INDIRECT SOMATIC EMBRYOGENESIS ON MUTANTS OF *Agave tequilana* WEBER CULTIVAR BLUE INDUCED WITH CO^{60} GAMMA RAYS†

[EMBRIOGÉNESIS SOMÁTICA INDIRECTA EN MUTANTES DE *Agave tequilana* WEBER VAR. AZUL INDUCIDOS CON RAYOS GAMMA CO^{60}]

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SUMMARY

Background: Blue agave (*Agave tequilana* Weber var. azul)**,** it is commonly known as "Agave azul or agave tequilero". The Agave crop is fundamental part of the productive chain Agave-Tequila, since is the distinctive source of reducing sugars for the elaboration of the liqueur known as Tequila, according with the Appellation of Origen Tequila to fulfill the National Normativity (NOM-). Besides, Tequila is the most spirit drink exported from Mexico worldwide. According with the latest evaluation over this crop, the susceptibility to pathogens generate in cases total lost of the agave fields. **Objective.** to achieve somatic embryos that could be resistant, generated from plantlets irradiated with Co^{60}gamma rays; which were obtained from axillary buds. **Methodology:** The arisen of embryo was reached in the modified Murashige and Skoog (MS) medium supplemented with of 2,4-D combined with BA or KIN. The plantlets were radiated 12 weeks after the somatic embryo induction with Co^{60} gamma rays. The doses were 0 (test control), 10, 20 and 30 Gy. **Results:** There was a significant difference in the induction of embryonic callus, development and mature somatic embryos, which were reduced as the doses of radiation increases. Since only somatic embryos were achieved in tissues treated with 10 and 20 Gy doses, in higher doses induction of embryonic callus was inhibited. The regression confirmed the negative results with the increase of radiation. **Implications:** There were made protocols to achieve somatic embryos and plants from tissues irradiated with 10 and 20 Gy. **Conclusions**: The radiation effect over somatic tissue was crucial as the doses affect the conversion into plantlets, nevertheless such radiation allowed the conversion of the embryos to complete agave plantlets

Key words: Agave; tissue culture; somatic embryogenesis; mutagenesis.

RESUMEN

Antecedentes: El agave azul (*Agave tequilana* Weber var. azul), es comúnmente conocido como "Agave azul o agave tequilero". El cultivo del Agave es parte fundamental en la cadena productiva Agave-Tequila, ya que es la Fuente de azúcares reductores que distinguen la elaboración del licor conocido como tequilla, de acuerdo con la Denominación de Origen del Tequila que cumple con la Norma Oficial Mexicana (NOM-). Además, el Tequila que es la bebida alcohólica que más se exporta desde México a todo el mundo. De acuerdo con las últimas evaluaciones de este cultivo, la susceptibilidad a patógenos genera en algunos casos la pérdida total de los campos de agave. **Objetivo.** Obtener embriones somáticos que pudieran ser resistentes, a partir de plántulas irradiadiadas con rayos gamma Co^{60}; que se obtuvieron a partir de yemas axilares. **Metodología**: Los embriones se obtuvieron en medio modificado de Murashige and Skoog (MS) suplementado con 2,4-D combinado con BA y KIN. Las plántulas se irradiaron a las 12 semanas después de la siembra con rayos gamma Co^{60}. Las dosis fueron 0 (tratamiento control), 10, 20 and 30 Gy. **Resultados:** Hubo diferencia significativa en la inducción de los callos embriogénicos, Desarrollo y maduración de los embriones somáticos, los que se redujeron conforma la dosis de radiación se incrementó. Los embriones somáticos se obtuvieron

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en el tejido tratado con 10 y 20 Gy, ya que en dosis mayores la inducción de callo embriogénico fue inhibida. La regresión confirma los resultados negativos cuando se incrementa la dosis de radiación**. Implicaciones:** Se estableció el protocolo que permitió obtener embriones somáticos y plántulas a partir de tejido irradiado con dosis de 10 y 20 Gy. **Conclusiones:** El efecto de la radiación sobre el tejido somático fue crucial de como las dosis afectaron la conversión en plantas, sin embargo, esas radiaciones permitieron la conversión de los embriones en plantas completas de agave
Palabras clave: Agave; cultivo de tejidos; embriogénesis somática; mutagénesis.

