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Induced mutagenesis in microbulbs of *Allium sativum* L.

Mutagénesis inducida en microbulbos de *Allium sativum* L.

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Abstract

A protocol for induced mutagenesis in garlic (*Allium sativum*) microbulb Boronó clone cultured *in vitro* was established. For this, two assays were performed: radio sensitivity assays were performed to determine the appropriate gamma radiation dose and mutagenesis assays to determine the behavior of the plant material up to storage stage. In order to determine radio sensitivity, grown microbulbs were irradiated with four gamma ray doses (6, 8, 10 and 12 Krad) plus control. Optimal dose was established considering 50% microbulb survival (LD_{50}). For this assay a randomized block design with 5 treatments and 20 replications per treatment was used. After mutagenic treatment, microbulbs were irradiated with 8 and 10 Krad and then stored at 10 °C in darkness for 45 days. In this assay a randomized block design with 3 treatments consisting two doses (8 and 10 Krad) plus control and 20 replications per treatment was used. In both trials, higher mean weight and diameter was observed in microbulbs irradiated with 8 and 10 Krad, suggesting that gamma irradiation of 8 and 10 Krad should be applied in order to facilitate mutant production with desirable agronomic traits in the clone Boconó.

Key words: Tissue culture, bulb induction, *Allium sativum*, gamma irradiation, radio sensitivity.

Resumen

Se estableció un protocolo de mutagénesis en microbulbos de ajo (*Allium sativum* L.) clon Boconó cultivado in vitro. Para el efecto se realizaron dos ensayos, uno de radiosensibilidad para establecer la dosimetría apropiada de radiación gamma y otro de mutagénesis para determinar el comportamiento de los materiales hasta la etapa de almacenamiento. En el primero los microbulbos fueron tratados con cuatros dosis de radiación gamma (6, 8, 10 y 12 Krad), más un control. Para establecer la dosis óptima se consideró la sobrevivencia del 50% de los microbulbos (DL_{50}). Se empleó un diseño de bloques al azar con cinco tratamientos y 20 repeticiones por tratamiento. En el ensayo mutagénico los microbulbos fueron irradiados con 8 y 10 Krad y almacenados durante 45 días a 10 °C en condiciones de oscuridad En este caso se utilizó un diseño de bloques al azar con tres tratamientos (0, 8 y 10 Krad) y 20 repeticiones por tratamiento. En ambos ensayos, los microbulbos irradiados con 8 y 10 Krad registraron los mayores promedios para peso y diámetro, lo cual permite concluir que estas dosis son adecuadas para favorecer la producción de mutantes con características agronómicas deseables en el clon Boconó.

Palabras claves: Cultivo de tejidos, ajo, *Allium sativum*, bulbificación, radiación gamma, dosimetría.

