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Effect of Gamma Irradiation and Selection with Fungus Filtrate (*Rhizoctonia solani* Kuhn) on the *in Vitro* Culture of Common Bean (*Phaseolus vulgaris*)

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Abstract

The present investigation was undertaken to study the effect of gamma irradiation (dose from 10 to 100 Gy) and *in vitro* selection with fungus filtrate as selecting agent (concentration from 20% to 100%) on the susceptibility of the common bean to *Rhizoctonia solani*. The best results were found with a dose of 20 Gy or a concentration of 20% of fungus filtrate applied separately. These conditions were used to evaluate the combined effect of both approaches in a second experiment. The combined effect of irradiation and then selection adversely affected growth (height and roots) and survival of the *in vitro* plants. It may not be necessary to combine the variation generated by irradiation with the selection technique. For future assays we propose the application of: 1) gamma radiation, thereby inducing not only mutants with pathogen resistance, but also with other agronomic traits of interest. Later in the subculture MV4 potential fungus-resistant mutants will be evaluated in the field; or 2) selection pressure using fungus filtrate during three subcultures, which may be sufficient to induce the variation necessary to obtain *in vitro* plants resistant to fungus.

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Keywords

Mutagenesis, Radiosensitivity, *In Vitro* Selection, *Rhizoctonia*, Common Bean

1. Introduction

The common bean (*Phaseolus vulgaris* L.) is one of the legumes of greatest consumption in Africa, India, the Caribbean, and Latin America [1] [2]. It is one of the basic foods in the Costa Rican diet [3], not only as a source of iron and vegetable protein, but also as a source of fiber, folic acid, thiamine, potassium, magnesium, and zinc [1].

Common bean yields are greatly affected by edaphic and climatic factors, pests and diseases, including those caused by the pathogenic fungi *Fusarium* spp., *Rhizoctonia* spp., *Pythium* spp., *Macrophomina phaseolina* and *Sclerotium rolfsii*. *Rhizoctonia* diseases have been reported frequently in Central, South America and the Caribbean, and cause seed disintegration, including pre- and post-emergence damping-off, stem and root rot, reduced growth and foliar damage [4]-[7]. Root rot is one of the principal limiting factors in common bean production. The causal agent is *Rhizoctonia solani* Kuhn (teleomorph *Thanatephorus cucumeris* (A.B. Frank Donk) [7] [8], which is widely distributed in all zones of reproduction [4]. *R. solani* is soil-borne and survives in the absence of a host as asexually produced sclerotia, which can survive in the soil for two years, and may accumulate and be rapidly dispersed by tillage or flooding [9]. The presence of root rot in favorable environmental conditions for development (22˚C to 28˚C temperature) and high soil moist, causes rot and/or destruction of the root, as well as restricted absorption of water and nutrients, reducing plantlet growth to 53% [4].

In Costa Rica, the common bean variety Bribri was introduced and evaluated between 1996 and 1999. This variety characterized by a small shiny dark red seed, type II-B (bush) growth habit, and a vegetative cycle of 76 to 80 days. Additionally, it presents intermediate tolerance to web blight [3], which is not sufficient to allow planting without the use of costly fungicides in areas where inoculum pressure is expected to be high [10]. The conventional fungus pathogen control method is mainly dependent on the intensive and extensive use of chemical fungicides, which have drawbacks such as doing harm to the ecological system, and its expensive cost [11]. Chemical control is also made difficult by the fact that species and the anastomosis groups (AGs) of *Rhizoctonia* show different sensitivities to fungicides [12].

Field identification of individuals tolerant to this disease is limited to seasons favorable for disease development, and plants that avoid the disease because of architectural characteristics cannot be differentiated from those that possess physiological resistance [10]. It is therefore necessary to identify strategies that allow the development of common bean lines with greater levels of resistance to web blight in order to improve common bean production in warm, humid tropical zones [10]. *In vitro* mutagenesis, which consists of *in vitro* culture and the induction of mutations, offers an alternative for increasing variability in cultivars of economic importance that can complement conventional breeding programs [13]-[15]. This technique has been used successfully to induce resistance to pathogens [16]. However, this and other species from the *Phaseolus* genus is recalcitrant to *in vitro* regeneration [17]-[19].

In the case of *in vitro* selection techniques, which use different selective agents, the resulting changes in phenotypic characters (e.g., resistance/tolerance to diseases) can be interpreted as potential mutations in the plant DNA sequence [20]. Selection with phytotoxins and culture filtrate appears to be more effective than use of the pathogen itself [20]-[22]. The use of *in vitro* methods for the evaluation of resistance is dependent upon a positive correlation between *in vitro* culture filtrate resistance and whole plant disease resistance [23].

More recently, *in vitro* techniques have been combined with mutation induction for generating genetic variation, including novel disease-resistant mutants [23]. Variability in somaclones can be induced and enhanced by exposure to physical mutagenic, by irradiation with non-ionizing (e.g. ultraviolet radiation UV) or ionizing (e.g. X and gamma rays, alpha and beta particles, protons and neutrons) radiation [20] [24]-[27]. Therefore, every experiment should start with radiosensitivity tests to determine the optimal irradiation dose for the plant material being used [23] [28]. The LD_{50} (*i.e.*, the dose that will kill 50% of the test organisms within a designated period) is often used as the optimal dose for mutation induction [23].