INTRODUCTION

The crop of Tequila's blue agave (*Agave tequilana* Weber var. Azul) has a great economic and social importance due to the plant fields in the zone of Appellation of Origin Tequila (DOT for its initials in Spanish). Besides, many people depend of the generation of jobs into the production chain known as Agave-Tequila from plants to bottled Tequila, in Mexico or in abroad (González *et al*, 2004). Traditionally, the blue agave has been cultivated for more than three centuries in Jalisco State; now in the States that are allowed to produce Tequila (in the zone of Appellation of Origin), however the culture method continues low technified, as well the most industrialized method applied until today. There is not a project via conventional genetic breeding, means sexual reproduction to find variation in the progeny. Contradictorily, the asexual propagation traditionally has increased undesirable clonation of the few selected genotypes, since is decreasing the genetic variation into the blue agave resource, which is considered as cause of the incidence and spreading of diseases (Rodríguez, G.1995). The same author assures that the conventional genetic improvement in *Agave tequilana* make complications since reproductive flowering and seed structure, polyploidy, life cycle from 6 to 12 years, short time of blooming and difficulties for breeding manipulation. So, this give an opportunity to apply biotechnology tools as the plant tissue *in vitro* culture, somatic embryogenesis method joined with manipulation of the actual genetic variability, in order to reach massive propagation protocols of desirable blue agaves, with better agronomic and industrial gains.

There are molecular researches with the purpose to determine the genetic 56 variability among agave phenotypes. Gil-Vega *et al*. (2001) reported studies among 40 field selections of *A. tequilana cultivar blue*, only 124 random amplified polymorphic DNA (RAPD) products (0.8%) were polymorphic and 39 of 40 were completely isogenic. This is one of the lowest levels of polymorphism detected to date for the analysis of a crop species. The asexual reproduction that is carried out, since the stolons naturally produced by the plant, tend to be a clone of the genotype which it comes from, and only the variability is caused by spontaneous mutations that are presented in the somatic tissue in the form of chimeras (González *et al*., 2007). The species that have asexual process as main way to propagate as blue agave, make more difficulties for genetic improvement; where the induction of mutations is a desirable process in order to reach genetic variation. One of this method is the exposition of somatic tissue to ionizing radiation (Robles, 1986).

Somatic embryogenesis is the desirable *in vitro* culture method since the potential application in clonal propagation, among other aims as genetic edition, genetic transformation or embryogenesis research. This biotechnology tool has been applied in plants of economic interest, and also shows great results to achieve massive somatic embryos of forest tree plants, particularly conifers (Ramírez-Serrano 2005 add). The somatic embryogenesis is a tool that allows clones with a normal ontogenesis, which also give rise a root system of high anchoring; moreover, this technique allows plant breeding potential, exchange and germplasm preservation (Medina *et al*., 2007).

At this time, there are several protocols to assure massive propagation of blue agave as Fernanda 1997 y Portillo et *al*. (2000), Rodríguez-Garay *et al*. (1996) and Martínez-Palacios *et al*. (2003) reported somatic embryogenesis in *A. Victoria-reginae* Moore, generated from both, cell of young leaf and segments of stem. In the other hand, the axillary bud stimulation is a developed technique according with the results of Santacruz-Ruvalcaba *et al*. (1999) in *A. Parrasana* as an example.

The importance of this research is based on three aspects, 1) Somatic embryogenesis generate plants that arisen from one single cell that means without the fusion of gametes (Tisserat *et al*., 1979). 2) Ionizing radiation in low doses induces randomly DNA mutation in each cell; and, 3) the mutations that were randomly induced in that single cell, on which will give rise an unique embryo that potentially becomes into plant, with unique characteristics, preserving the particular characters of the *Agave tequilana* cultivar blue according with the DOT normativity.