Introduction

Garlic (*Allium sativum* L.) is a widely used product because of its cooking and medicinal properties (Brewster, 2008). During the domestication process of the species the selection towards vegetative development and bulb yield has been favored at the expense of the reproduction system and flower formation in the plants. This explains why most of the garlic cultivars developed worldwide are strictly for asexual or vegetative propagation, being this one the only production system for commercial use, limiting the selection to use the preexisting genetic variability, induced mutations and tissue culture techniques as strategies to get new variants. (Zheng *et al.*, 2007; Matijevic *et al.*, 2013). Mutagen application implies the evaluation of biological effects produced on a specific cell or tissue, therefore, factors such as mutagen type, radiation dose used, and size and origin of the explants and their radio-sensitivity have to be considered (Taner and Kunter, 2004). Since the response to a specific mutagen is mainly measured in function of the survival rate and the regeneration rate and/or multiplication of the irradiated material, it is needed to determine the radiation dose that allows survival of the material for later selection of the mutants with the desired characters. Ahloowalia and Maluszynski (2001) performed radiosensitivity assays in order to establish the radiation range that can be applied to determined crop. Among the physical mutagens, gamma rays are used to get variability in the agricultural crops, also to reduce post-harvesting losses, avoid early sprouting, reduce contamination, control pest and diseases, extend the shelf life of products, etc. (Piri *et al.*, 2011). In this field, the gamma rays are considered as ionizing radiation obtained mainly by radioactive isotopes with cobalt 60 (^{60}Co) and cesium 137 (^{137}Cs) as main sources of radiation; the most common unit used to measure the radiation exposition are Grays (Gy) or their equivalents the rads (1 rads = 0.01 Grays) and/or the Krad (1 Krad = 1000 rads) (IAEA, 2005). For the garlic crop, research has been done using bulbs treated with gamma radiation in order to improve characteristics such as disease resistance (Al–Safadi *et al.*, 2000; Nabulsi *et al.*, 2001), inhibition of sprouting (Croci *et al.*, 1987; Taner and Kunter, 2004; Pérez *et al.*, 2007), weight gain (Croci y Curzio, 1983), storage durability (AL–Safadi, 2000), morphological changes (Pellegrini *et al.*, 2000; Wi *et al.*, 2007) and flavor modifications (Ceci *et al.*, 1991), among other characteristics. On the other hand, combination of techniques such as tissue culture and mutagenesis have facilitated, in several agricultural crops, the regeneration of a high number of desirable variants, development of materials free of pests and diseases and the possibility to irradiate a high number of explants (Zhen, 2001; Zheng *et al.*, 2007). Due to the above reasons, this study aimed to develop a protocol for *in vitro* mutagenesis in the Boconó clone of *A. sativum*, through techniques of tissue culture and gamma rays irradiation.

Materials and methods

Establishment of the *in vitro* culture

The initial and later stages for bulb propagation of *A. sativum* by *in vitro* culture were done in the Laboratory of Biotechnology of the Postgraduate in Horticulture of the Universidad Centroccidental Lisandro Alvarado (UCLA), in Tarabana, State of Lara, Venezuela. At the start, the shoot apex were grown in Murashige and Skoog medium (MS) with 0.1 mg/l naphthalene acetic acid (ANA) and 0.5 mg/l 2-isopentenyladenine (2ip). To reach the propagation phase (bulb formation), the buds coming from the shoot apex culture were grown in MS media with 2 mg/l 2ip, 90 g/l sucrose and 8 g/l agar according to the protocol of Mujica *et al.* (2008). For the radiosensitivity and mutagenesis assays garlic microbulbs of the Boronó clone, with weight between 0.5 and 0.8 g and equatorial diameters of 0.5 and 1 cm were used.

Radiosensitivity assay

The experiment was performed at the Unit Pegamma of the Venezuelan Institute of Scientific Research (IVIC) located in Altos de Pipe, los Teques, State of Miranda, Venezuela. Microbulbs were placed at 75 cm distance and 8 cm height of the gamma radiation source (^{60}Co). The power and velocity of the source was 9813 Curie and 195 Krad/h, respectively. According to these values, five doses of radiation were applied: 0, 6, 8, 10 and 12 Krad, corresponding to exposure times of 0, 1 min 50 s, 2 min 28 s, 3 min 41 s, and 4 min 16 s, respectively.

Once irradiated, the microbulbs were transfered to a fresh MS medium for bulb formation with 2 mg/l 2ip, 90 g/l sucrose and 8 g/l agar, with two subcultures at 45 and 90 days. In both timings (age) variables evaluated in the microbulbs were: dry weight (g), equatorial diameter (cm) and polar diameter (cm). At the end of

the experiment, the survival percentage according to the scale 1 – 0, where 1 = viable/turgid microbulbs and, 0 = inviable/dry microbulbs was determined (Figure 1). To determine the radiosensitivity of the materials the optimal dose was established as the one that allows the survival of 50% of the microbulbs (LD_{50}).

Figure 1. Model for survival used in garlic microbulbs. Left = viable/turgid bulbs and right = unviable/dry bulbs.