Mutagenesis *in vitro* is a relatively simple, inexpensive and efficient technique, with the advantages associated with *in vitro* culture, such as 1) manipulation of different types of explants (axillary buds, organs, tissues

and cells); 2) management of a large number of individuals in a small space; 3) ability to separate (subculture) mutated and non-mutated sectors (dissolving a chimera to obtain homo-histonts); 4) the entire process is performed under highly phytosanitary conditions and 5) the differences in polygenic characteristics can be evaluated easily and precisely [16] [21] [29] [30].

The objective of the present study was to evaluate the effect of gamma irradiation and selection with fungus filtrate (*Rhizoctonia solani* Kuhn) on the *in vitro* culture of the common bean (*Phaseolus vulgaris*). The optimum mutagenic dose of gamma rays was determined, based on radiosensitivity (LD_{50} in survival and height increase of *in vitro* plants). The concentration of pure filtrate of *R. solani* Kuhn to be used as selecting agent *in vitro* was determined. The selected dose and/or concentration were applied and the effects of gamma irradiation and selection *in vitro* on cultures of *P. vulgaris* var. Bribri were evaluated.

2. Materials and Methods

P. vulgaris var. Bribri [3] seeds are small and bright red, these were provided by the Fabio Baudrit Moreno Experimental Station (EEFBM), University of Costa Rica, located in La Garita of Alajuela, Costa Rica.

Seeds of *P. vulgaris* var. Bribri were disinfected following the method of Gatica *et al*. [18]. Embryonic axes (EAs) were excised using a scalpel and tweezers under a microscope stereo. The EAs were disinfected with a solution of 0.1% bleach (commercial sodium hypochlorite) for 10 minutes, washed three times with sterile distilled water, and placed in Petri dishes on basal semisolid MS media (25 EAs per Petri dish).

2.1. Basal Semisolid MS Media (BMS)

The EAs were cultured in semisolid MS media [31] supplemented with 4.44 µM BAP, with added sucrose 3% (w/v) and 0.8% agar (BMS). The pH was adjusted to 5.7 prior to autoclave sterilization (120˚C for 20 min). Cultures were incubated for three months at 26˚C ± 1˚C with a photoperiod of 12 h light (30 µmol/m^2·s) and 12 h darkness. The cultures were subculture every 30 days using the same type of media, until the third subculture was obtained (MV_1, MV_2 and MV_3). This composition media allow the regeneration of EAs of the common bean through direct organogenesis, so that in each subculture separation of mutated from non mutated sectors of *in vitro* plants was achieved.

2.2. Radiosensitivity of *in Vitro* Embryonic Axes to ^{60}Co

The EAs were irradiated with a cobalt 60 source (^{60}Co) in the Radiotherapy Unit of the San Juan de Dios Hospital in San Jose, Costa Rica (Theratron 780E), to a distance of 80 cm of source. Two experiments were performed in order to estimate appropriate radiation dose: I) the EAs were irradiated with the following doses: 10 Gy, 30 Gy, 50 Gy and 70 Gy. A non-irradiated control was included. Fifty EAs were used for each treatment; II) based on results of the first experiment, EAs were irradiated with the following doses: 20 Gy, 25 Gy, 30 Gy and 35 Gy. A non-irradiated control was included. Fifty EAs cultured in BMS media were used for each treatment, as in Experiment I. The EAs were cultured in BMS before irradiation and after the EAs were transferred immediately to fresh BMS media.

Radiosensitivity of the EAs was evaluated by measuring the increase in height (%) and survival 30 days after exposure to gamma radiation. The lethal dose (LD_{50}) was defined as the dose that eliminated 50% of the explants 30 days after exposure [16].

2.3. Selection of *in Vitro* Plants Using Fungus Filtrate

Leaves samples of bean plants with symptoms of infection by *R. solani* in the field were collected. The pathogen was isolated and identified according to Mora-Umaña [32] [33]. The fungus was isolated (3242) from Buenos Aires, (longitude W0 83 31 37 7” and latitude N 09 07 41 1”). The molecular identification of the anastomosis group and subgroup isolation was performed using specific primers 3224 [8] and the fungus was found to belong to AG1 group and the subgroup AG1-IF (GenBank accession number JX294319) [33].

The liquid media for inoculation and growing was prepared using AC agar (All culture agar) of Sigma (20 g/L protease peptone, 3.0 g/L beef extract, 3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L dextrose, 0.20 g/L ascorbic acid, 1 g/L agar, pH 7.2) in the Erlenmeyer flasks 250 mL (shaken culture). The culture media into Erlenmeyer flasks was inoculated with small pieces of PDA (potatoes-dextrose-agar) media with mycelium of *T.*

cucumeris (about 1 cm in diameter). The culture was incubated at 24˚C on an orbital shaker (2000 rpm) for five days under a 14 h photoperiod. The fungus filtrate of *R. solani* was provided by the Cellular and Molecular Investigation Center (CIBCM) of the University of Costa Rica.

2.4. Sensitivity of the EAs to the Fungus Filtrate

The concentration of fungus filtrate to be used was optimized by culturing non-irradiated EAs in BMS media with 20%, 40%, 60%, 80% or 100% (v/v) fungus filtrate or BMS media without filtrate. Four EAs were placed in each culture vessel, with three repetitions of each treatment. Preliminary test using not irradiated EAs was made for determine one filtrate dose to be used later in the irradiated ones. The sensitivity of the EAs was evaluated using the LD_{50} for survival after 30 and 60 days of culture. The effect of filtrate concentration in the media was determined through survival and height (cm) of *in vitro* plants. In this step the plantlets in the greenhouse were not evaluated. This was a preliminary test using non-irradiated embryonic axes to determine one filtrate dose to be used later in the irradiated axes. To discard any effect of the AC liquid media used for growing *R. solani* on the development of explants, in this research were included the results of the evaluation of non-irradiated EAs cultured in BMS media with different concentrations of the liquid media used for inoculation and growing of fungus filtrate of *R. solani*. The treatments were: only BMS media or BMS media added with 20%, 40%, 60%, 80% or 100% (v/v) of liquid media AC without fungus filtrate. The objective was evaluated the response of plants in this conditions.