The objective of the research was to develop a somatic embryogenesis protocol to achieve plantlets of blue agave (*Agave tequilana* Weber cultivar blue) from micropropagated plantlets that were obtained from axillary buds and irradiated with Co^{60} gamma rays.

MATERIALS AND METHODS

Induction of embryogenic callus *in vitro*

Biologic Material. The explants were obtained from *in vitro* plantlets of agave developed from axillary buds with 12 weeks of growth and a size from 8 to 10 cm. Full plantlets were irradiated with Co^{60} gamma rays, acquiring the mutant generator M_1V_1.

Plantlets Irradiation with Co^{60} gamma rays

In the "National Institute of Nuclear Investigation's" (ININ for its initial in Spanish). The radiation protocol was applied by a Gammacell radiator Model GO-220 (Ontario, Canada), *in vitro* plantlets of blue agave were treated with Co^{60} gamma rays: 0 Gy (control), 10 Gy, 20 Gy, and 30 Gy based on the lethal doses (DL_{50}) reported by (Angeles-Espino *et al.*, 2013); assuming that the probability of inducing positive mutations increases (Morela, 2002). The radiation of the *in vitro* plantlets was fully applied before the induction of embryogenic callus.

Establishment of *in vitro* culture and induction of blue agave somatic embryos

The media culture was a MS (Murashige and Skoog, 1962) modified and supplemented with vitamins (Phillips and Collings, 1979), modified ammonium to nitrate ratio 10:90 (Ramirez-Serrano, 2003), 13.57 µM of 2,4-Dichlorophenoxyacetic acid (2,4-D), 8.87 µM of Benzyladenine (BA), 9.27 µM of Kinetin, 30 g L^{-1} of sucrose as carbon source, and 10 g L^{-1} of agar in order to provide cell and tissue support (Soltero *et al*, 1999 and Angeles-Espino *et al*, 2018). In the laminar flow chamber (VECO, U.S.A.) the flasks with plantlets were opened, then leaves from them were cutted above the Petri dishes previously sterilized, the explants were square cutted and transferred into induction medium, the squares of leaves were of approximately 0.5 cm^2, and six of them were placed into each container; three repetitions by treatment. Finally, the flasks were sealed and labeled properly before all them were placed in the growth chamber. The culture conditions were at 24° C ± 3° C and 16 h lighting with intensity to 1000 lux and 8 h dark.

Treatment for maturation of somatic embryos

Once the callus reached the globular stage in 28 days after their induction, the embryogenic masses were transferred into the same modified MS medium lacking of growth regulators, supplemented with 500 mg L^{-1} of casein hydrolyzed, 250 mg L^{-1} of glutamine, 40 g L^{-1} of sucrose and, 10g of agar in order to promote maturation of the somatic embryos.

Evaluated variables and statistical analysis

Induction of embryogenic callus

A simple experiment was designed as completely random distribution with four treatments (0, 10, 20 and 30 Gy) and three replications (Steel and Torrie 1960, and Reyes 1978). The experimental unit consisted in three flasks per treatment; six irradiated explants were sowing in each flask (18 in total). The data were token after 14, 21, and 28 days of culture.

The explants were the embryogenic callus, then as soon it appeared was register the date of it and the number of explants under that condition. For the second one data, the appearance of embryogenic callus in the new explants were noted too, and correspondingly were performed for the third. The statistical analysis was evaluated with the total of the explants that generated callus until the 28th day, they were processed via variance analysis ($\alpha = 0.01$) by means of orthogonal components to determine the differences among treatments.

Callus culture

This evaluation was carried on under the completely random design with six replications. The experiment was carried out with only two explants per container, which means two callus per sample. The callus development was generated after 14 days that was the first data noted, and successively two additional measurements after 21 and 28 days. The experimental unit was the callus mass per replication; considering the procedure to measure weight, which was followed up in the three times (14, 21 and 28 days) as it was carried out.

