	g					
M_{29}**	8,83					e	f	g	h				
M_{32}**	8,35						f	g	h	i			
M_{36}**	8,18						f	g	h	i			
M_{77}S	6,84							g	h	i			
M_{62}**	6,55								h	i	j		
M_{55}**	6,5								h	i	j		
M_{65}*	6,45									i	j	k	
M_{87}*	6,3									i	j	k	
M_{40}S	6,06									i	j	k	
M_{111}S	6,03									i	j	k	
M_{22}S	4.41										j	k	l
M_{85}S	4,14											k	l
M_{129}S	3,69												l

TT: Tukey's Test: Averages followed by the same letter do not differ from each other, Averages followed by a different letter differ from each other, at a 5 % level of significance.
**: Plants that did not show symptoms;
*: Plants with symptoms that finished the cycle; S: Plants with symptoms that did not finish their cycle. M_1, M_2, M_x: code of each genotype evaluated.

tolerant ones such as M_{89}, M_{27}, M_{79} with 10.87; 9.96 and 9.48 mg/g, respectively, that were shown to be susceptible. Genotypes with low phenol content such as M_{62} and M_{55} with 6.5 mg/g were tolerant to charcoal rot.

Determination of the variation of total phenols in the reaction of soybean genotypes induced by mutation to M. phaseolina

It is known that plants can present mechanisms of defense against pathogens such as structural or physical barriers as well as the synthesis of substances in response to the presence of the pathogen, such as phenolic compounds [17]. However, when evaluating the relationship between this accumulation and tolerance to the disease caused by this fungus, disparate responses have been observed. It has been seen that plants have increased the phenol content to a high level that showed severe symptoms of the disease, as well as plants with a similar phenol level that showed tolerance. In relation to this increase, it can be attributed to the activation of phenolic metabolism and the accumulation of certain phenols usually appear associated with induced systemic resistance, SIR (Systemic Induced Resistance), as a defense mechanism to various types of stress, both abiotic and biotic, which decreases or increases depending on the susceptibility or resistance of the host. However, reports on the direct relationship of higher concentrations of phenolic compounds in resistant plants than in susceptible ones sometimes do not specify this dynamic.

Final considerations

Mutant genotypes with traits demonstrating tolerance to charcoal rot were observed.

The mutation induction technique can be complementary to other biotechnological techniques for plant improvement.

Limitations

The limitations of this study include that the mutations that can be generated by seed exposure are random, and may not generate the desired characteristics, in addition to the fact that the evaluation of the generated materials can be complex.

Ethics statements

In this research, the data used does not compromise the safety or integrity of people or animals.

Declaration of competing interest

he authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

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Data availability

No data was used for the research described in the article.

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