2.5. The Effect of Gamma Irradiation and *in Vitro* Selection on Common Bean

Based on results obtained in the first experiments, a dose of 20 Gy and concentration of 20% fungus filtrate in BMS were applied in the following treatments: T1: irradiated EAs with fungus filtrate in BMS; T2: irradiated EAs initially grown without fungus filtrate, with filtrate in subculture MV_2; T3: irradiated EAs without fungus filtrate in BMS; T4: non-irradiated EAs with fungus filtrate in BMS; T5: non-irradiated EAs, initially without fungus filtrate, with filtrate in subculture MV_2; T6: non-irradiated EAs without fungus filtrate in BMS.

Four EAs were cultured in BMS media with 20 repetitions per treatment. The subcultures of irradiated material and *in vitro* selected material were made every 30 days for separation of mutated sectors from the non mutated. The *in vitro* plants (90 days MV_3) obtained in all six treatments, with 1.5 cm height and root development were evaluated (shoot height, number of leaves, presence of roots, number of branches, height branches). They were then moved to the greenhouse and maintained for four days for hardening off before planting. Roots were then washed carefully with water and planted in large pots in universal soil-organic substrate (3:1) and watered daily near the stem. The pots were maintained in the greenhouse at an average temperature of 24˚C. Plants were fertilized with 10-30-10, 30 days after transplanting and then every 22 days. They were watered every three days and fertilized until the being harvest, from which was increased in the following generation (MV_4).

2.6. Statistical Analysis

Lethal dose (LD_{50}) was determined in two experiments using logistic regression models with explants survival as the response in both cases, and the irradiation dose (Gy) or the percentage of fungus filtrate culture as the predictor. The analysis was performed using the GLM function of the statistical software R (version 2.13.0, R development Core Team, 2011). Confidence intervals for LD_{50} were calculated using the variance obtained by the delta method [34]. The observed survival probabilities were plotted and the regression curve and confidence intervals for the LD_{50} were added. A simple linear regression model was used to analyze the increase in height of EAs irradiated. Similarly, treatment effect on the survival probability and the probability of green shoots was analyzed using a logistic regression models, and the corresponding analysis of variance and Tukey multiple comparisons between pairs of treatments. Box plots were used to compare the distributions among the different treatments.

3. Results

3.1. Radiosensitivity of *in Vitro* Embryonic Axes to ^{60}Co

An increase in sensitivity to radiation was observed with a rise in the radiation dose. It was observed that the

higher the dose, the greater the physiological effect in terms of survival and growth rates of explants (**Figure 1**). For doses > 40 Gy (50, 60 and 70 Gy) the survival rates were of 0%; and for the 10 Gy dose, the irradiation effects were not visible as the non irradiated explants (**Figure 1(a)**, **Figure 1(b)**).

In experiment I (0 - 100 Gy) the mean lethal dose LD_{50} of radiation at 30 days was approximately 22.56 Gy with a confidence interval of 18.17 to 26.95 Gy (**Figure 1(a)**). This was reduced to 21.26 Gy (with an interval of 15.17 to 27.35 Gy) after 60 days of culture (data not shown). In experiment II (20 - 35 Gy) the LD_{50} at 30 days of culture was 23.8 Gy with a confidence interval of 22.41 Gy to 25.19 Gy (**Figure 1(b)**).

For increment in height the determination coefficient (R^2) was 0.82 and 0.99 for the correlation, it should be noted that the most accurate correlation per *in vitro* plant was observed in second experiment (**Figure 1(c)**, **Figure (d)**). Irradiated EAs reached 50% height when the administered dose was 30 Gy, as interpolated from the graph in (**Figure 1(d)**).

Necrotic tissue was noted with doses of 50 and 70 Gy (**Figure 2(a)**). Explants irradiated with 70 Gy showed bacterial growth at 30 days (**Figure 2(a)**), which resulted in the nearly total loss of the cultures. Explants irradiated with doses from 10 to 35 Gy showed no necrotic tissue; however, at 30 days, growth was reduced, with respect to the control plants, principally with doses from 25 - 35 Gy (**Figure 1(d)**, **Figure 2(b)**).

3.2. Selection of *in Vitro* Plants Using Fungus Filtrate

Sensitivity of the EAs to the Fungus Filtrate

After 30 days of culture (MV_1), the LD_{50} for the concentration of fungus filtrate in BMS was 31.46% with a