In the laminar flow chamber the callus of each treatment was transfer from container to petri boxes (previously tare), and weight was measured with an analytical balance, following all callus were transfer to container. To determine the net growth of calluses in seven days, in the second weight (21 d), it was reduced the weight of the previous measure (14 d), and in the same way to the third weight (28 d) it was reduced the weight of precious measure (21 d). The statistical analysis was made by variance analysis and orthogonal components for the different treatments. Besides, the regression components were achieved, as well as the correlation between the radiation doses and the weight of the callus.

Development of somatic embryos

The data collected was only from the explants that induced and developed embryogenic callus, consequently mature somatic embryos. The

experimental design was performed with three treatments of radiation and six replications each. A variance analysis was performed, and the treatments were compared through orthogonal components, as well as the correlation analysis and the simple lineal regression to establish the effect of radiation doses over the regeneration of somatic embryos that were capable to became into plants.

Plantlet conversion

Only the somatic embryos which developed as plantlets were consider into the evaluating data, maintaining the experimental design with three radiation doses and six replications each. A variance analysis of the design was performed, also the effect done by radiation were compared through orthogonal components. Finally, the percentage of plantlets was determined from each treatment.

The statistical analysis was performed by the MINITAB-16 program.

RESULTS AND DISCUSSION

Induction of embryogenic callus

The induction of embryogenic callus was achieved after the period of culture (Table1). Respectively, in the control treatment in the second week (14 days), was observed 11 out 18 explants which correspond to 61%, while in irradiated explants with 10 Gy was obtained 5 explants (28%) and 20 Gy 4 explants (22%). This result has shown the proportionally effect of the absorbed radiation by the somatic tissue, which are detailed below according to the statistical analysis that were made. The treatment with 30 Gy doses was lethal for the somatic cells.

Moreover, on the third week (21 days), in the test control three more explants had the induction of embryogenic callus; also, was observed in three explants treated with 10 Gy, and only two explants with 20 Gy doses. This data shown that more of 50% of the explants generated embryogenic callus after two weeks in induction medium (**Table 1 and Figure 1)**. Them on the fourth week it was observed 16 out of 18 explants untreated (test control), which correspond to 89%, while in the irradiated explants with 10 Gy was obtained 61%, and with 20 Gy only 44%. The highest induction of embryogenic callus was obtained 14 days in all treatments, while there was not statistic difference in second and thirty measure (figure 2 A and B).

The induction of embryogenic callus coincides with the report of González *et al.* (2005); they induced the embryogenic callus in leaf blades of sweet potato (*Ipomoea batatas* L. Lam.) in cells of the perivascular parenchyma which developed from germinated zygotic embryos at 21 days in induction medium, with the rupture of the epidermis, which permitted the emergence of the bipolar cells.

The evaluation of cell competence after radiation showed a significant difference ($P<0.01$) among treatments, as increases irradiation treatment in the explants that were irradiated with 10 and 20 Gy, so decrease proportionally such competence, which means less induction of somatic embryogenesis until it is inhibit at 30 Gy radiation dose. Similar results were reported by Kleiffer *et al.* (1985) in *Euphorbia pulcherrina* somatic embryos, such embryos were irradiated with X-rays with doses of 10 to 60 Gy, where the survival rate decreased as the dose increased, from 50% with 30 Gy (DL_{50}) to 11% with 60 Gy.

The regression analysis applied to this evaluation demonstrates the radiation doses (X) and the explants that induced embryogenic callus (Y) have shown a highly significant difference in the lineal behavior, and significant in the quadratic component. The correlation is an indicator of the grade of association between the radiation doses effect on the cell´s DNA structure (mutations), which confirms the lineal behavior. In the other hand, the quadratic element demonstrates the mutations are randomly acquired and the changes are expressed by active or inactive genes mutated for the effect of radiation in each cell, in which differs from one to another.

Table 1. Number of explants and percentage values in the induction of embryogenic callus at the 14th, 21st and 28th days after culture.