Mutagenesis assay

Once the radiosensitivity was determined, the microbulbs coming from the *in vitro* culture selected based on their weight (0.5 to 0.9 g) and equatorial diameter (0.8 to 1 cm), were treated with 8 and 10 Krad of gamma radiation following the protocol previously described.

Once irradiated, the microbulbs were transferred to fresh MS bulb formation medium with 2 mg/l 2ip, 90 g/l sucrose and 8 g/l agar, with two subcultures at 45 and 90 days to evaluate in the microbulbs the variables dry weight (g), equatorial diameter (cm) and polar diameter (cm). Once the *in vitro* culture stage was finished, the microbulbs were placed in plastic trays 20 x 10 x 10 cm with lid before storing them at 10 °C in the darkness for 45 days, after that the variables dry weigh (g), equatorial (cm) and polar diameter (cm) of the microbulbs were measured again. Both, during the bulb formation stage, and in the storage stage, a completely randomized blocked design with three treatments (0, 8 and 10 Krad), 20 replicates per treatment and one microbulb per test tube as experimental unit were used.

Data analysis

Survival data were analyzed by non-parametric method (Krustal-Wallis, 1952) with a multiple range mean comparison test, indicating their corresponding means; whereas for weight and diameter of microbulbs the Tukey{s test was applied. In all the cases, the significance level was 5%. For the analysis the Infogen v. 2012 statistical software was used.

Results and discussion

Establishment of the *in vitro* culture

Initiation happened in 98% of the apices grown on MS medium with 0.1 mg/l ANA and 0.5 mg/l 2ip, allowing the development of only one bud per test tube, height averages were between 4 and 4.6 cm. On the other hand, the microbulb formation from buds grown on MS supplemented with 2 mg/l 2ip and 90 mg/l sucrose, allowed the development of only one microbulb per test tube, showing the efficiency of the regeneration protocol used for the *A. sativum* microbulbs.

Radiosensitivity assay

During the determination of the radiosensitivity to the gamma radiation by the *A. sativum* microbulbs, both the controls and the irradiated bulbs with 6, 8 and 10 Krad showed survival percentages above 50% (LD_{50}), whereas the ones treated with with 12 Krad had a lower percentage (320%) at the end of the experiment (Table 1). The similar behavior of the 6, 8 and 10 Krad gamma radiation treatments vs. control, indicate that they favor the development and/or microbulb growth of garlic Boconó clone.

Table 1. Survival average of the garlic (*A. sativum* L.), microbulbs treated with gamma radiation.

Treatment (Krad)	Survival (%)
0	100 a*
6	70 ab
8	70 ab
10	55 bc
12	20 c
KW	18.5

* Values followed by similar letters do not differ statistically according to Krustal-Wallis test (P > 0.05).

Al–Safadi *et al.* (2000); Nabulsi *et al.* (2001); Zheng *et al.* (2007) and Wi *et al.* (2007) found in *A. sativum* cloves that the optimal doses of gamma radiation varied between 0.1 to 0.7 Krad. For the materials coming from tissue culture, Zhen (2001) found that an optimal range between 0.5 and 1 Krad on gamma ray irradiated calluses. In other researches (Croci and

Curzio, 1983; IAEA, 1997; Taner and Kunter, 2004 and Pérez *et al.*, 2007) were found that doses between 3 and 9 Krad stimulate the development and/or growth of formed bulbs from *A. sativum*, indicating that each type of structure, cloves, microbulbs or bulbs, requires a specific radiation dose for survival and later regeneration.

The microbulb dry weight analysis showed differences ($P < 0.05$) among the treatments. In this case, by using the Tukey´s test it was possible to make three groups at 45 days (Table 2). The microbulbs irradiated with 8 Krad presented the highest average value followed by the 10 and 12 Krad treatments. To the contrary, the control microbulbs and the ones irradiated with 6 Krad had the lowest values for this parameter. In comparison to the control, the irradiated materials with 8, 10 and 12 Krad had a weight gain of 0.35, 0.18 and 0.18 g, respectively.