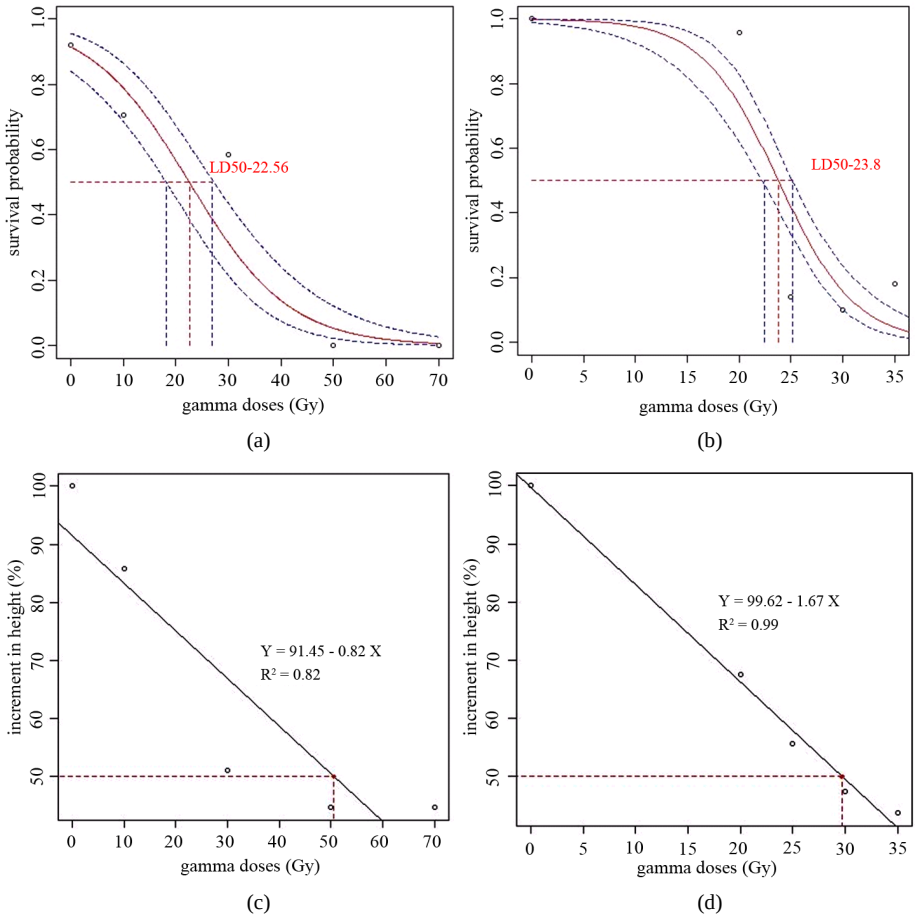

Figure 1. Evaluation of the sensitivity of *Phaseolus vulgaris* var. Bribri to different doses of radiation with ^{60}Co, after 30 days of *in vitro* culture (MV1). (a), (c) doses of 0 - 70 Gy; (b), (d) doses of 0 - 35 Gy; (a), (b) probability of survival by dose with 95% confidence intervals; (c), (d) increase in height (%).

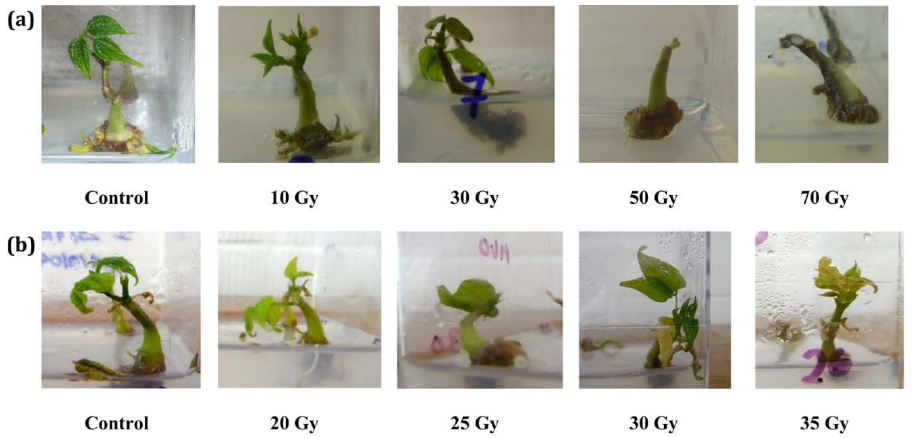

Figure 2. *Phaseolus vulgaris* var. Bribri *in vitro* plants after 30 days of culture, obtained from irradiated EAs. (a) evaluation of doses from 0 to 70 Gy; (b) evaluation of doses from 0 to 35 Gy.

confidence interval of 24.31% to 38.60% (**Figure 3(a)**). After 90 days of culture (MV_3), the LD_{50} was 23.71% with a confidence interval of 17.61% to 29.81% (**Figure 3(b)**).

The effect of the selection agent (culture filtrate) demonstrated in the experiment a suitable concentration range which allows for a comparison of the toxic effects on susceptible and tolerant/resistant *in vitro* plants. The outcome of such preliminary experiments was to ascertain the precise dosage of selection agent that was optimal for screening resistant material, while killing or drastically reducing growth of susceptible material. One of the most evident morphological changes was the reduction in the proportion de survival *in vitro* plants as the concentration of fungus filtrate in BMS was increased. At 30 days of culture, 50% of the non-irradiated *in vitro* plants survived in presence of 30% fungus filtrate in BMS media. In contrast, the cultures with 60% fungus filtrate in BMS showed 60% necrotic (dark brown) *in vitro* plants (data not shown). The explants cultured in BMS with 100% fungus filtrate showed total mortality from the first week of culture. This treatment was considered to be a positive control, since it showed that the explants did not survive in presence of *R. solani* filtrate.

EAs length was affected by the presence of fungus filtrate in the culture media, with a tendency towards reduced growth as the filtrate concentration was increased, both at 30 and 90 days of *in vitro* culture (**Figure 3(c)**, **Figure 3(d)**). Therefore, the optimal concentration of culture filtrates to be use as selection agent for *in vitro* selection of the common bean was 20%.