	14 d		21 d		28 d		Total		
	No. of Explants	%	**No. of Explants**	%	**No. of Explants**	%	Total	S. Dev.	%
0 Gy	11	61.11	3	77.78	2	88.89	16	± 4.93	88.89
10 Gy	5	27.78	3	44.44	3	61.11	11	± 1.15	61.11
20 Gy	4	22.22	2	33.33	2	44.44	8	± 1.15	44.44
30 Gy	0	0.00	0	0.00	0	0.00	0	± 0.00	0.00
Total	**20**	**37.04**	**8**	**14.81**	**7**	**12.96**	**35**		**64.81**

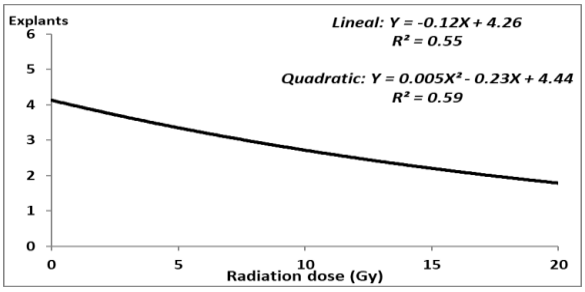

Figure 1. Correlation, lineal and quadratic regression between radiations doses (Gy) and number of explants with embryogenic callus induction.

As somatic embryogenesis requirement, the nucleus must be reprogramed in order to change the physiology pathway to generate a new organism. So, is clear the induction was decreased by the effect of the radiation into the cells by the mutations individually acquired; however, the somatic embryos can be achieved from the irradiated tissue with 20 Gy or minor doses, also the effect coincides with the reductive doses medium (GR_{50}) reported for Angeles-Espino *et al* (2013). Morela, (2002) and Valdez (2004) report mean lethal dose (LD_{50}) at 30 Gy in calluses irradiated with Gamma Rays Co^{60}, increasing the probability of obtaining benefic mutations to induce resistance to rust *Puccinia melanocephala* in sugarcane

By the evaluation of the induction of somatic embryogenesis, obviously it was found that the best result in the control treatment after 14th days of culture, where were stimulated 11 out of 18 explants, while in the irradiated treatments, the induction was drastically diminished, with only five explants in a doses of 10 Gy, and four in 20 Gy; however the data analysis demonstrate no differences in induction by the radiation doses; however the quality of the plantlets was the evaluated key factor to assume the consequences of that effect in cell competences.

Callus development

The evaluation of radiation on embryogenic callus development is shown in **Table 2**. The data of cell division (embryogenic callus) demonstrates that callus growth continued throughout incubation period, increasing net weight in 18% in period of seven days, from the first measurement (14 days) to second measurement (21 days) in control treatment, 11% in 10 Gy and 9% in 20 Gy doses respectively. While higher weight increased was in the third measurement that include a period from 21 to 28 days, overtaking 31% of weight in the control, 18% in 10 Gy and 14% in 20 Gy.

At 28 days that was into culture conditions. The analysis demonstrates the growth of damaged and healthy cells, where was found a significative difference of 95% among treatments, which means increases of each 10 Gy give rise mutations that affect both cell totipotence and competence. Results indicate that the mutations acquired, give a lot of changes in cell division capacity, which increases with radiation doses (10 Gy and 20 Gy). With basis on the weight that the test control reached on the 28th day (85.5 mg), it was taken as the 100% of development, so the growth in the 10 Gy dose was of 48.65% (GR_{50}) with regard of the control, while in the 20 Gy doses the average development of the callus reached the 38.12%, which explains the statistic difference between treatments (**Table 2**). The values of reduction in volume that were presented in the development of calluses, was due to the fact that it is a tissue made up of embryogenic cells with a high capacity for differentiation (morphogenesis) and are very susceptible to changes, especially when DNA is altered by have a physiological and cytological effect. in in addition to the fact that embryogenic cells genetically have a bipolar structure, however any changes in DNA would be expected by losing the differentiation capacity that characterizes the bipolar cells, which explains the high rates of decay that still occur in doses 10 Gy and 20 Gy. (Freire, 2003).