Table 2. Dry weight (g) average of garlic (*Allium sativum* L.) microbulbs at 45 and 90 days after gamma ray irradiation.

Treatment (Krad)	45 days (g)	90 days (g)	Difference (g)
0	0.64 b*	0.83 c	0.19
6	0.63 b	0.66 c	
8	0.99 a	1.38 a	0.39
10	0.82 ab	1.11 b	0.29
12	0.82 ab	0.76 c	
CV (%)	29.63	27.81	

* Values followed by similar letters do not differ statistically according to Tukey´s test ($P > 0.05$).

Ninety day after the mutagenic treatment the Tukey´s test allowed the conformation of three groups, with a maximum observed average in the microbulbs irradiated with 8 Krad, followed by the 10 Krad treatment. In the last group were found the control microbulbs and the ones irradiated with 12 and 6 Krad. Similarly as in the previous case, the materials irradiated with 8 and 10 Krad had a weight gain of 0.55 and 0.28 g, in comparison with the control.

In the materials treated with 6 Krad the weight gain was low (0.03 g) and in the ones irradiated with 12 Krad there was a reduction of 0.06 g. Pellegrini *et al.* (2000) and Wi *et al.* (2007) found that low radiation doses (6 Krad) do not affect the radiated materials, whereas high doses (12 Krad) drastically reduce the development and growth due to alteration in the mitosis process in damaged cells. Wi *et al.* (2007) determined that high radiation doses can affect cells, tissues and also organelles such as chloroplast. It is also widely known that the gamma radiations frequently produce both, genetic and chromosomic, mutations (Donini, 1997), that not only inhibit cell division processes but, also the multiplication and growth or development of tissues.

In this study, the equatorial diameter of the microbulb did not show differences ($P > 0.05$) for the treatment effect; which was opposite to the observations for the polar diameter (cm) at 45 and 90 days of evaluation (Table 3). In this case, at 45 days, the Tukey´s test showed three groups, with a maximum observed value in the materials that were irradiated with 8, 10 and 6 Krad. In relation with the control treatment, the microbulb elongation varied between 0.13 and 0.16 cm. Ninety days after irradiation there were four groups formed for the same variable, they correspond to the microbulbs treated with 8, 10, control and 6 and 12 Krad. It is important to highlight that the materials treated with 8 and 10 Krad grew in length 0.27 and 0.21 cm, respectively, in comparison to the control.

Table 3. Equatorial and polar diameters of garlic (*Allium sativum* L.) microbulbs 45 and 90 days after gamma ray irradiation.

Treatment (Krad)	Equatorial diameter (cm)		Polar diameter (cm)	
	45 days	90 days	45 days	90 days
0	1.00 a*	1.09 a	1.28 ab	1.43 bc
6	1.02 a	1.02 a	1.41 a	1.38 c
8	0.99 a	1.15 a	1.44 a	1.70 a
10	0.98 a	1.10 a	1.42 a	1.64 ab
12	0.92 a	1.05 a	1.19 b	1.32 c
CV (%)	15.56	18.80	16.59	16.82

* Values followed by similar letters do not differ statistically according to Tukey´s test ($P > 0.05$).

At the same age of evaluation, there was more elongation on the materials irradiated with 8 and 10 Krad (0.26 and 0.22 cm), in comparison to the control, which was not observed in the in the 6 and 12 Krad treatments. These results indicate that the application of 8 and 10 Krad of gamma radiation stimulated the mitotic activity in the apical buds located at the base of the stem, leading the bulb formation process towards elongation and/or reserves production for development or later emergence of bulbs in the detriment of the volume or thickness of the microbulbs.