The results of the evaluation of non-irradiated EAs cultured in BMS media with different concentrations of the liquid media used for inoculation and growing of fungus filtrate of *R. solani* (AC agar of Sigma), did not showed statistical significance for the survival among different treatments. After 30 days, the length of the shoots (cm) growing under this conditions was 2.6 ± 0.09c (BMS without AC), 3.1 ± 0.07d (20% AC), 2.8 ± 0.09cd (40% AC); 2.55 ± 0.15bc (60% AC); 2.12 ± 0.09ab (80% AC) and 2.06 ± 0.10a (100% AC) with significance between treatments (letters adjacently to the standard error).

The EAs cultured in BMS with 20% of AC media had a small increase in shoots length compared with the control. It is important, because in this research we determined that 20% of the fungus filtrate in AC media has an effect as selective agent for *in vitro* selection.

3.3. The Effect of Gamma Irradiation and *in Vitro* Selection on Common Bean

A dose of 20 Gy of gamma rays of ^{60}Co was considered to be adequate for generating mutations in EAs of coomon bean var. Bribri, without affecting the growth of *in vitro* plants. The concentration of 20% fungus filtrate in BMS media was used to pre-select only those possibly mutated individuals showing resistance to the *Rhizoctonia solani* filtrate, since after the third subculture (MV_3). The plantlets would be acclimated to obtain the MV_4 for further increase of possibly mutated plant material.

In order to evaluate both criteria in the methodology for the induction of mutations and/or selection *in vitro* of *Phaseolus vulgaris* var. Bribri, experiments were conducted using 20 Gy and 20% fungus filtrate in BMS media. At 90 days, a severe effect was observed in the survival of irradiated explants immediately selected for possible

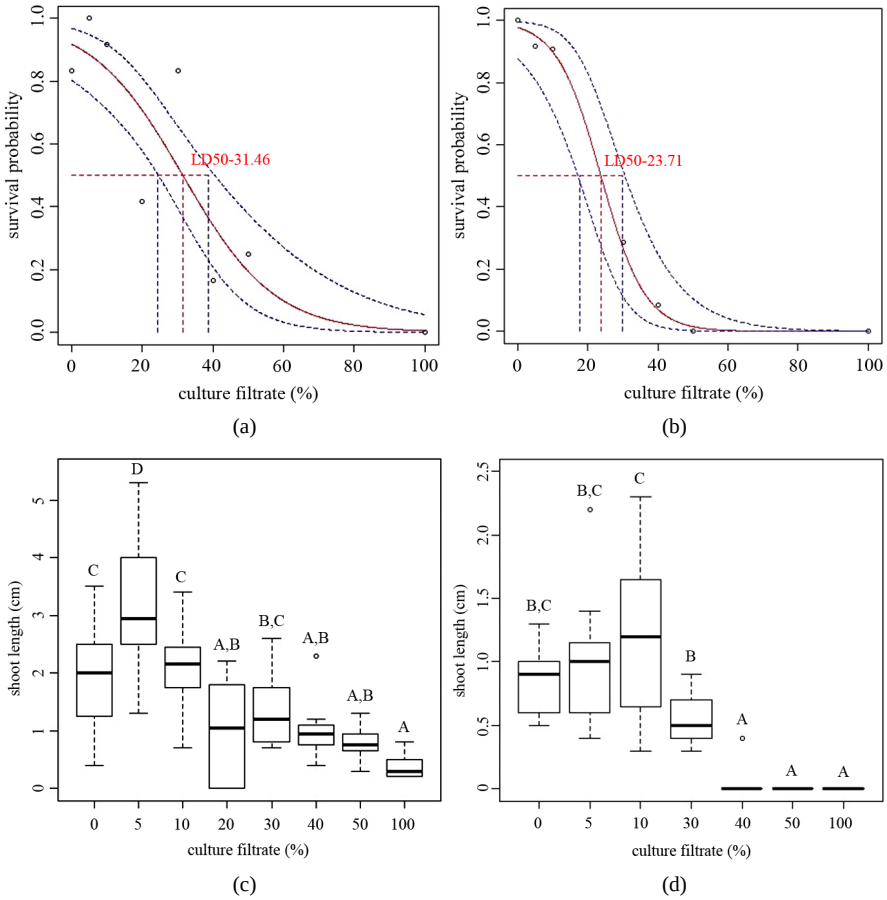

Figure 3. *In vitro* selection of *Phaseolus vulgaris* var. Bribri, obtained from non-irradiated embryonic axes grown in *Rhizoctonia solani* culture filtrate. (a), (c) Evaluation after 30 days of culture (MV1); (b), (d) evaluation after culture for 90 days (MV3); (a), (b) survival probability by dose, with 95% confidence intervals; (c), (d) evaluation of plant height (cm). Different letters indicate significant difference ($p < 0.05$).

resistance to the fungus. Mortality was high (less than 50% survival) (**Figure 4(a)**). *In vitro* plants that survived showed stunted growth (**Figure 4(b)**), foliar tissue in rosette form, and little or no root development. None of the roots that were formed were functional (necrotic), which limited their acclimation (**Figure 5(a)**, **Table 1**).