Table 2. Growth of embryogenic callus under the effect of radiation doses.

	14 d		21 d		28 d	
Doses	Weight (mg)	**Net Growth (mg)	Weight (mg)	Net Growth (mg)	Weight (mg)	S. Dev
0 Gy	38.1 45 %	15.2 18 %	53.3 62 %	32.1 38 %	85.5 a* 100 %	± 24.2
10 Gy	23.5 27%	9.3 11 %	32.8 38 %	15.7 18 %	48.6 b 57 %	± 12.6
20 Gy	18.8 22 %	7.6 9 %	26.4 31 %	11.6 14 %	38.1 c 45 %	± 9.72

*Values with the same letter inside column are not statistically significant difference (P>5%)
**Net weight Gain in seven days of growth

On the other side, comparing the growth of the callus in each date (**Table 2**), on the 14th day the growth of the test control was the 45% of the callus weight, in comparison to 29% and 22% of the treatments with 10 and 20 Gy respectively (Figure 3A). Difference in weight is a response of the explants to the mutations that were produced by the absorbed radiation, which not only affected the induction of callus, also it had negative effects in the development, since the weight average of the callus in the test control (85.5 mg) was 43% superior to 10 Gy doses (48.6 mg); and 55% to the 20 Gy doses (38.1 mg). as shown in figure 3. The increment of weight was proportionally according to increase the radiation dose, and it was significant lower in irradiated treatments (10 and 20 Gy) according to control test (figure 2 A, B and C). Similar results were found in sugar cane when callus size was reduced 50% when dosage reached 30 Gy, while higher dosages (40 to 80 Gy) callus size was drastically inhibited (Valdez *et al*. 2004).

Additionally, the regression applied (lineal) ($y = -0.0375x + 1.2306$) was reached, which indicates as in induction of embryogenic cells; their development was affected similarly by the radiation doses, reducing growth according with radiation level. In other hand the coefficient of determination indicates that the 86% of the inhibition of such cell competence was caused by the mutations, altering the development until bipolar cells that is the signal of the initiation of life program of any organism. Is absolutely predictable that the radiation give rise undesirable changes on cell´s DNA, on which was demonstrated by the low competence of the callus generated, which decreases as radiation dosage increases as effect on cells genetics and physiology pathways, in such a way of the amount of cells reprogramed to initiate an asymmetric division as the via to develop an organism, and consequently such cells have no connection with the vascular tissue or other cells, therefore the callus is produced by the division of pre-embryogenic cells that had a window to generate bipolar structures that is limited by apoptotic phenomena.

As it has been discussed, the radiation effect into the somatic tissue is the mutations of the genetic content (DNA), and consequently the expression paths. Under this sight, such effect damages the induction, proliferation, and development of the embryogenic cells. When comparing these results with the callus induction, it was found a high similarity with the behavior, because the inhibition was present in the induction and in the callus development. Also considering that in both variables the correlation is negative and significative, confirms that the mutations that were originated in the somatic tissue, propitiated changes that altered the DNA of the competent cells, inhibiting the induction of embryogenic callus, while the mutations that were presented in the induction; however the development decreased in the irradiated treatments regarding the test control. Similar results were reported by Valdez *et al*. (2004), who have found that the callus cells capacities of sugarcane were affected when radiation dosage was increasing.