Mutagenesis assay

In this experiment the analysis for the microbulb weight did not show differences 45 days after irradiation (P > 0.05); however, at 90 days there were differences at this probability level. According to this result the Tukey´s test allowed the grouping in two, observing the highest averages with 10 Krad use, followed by 8 Krad and the control (Table 4). In this case, the irradiated microbulbs with 8 and 10 Krad had a weight gain of 0.24 and 0.88 g, respectively.

Table 4. Dry weight of garlic microbulbs (*Allium sativum* L) 45 and 90 days after gamma radiation treatment.

Treatment (Krad)	Dry weight (g)	
	45 days	90 days
0	0.63 a*	0.86 b
8	0.66 a	1.10 b
10	0.75 a	1.74 a
CV (%)	6.53	12.64

* Values followed by similar letters do not differ statistically according to Tukey´s test (P > 0.05).

The results in Table 4 show that between 45 and 90 days the microbulbs showed weight gains of 0.44 and 0.99 g, when irradiated with 8 and 10 Krad, respectively, whereas in the control the gain weight was 0.23 g. Pellegrini *et al.* (2000) and Al–Safadi *et al.* (2000), reported minimum weight loss in garlic bulbs irradiated with gamma rays in comparison to the control. Similarly, the "Rosado Paraguayo" and "Red" of *A. sativum* experimented a minimum weight loss when where irradiated with 5 and 3 Krad of gamma rays, respectively (IAEA, 1997). For the equatorial and polar diameters there were no differences at the 45 and 90 days between the treatments and the control (Table 5). For the results already mentioned and for practical reasons, the 8 and 10 Krad doses were selected as optimal doses for garlic microbulbs, since with those doses the penetration in the tissues is granted (Donini, 1997; IAEA, 1997; Piri *et al.*, 2011).

Table 5. Equatorial and polar diameter of garlic microbulbs (*Allium sativum* L.) 45 and 90 days after gamma irradiation.

Treatment (Krad)	Equatorial diameter (cm)		Polar diameter (cm)	
	45 days	90 days	45 days	90 days
0 Krad	1.22	1.49	1.10	1.45
8 Krad	1.35	1.53	1.26	1.50
10 Krad	1.19	1.48	1.19	1.48
CV (%)	22.68	16.36	18.61	14.94

* Values followed by similar letters do not differ statistically according to Tukey´s test (P > 0.05).

During the microbulbs storage stage under *ex vitro* conditions (10 °C and darkness) there were differences (P < 0.05) for the dry weight and diameter variables, observing the highest averages with the use of 10 Krad, followed by the 8 Krad and the control (Table 6).

Table 6. Dry weight, equatorial and polar diameter of garlic microbulbs (*Allium sativum* L.) after 45 days of storage at 10 °C in the dark.

Treatment (Krad)	Dry weigth (g)	Equatorial diameter (cm)	Polar diameter (cm)
0	0.72 b*	1.09 b	1.27 c
8	0.96 b	1.35 a	1.62 b
10	1.64 a	1.34 a	1.98 a
CV (%)	12.59	25.10	20.90

* Values followed by similar letters do not differ statistically according to Tukey´s test (P > 0.05).

These results show the advantages of the storage conditions on garlic in this study and agrees with the results of Donini (1997) on the importance of evaluating the periods and storing temperatures in the materials subjected to mutagenesis treatments.

Conclusions

With the use of gamma radiation, the garlic microbulbs as pre-basic seed, can be directly carried to controlled greenhouse conditions, reducing the input and labor costs required for the regeneration by other culture techniques.

The protocol has the additional advantage since it facilitates the selection and maintenance by vegetative means of solid or stable mutants, with the desirable characteristics in short time (1 – 2 years).

A protocol for *in vitro* mutagenesis in the Boconó clon of *A. sativum* was established. In this way, the radiation in the microbulbs coming from tissue culture with 8 and 10 Krad doses, favors the development and growth of the garlic microbulbs that after a selection process can be part of genetic breeding programs in production of basic seeds of *A. sativum*.

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