The irradiated EAs cultured initially in BMS without fungus filtrate, with filtrate added in the second subculture, showed a greater than 50% survival rate (**Figure 4(a)**, **Figure 5(b)**). Survival of irradiated EAs in absence of filtrate was greater than that of irradiated EAs immediately exposed to fungus filtrate (**Figure 4(a)**, **Figure 5(c)**). Delaying the addition of filtrate to BMS until the second subculture (MV_2) increased the proportion of non-irradiated explants that survived in presence of fungus filtrate (greater than 80%) (**Figure 4(a)**, **Figure 5(e)**). At 90 days, no significant differences were observed for survival with respect to non-irradiated axes cultured without fungus filtrate (controls) (**Figure 4(a)**, **Figure 5(e)**, **Figure 5(f)**).

Survival of non-irradiated EAs in presence of fungus filtrate was low, and differences between non-irradiated treatments and treatments 1 and 2 (irradiated EAs with filtrate and irradiated EAs with filtrate in the second subculture, respectively) were not significant. The observed negative effect on survival may be due to the presence of fungus filtrate in the culture media, and not only a product of irradiation (**Figure 4(a)**, **Figure 5(a)**, **Figure 5(b)**, **Figure 5(d)**). After 90 days of culture (MV_3), *in vitro* plants were obtained from irradiated or non-irradiated EAs, cultured in BMS with or without fungus filtrate (**Table 1**).

At planting, many roots of *in vitro* plants were lost or broken when washed to remove culture media, since they were not functional (necrotic). After 40 days in pots, one non-irradiated plant selected *in vitro* with fungus filtrate had survived and had abundant foliar tissue and good development. It is important to note that some of

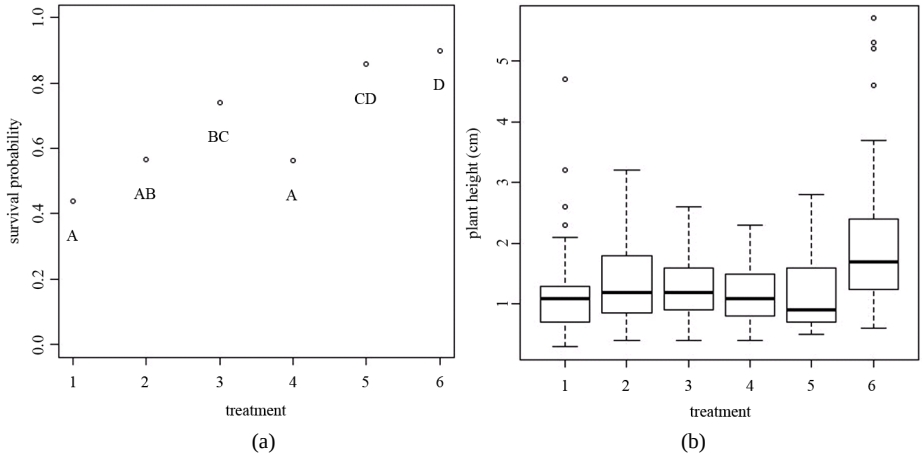

Figure 4. Effect of gamma irradiation and *in vitro* selection on *Phaseolus vulgaris* var. Bribri, 90 days after treatment. T1: Irradiated, with fungus filtrate. T2: irradiated, initially grown without fungus filtrate, with filtrate added in subculture MV2. T3: irradiated, without fungus filtrate. T4: non-irradiated, with fungus filtrate. T5: non irradiated, without fungus filtrate initially, filtrate added in subculture MV2. T6: non-irradiated, without fungus filtrate. A: survival, B: evaluation of plant height (cm). Different letters indicate significant difference ($p < 0.05$).

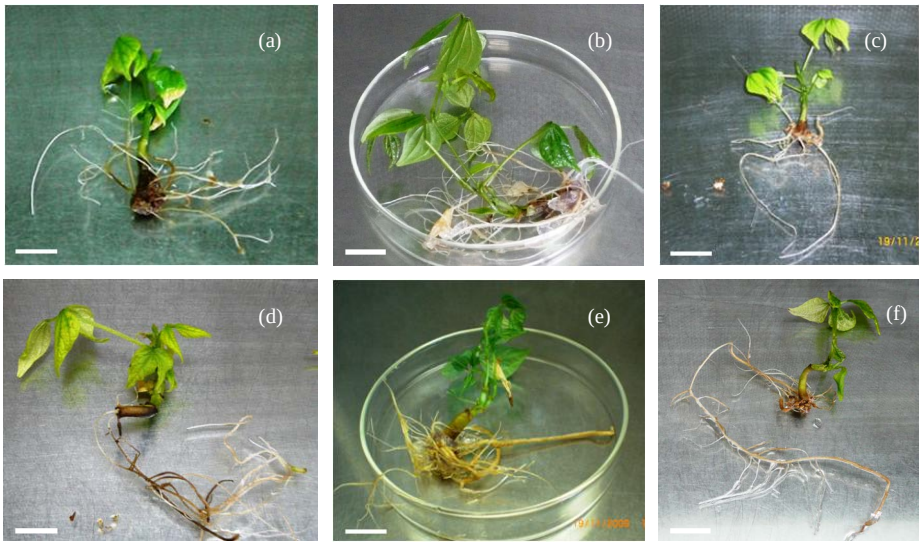

Figure 5. Effect of gamma irradiation and *in vitro* selection on *Phaseolus vulgaris* var. Bribri, 90 days after treatment. (a) irradiated with fungus filtrate; (b) irradiated without fungus filtrate initially, with filtrate in subculture MV2; (c) irradiated without fungus filtrate; (d) non-irradiated with fungus filtrate; (e) non-irradiated without fungus filtrate initially, with filtrate in subculture MV2; (f) non-irradiated, without fungus filtrate. Scale bars: (a) 0.5 cm, (b) 1 cm, (c) 2 cm, (d), (e) 1 cm and (f) 2 cm.

the negative control plants (T4: irradiated, grown without fungus filtrate) as well as positive controls (T6: non-irradiated, grown without extract) were acclimated. The remaining *in vitro* plants (**Table 1**) were maintained in MV_3 media for 60 more days and were acclimated after the appropriate height was reached and roots were present. Plants irradiated and selected *in vitro* did not survive to the acclimatization stage.