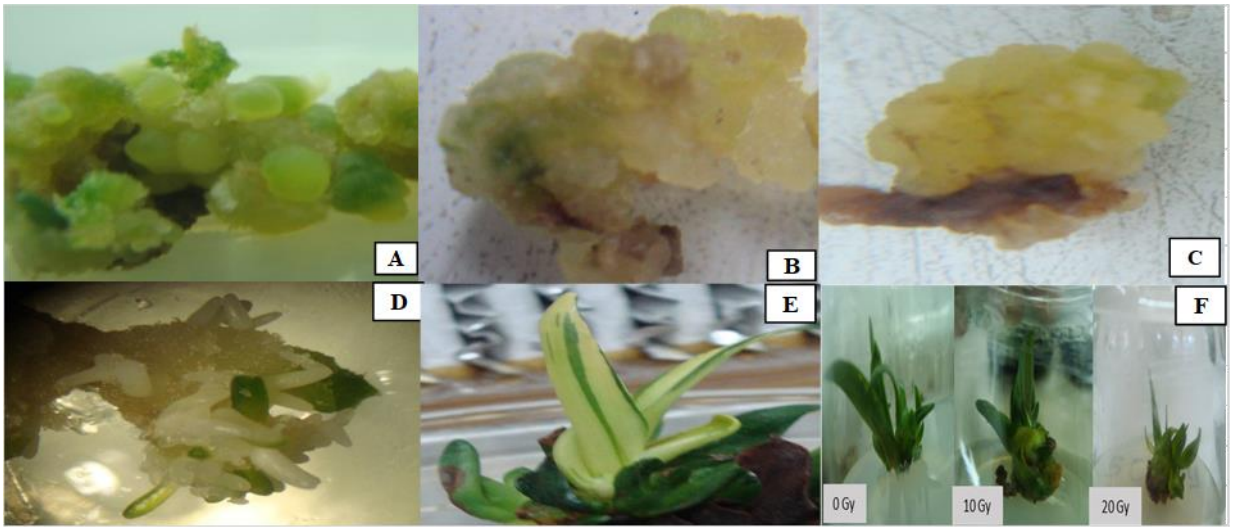

Figure 2. Somatic embryogenesis in *Agave tequilana* Weber cultivar blue. A) Callus development (control) after 28 days. B) Callus development with 10 Gy dosage; C) Callus development with 20 Gy dosage. D) Embryos in cotyledon stage E) Mutant Plantlet 20 Gy regenerated from a somatic embryo 56d after transferred to medium for germination. F) Plantlet with 0 Gy (control), 10 Gy and 20 Gy dosage regenerated from a somatic embryo 56d after transferred to medium for germination.

Table 3. Number and size of somatic embryos developed into plantlets, and plant conversion means achieved by the effect of radiation doses in *Agave tequilana* cultivar blue.

	Embryos		Length		Plant conversion	
Doses (Gy)	Number	%	(cm)	%	Number	%
0	18 a*	48.00	9.62 a	100	91 a	47.6
10	12 b	31.56	8.11 b	84.3	61 b	31.9
20	8 c	20.44	6.92 c	71.8	39 c	20.4

*Values with the same letter inside column are not statistically significant difference (P>5%)

Development of somatic embryos

In the Table 3, it is shown the results over the evaluation of the somatic embryos developed into plants, as well as their length, and plant conversion

The number of developed embryos give rise the same behavior with highly significance ($p < 1\%$). The differences between treatments were determined through orthogonal tests, being statistically superior in the test control with regard to the doses of radiation, such as the significant difference between the 10 Gy and 20 Gy doses. As it is observed in the **Table 3 and Figure 2A.** With this analysis again is demonstrated the radiation effect the conversion of somatic embryos into plants; as in all embryogenic process in blue agave.

The length of the somatic embryos showed again large statistic differences ($P \leq 1\%$) among treatments. As was expected, the control presented the lengthiest with an average of 9.62 mm, followed by the 10 Gy dose with 8.11 mm and 6.92 in the 20 Gy dose (Table 3 and Figure 2 A, B and C) on which is assumed less capacity for embryo development. Consequently, radiation dosage affects the development of embryos during the globular, scutellum and coleoptile stages, that means somatic embryo size. Although, all embryos achieved became into plantlets.

As is described above, there is effect of the radiation on the maturation and the development of the somatic embryos, and had a negative and statistically significance implication, since the number of embryos decreased according to the increase of the radiation doses (y= -0.425x+14.528; and r=-0.95). Contrasting the performing effects of radiation on cells to became into embryos with the test control, the mutations caused a negative behavior, so the coefficient determination (R^2 = 90%) indicate the influence that the absorbed radiation give rise unknown mutation expressed as less cell totipotency and competence to regenerate such cell into a whole organism.

Similar result was published by Portillo *et al.* (2007), who evaluated different genotypes, and the effect of plant growth regulators combinations, on which is important remark the generation of a colored friable embryogenic callus, little and lengthened globular cells. From the callus arisen somatic embryos capable to maturate, to germinate, and to develop into well typical plants, demonstrating the successful process of indirect somatic embryogenesis in blue agave.

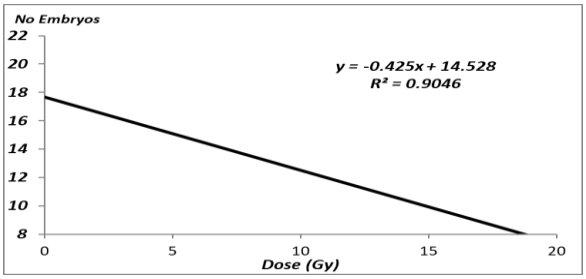

Figure 3. Correlation and lineal regression between radiations doses (Gy) and number of embryos.

In order to understand the meaning of both cell totipotency and competence, must be remarqued that all plant cells give rise a new division (totipotent cell), are the best in getting linage or reprograming their own function until generate a new organism, such cell could be both meristematic or parenchyma cells. Under this sight, not all cells have the capacity to generate embryos, it could depend the genotype, cell development, etc. (Fiore et al., 2002). The results have shown an induction of wide genetic variation which was achieved by the effect of the radiation absorbed by the tissue, where were arisen mutations that have altered heredity of DNA since the lacking expression of those genes responsibly of the somatic embryogenesis according to Yao, *et al* (1993). Similarly, in the measure that was made to determine the length of the somatic embryos, there was a significative difference between the treatments, decreasing the length according the increase of radiation doses. As control treatment was consider as normal growth and development of the embryos, it was observed that the length achieved after a 10 Gy was 16%, and 29% in the 20 Gy, respectively smaller than the test control, which explains the statistic difference among treatments; moreover, the growth decreased with radiation in all treatments the conversion of embryos into plantlets were observed, however the mutations could affect the *in vivo* development. The most important achievement by this research is the

generation of somatic embryos capable to become into plantlets from irradiated germplasm.

Plantlets conversion

Complete plantlets were achieved after 56 days after treatment on the maturation medium (Figure 2 E and F). The table 3 shows the number of plantlets regenerated by agave somatic embryogenesis and significative difference ($p < 1\%$). The differences between treatments were determined through orthogonal tests being statistically superior the number of plantlets obtained in the control with regard to the doses of radiation, such as the difference between the of 10 Gy and 20 Gy doses.

The conversion of plantlets in the control treatment was 15.7% higher that the dose of 10 Gy and 27% with regards to the 20 Gy doses as shown in table 3 and figure 2 F. The effect of the radiation over the plantlet's conversion decreased according to the increase of the radiation doses. However, is important to consider that with the radiation treatments evaluated in this experiment, plantlets well-developed of Tequila´s blue agave can be reached from somatic embryos supplemented with 500 mg L^{-1} of casein hydrolyzed, 250 mg L^{-1} of glutamine, 40 g L^{-1} of sucrose, while Arzate-Fernandez and Mejia-Franco (2011) have assuring germination of all somatic embryos and the conversion into whole plants of *A. angustifolia* without in medium lacking growth regulators and supplemented with higher amount of sucrose. The difference in sucrose concentration could be because they did not add hydrolyzed casein.

CONCLUSIONS

According to the results achieved, indirect somatic embryogenesis process is demonstrated in all steps, which is a protocol to get agave plantlets from irradiated micropropagated plantlets.

The radiation effect over somatic tissue was crucial as the doses affect the conversion into plantlets.

The radiation arisen mutations that altered the competence of cells, decreasing significantly the embryogenic callus, as well as development of somatic embryos in contrast of the control, however plantlets from one single genotype were obtained, and the genetic implications by the radiation.

Must be remarkable, such radiation allowed the conversion of the embryos to complete agave plantlets.

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