4. Discussion

Many factors affect the response of a plant to irradiation. It is therefore important to determine the effect of

Table 1. Morphological characteristics of *in vitro* plants of P. vulgaris var. Bribri (MV3) transferred to greenhouse (treatments No. 1 - 5). Small crosses indicate relative presence of roots (abundant).

No. plant	No. treatment	Shoot height (cm)	No. leaves	presence of roots	No. branches	Height branch (cm)
1	1	1.5	3	+++	1	2.5
2	2	4.3	9	+++	1	3.5
3	2	4	15	+	0	0
4	2	3	6	++	0	0
5	3	4.5	21	+++	1	4
6	3	4.5	3	+++	1	2
7	3	6	12	+	1	4.5
8	3	4	12	+	3	3.5 2.5
9	3	1.5	6	+	1	3.5
10	3	4.5	14	+	3	4.5, 4, 4.3
11	5	1.5	9	+	0	0
12	5	3	12	+++	1	4
13	5	1.5	6	+	1	2
14	5	2.5	6	+	1	3
15	5	1.5	6	+	1	2

radiation in each case before selecting the appropriate dose [35]. According to the results of this study, the radiosensitivity of plants of the common bean of the variety Bribri was strongly influenced by the dose of radiation applied. With doses between 10 - 100 Gy, growth and survival *in vitro* plants decreased significantly as the dose was increased. This dosage-dependent negative effect has been previously reported for height in the common bean [36] [37] and other crops [38]. In one particular study in the common bean, growth (dry and fresh weight), color and friability of calli were influenced by the dose of radiation. High doses of 20 - 30 krad drastically reduced growth, and cell death occurred with 40 krad [36]. Radiobiological effects of gamma radiation on the common bean were reported to be different in seeds, plants and calli [37] [39].

In breeding by mutation, generally the frequency of mutations increases with dose and the rate at which the dose is applied, but the rate of regeneration *in vitro* is reduced [35]. In other crops, the use of low doses of gamma radiation has been reported to stimulate growth *in vivo* and *in vitro* [35] [40]. In *P. vulgaris*, low doses (0.5 Krad) stimulated growth (dry weight and fresh weight) and growth of *in vitro* calli [37].

After estimating the induction dose, the most appropriate dose to apply for treatment was determined. For the experimental protocol, Predieri and Virgilio [24] suggest the initial establishment of a dose corresponding to the $LD_{50\neq}$ 10%, where lower doses stimulate the recuperation of the plant after treatment, and higher doses increase the probability of inducing mutations (positive or negative). Based on results in our study of height increase and the range of the LD_{50} for survival, a dose of 20 Gy was chosen for the induction of mutations in EAs of *Phaseolus vulgaris* var. Bribri.

This dose was considered to be one that would allow possible mutants to be obtained without negatively affecting the development of the *in vitro* plants. The irradiation always resulted in problems in the development of some treated *in vitro* plants. The doses which results in 100% of *in vitro* plants with problems in development must to be discarded. This coincides with reports by Lu *et al*. [35] that the critical level of irradiation with which mutations can be induced may be within the range of tolerance for regeneration *in vitro*. Breeding with ionizing radiation and/or chemical mutation in plant tissue culture has been reported in several crop plants, including the common bean [36] [37] [39], potato [40], pear [41] and peanut [42].

In this study the filtrate fungus of *R. solani* was used in the *in vitro* cultures, because the fungus can directly be applied to plants in field selections, but cannot be applied to plantlets *in vitro* [20] [21]. This is because the fungus grows much faster than plant tissues *in vitro* culture conditions and dominates the culture media and flasks, and the growth of the plant tissue is impeded by a lack of nutrients or space, rather than due to suscepti-

bility to the disease [20] [21] [43]. That is the biggest problem of *in vitro* selection; the fungus does not attack *in vitro* tissues equally, thus allowing susceptible plantlets to escape from the selection pressure. To overcome this problem, techniques using several selection factors (agents) have been created, such as fusaric acid, culture filtrates of the pathogenic fungus and other substances that cause similar effects as the fungus attack [20] [43]. The fungus culture filtrates may contain a spectrum of fungus metabolites like polysaccharides, oligosaccharides, proteins, glycoproteins, unsaturated fatty acids, growth regulators as auxin, cytokinin and gibberellic acid, along with toxins that may play a role as co-determinants of pathogenicity during disease development [20] [22] [43]. The application of filtrates to cultures *in vitro* can elicit various defence responses, e.g., phytoalexins; activity of certain enzymes; accumulation of phenolic acids total phenols, peroxidases and beta 1,3-glucanase; and chitinase [20].

In our study, the activity of toxic compounds present in the *R. solani* filtrate was determined on susceptible control *in vitro* plants derived in comparison with non treated *in vitro* plants. Also, our results suggest that the AC liquid media used for grown the fungus has not negative effects on the common bean regeneration. One of the most evident morphological changes was the reduction in growth *in vitro* plants (height) and finally led to the death of explants as the concentration of fungus filtrate in BMS media was increased, specifically in the explants cultured with 100% of fungus filtrate. Then, the morphological effects observed in the EAs and reduction in the survival proportion of *in vitro* plants cultured under different concentrations of fungus filtrate might be caused by *R. solani*. One spectrophotometric method described by Morpurgo *et al*. [44] provides a faster way of determining both the biological effects and the filtrate concentration to be added in the media. They showed the inhibition of shoot growth of both susceptible and tolerant banana clones cultured in media with different concentrations of fungus filtrate [44]. This is in agreement with previous reports of the effect of halo toxins in filtrates of *Pseudomonas phaseolicola* [45], *Colletotrichum lindemuthianum* [46] and *Pseudomonas syringae* pv. *phaseolicola* [47].

Non-irradiated explants cultured in presence of fungus filtrate (maximum 30%) showed good growth (determined in height), but necrotic roots and in some cases the root system was absent, which made survival difficult after planting in the greenhouse. The culture filtrate of *R. solani* gave phenylacetic acid, a well-known plant growth regulator, and the acid could inhibit the growth of roots of seedlings at concentrations above 0.05 per cent (sugar beet), and 0.005 per cent (rape and rice plant respectively). Phenylacetic acid, however, did not cause necrosis, which was a characteristic phenomenon of the root rots [48]. Different responses *in vitro* have been reported for calli and plantlets (leaf necrosis, inhibition development of secondary roots, and eventually induced stunting of plantlets), which were influenced by plant genotype, metabolite composition and filtrate concentration [46] [47].

The combined effect of irradiation and then selection adversely affected growth (height and roots) and survival of the *in vitro* plants in the third subculture. This situation was a limiting factor, because it reduced the mutated *in vitro* plants transferred to greenhouse. In contrast to conventional *in vivo* cultivation, *in vitro* cultivation allowed the separation of mutated sectors from non mutated plants (quick dissolution of chimeras) in a short time (3 - 4 cycles of subculturing). This has been shown to increase the frequency of solid mutants compared to *in vivo* radiation [41]. The number of selection cycles used and the duration of the selection pressure by exposure to filtrate *in vitro*, are essential factors for the isolation of tolerant material, since more than three cycles of continuous selection *in vitro* can give rise to undesirable alterations such as polyploidy, reduced regeneration capacity [49] and could induce secondary mutations [43].

The results of this study suggest that the selection pressure applied using fungus filtrate during three subcultures may be sufficient to induce the variation necessary to obtain *in vitro* plants resistant to *Rhizoctonia solani.* In a common bean breeding program with the objective of early selection of lines resistant to *Rhizoctonia solani* using *in vitro* culture, it may not be necessary to combine the variation generated by irradiation with the selection technique. The use of purified or partially purified toxins, or crude fungus culture filtrates has been effectively used as a tool for selection for disease resistance for more than 30 plant species, including herbs and woody plants from different families and geographical regions [21].

Successful cases of breeding for resistance using only *in vitro* selection have been reported for barley resistant to *Helminthosporium sativum*, sorghum resistant to *F. solani*, soybean resistant to *Glycine max* and bean calli resistant to *Colletotrichum lindemuthianum* [21] [46]. In many cases, the sensitivity to the *in vitro* selective agent and the resistance response in the plants *in vivo* showed a correlation [21]. In addition, tissue culture can generate a resource of genetically stable variation, useful in plant breeding, similar to that induced by physical

and chemical mutagenesis [13]. It is important to note that the *in vitro* selection economizes time in the development of resistance to diseases, tolerance to minerals and abiotic stress. However, the mutants selected *in vitro* should be tested in the field to confirm the genetic stability of the selected characteristics [13].

5. Conclusions

The tools used in this study may offer a new approach for national breeders of the common bean for developing material resistant to diseases, not only *R. solani*. The induction of mutations by irradiation and/or the use of effective selection agents are a scientific and social alternative to plant genetic engineering, since the public is not as averse to these techniques as to plant genetic transformation [16] [20] [21] [50]. However, field evaluation of selected and/or irradiated materials is important since common bean mutants may be identified that present variations in other morphological characteristics of interest, not only resistance to *R. solani*. The mutagenic agents (gamma irradiation) could increase the variability in traits as yield, habit growth, resistance to diseases, premature, color of flowers and seeds, allowing the selection of plantlets with new traits for the commercial production [39].

This study has led to the following conclusions: 1) a dose of 20 Gy of gamma radiation is appropriate for mutagenesis of *in vitro* cultivated EAs; 2) by comparison of the different treatments (different radiation doses) and the control (not irradiated), it was observed that there were significant alterations in the survival and *in vitro* plant growth for all the gamma doses; 3) the results suggest that 20% fungus filtrate culture in the BMS media allowed to *in vitro* selection common bean plants; 4) however, the application of gamma irradiation and *in vitro* selection affected survival and the *in vitro* growth of plants with increased doses and concentrations, respectively; 5) Selection pressure applied using fungus filtrate during three subcultures may be sufficient to induce the variation necessary to obtain *in vitro* plants resistant to *Rhizoctonia solani*.

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Abbreviations

BAP: 6-Benzylaminopurine;
BMS: basal semisolid MS media with 4.44 µM BAP;
Gy: Gray (equivalent to 1 $J \cdot kg^{-1}$);
EA: embryonic axes;
MV_0: *in vitro* plant subculture mutated zero;
MV_1: *in vitro* plant subculture mutated one;
MV_2: *in vitro* plant subculture mutated two;
MV_3: *in vitro* plant subculture mutated three,
MV_4: plantlet in greenhouse.