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Article

A temporary immersion system improves regeneration of *in vitro* irradiated recalcitrant rice embryogenic calli.

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Abstract: The development of gamma ray-mutated rice lines is a solution for introducing variability in rice varieties already being used by farmers. *In vitro* gamma ray (60Co) mutagenesis reduces chimeras and allows a faster selection of desired traits but requires optimization of the laboratory procedure. The objectives of the present work were the *in vitro* establishment of recalcitrant rice embryogenic calli, the determination of their sensitivity to gamma radiation (60Co), sequencing of MATK and Rubisco genes for identification purposes, and optimization of the generation procedure. The radiosensitivity of the embryogenic calli showed an LD50 at 110 Gy, while the 20% lethal dose was 64 Gy. All sequenced genes matched perfectly with previously reported MATK and Rubisco *O. sativa* genes, with a clear SNP that identified the local variety as being related to one in the Southeast Asia region. Callus induction improved in MS medium containing 2 mg/L 2,4-D, and regeneration was achieved with MS medium with 3 mg/L BAP and 0.5 mg/L NAA. The optimized radiation condition was 60 Gy, with 83% regeneration in a semisolid medium, allowing a balance between mutation and regeneration. When the radiation increased to 80 Gy, the regeneration rate fell to 29%. An immersion system (RITA®) of either 60 or 120 seconds every 8 hours allowed systematic and homogeneous total regeneration of the recalcitrant line, in contrast with the semisolid medium that resulted in positive but irregular regeneration. Other well-known recalcitrant cultivars, CR1821 and CR1113, also had improved regeneration in the immersion system, demonstrating their potential use as recalcitrant materials. To our knowledge, this is the first report on using an immersion system to allow regeneration of gamma-ray mutants from recalcitrant rice materials.

Keywords: Embryogenesis; Cobalt-60 radiation-induced mutagenesis; Temporary immersion systems (TIS).

1. Introduction

Rice is an important cereal that provides 20% of the world's energy, particularly in Asia, Africa, and Latin America. The *Oryza* genus consists of 22 species, but only two are commonly planted: *O. sativa* and *O. glaberrima*[1–5]. Of those, farmers prefer only a few cultivars, depending on the country. The limited variability of commercial materials can become an obstacle in increasing productivity given emerging conditions such as heat, salt stress, soil acidification, plague sensitivity, and weeds. Introducing variability with crossbreeding is slow and can result in the introduction of undesired traits. Radiation methods to generate variability in seeds are common techniques used since 1928 on vegetables, while other methods exist, such as the use of ethyl methanesulfonate (EMS) and new breeding techniques to introduce specific genetically engineered mutations [6–11].

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Plant tissue culture represents an opportunity to overcome time limitations, land requirements, and selection of the desired variability that results when using gamma ray mutagenesis in seeds. Once irradiated, the seeds must be planted several times (M0 to M4) to avoid chimeras and heterogenicity until exposed to stress selection conditions, as has been done in the past6,8. In contrast, rice tissue culture can produce a primitive cell aggregate or callus with embryogenic potential and consequently mutate and regenerate from one or a few cells with stressor selection from the beginning[12,13].

The latter is possible because plant cells are totipotent, which means that whole plants can develop from the single cells[14]. Mutation in the embryogenic callus using gamma radiation was previously developed in our lab for the cultivar CR5272 for salt tolerance; however, farmers no longer use this cultivar and instead use modern materials that are more recalcitrant to tissue culture[13]. The establishment of embryogenic rice calli is influenced by the germplasm of origin and the 2,4-D concentration[14,15]. Embryogenic cells result when exposed to Murashige and Skoog (1962) medium supplemented with 2 to 2.5 mg/l 2,4-D, resulting in pro-embryos and somatic embryos[16–18]. Costa Rican cultivars such as CR-201, CR-1707, CR-1821, CR-8334, and CR-8341 have unpredictable and variable behavior, while CR-1113 and CR-5272 have predictable induction and regeneration in 2.5 mg/L 2,4-D[19].

Here, we faced the challenge of identifying our material with the commonly used molecular markers MATK and Rubisco, improving our plant tissue culture methods, and determining the radiosensitivity of embryogenic calli and further regeneration. We present a simple method to induce mutations using gamma rays in embryogenic calli of a recalcitrant cultivar, with an alternative immersion method that allowed our material to fully generate homogeneous and predictable *in vitro* plants after irradiation.

2. Results

The MATK MZ558335 and Rubisco MZ558334 sequences showed perfect matches with the already published NCBI *O. sativa indica* accessions and demonstrated the identity of the material as expected. We detected three synonymous mutations in the Rubisco sequence that are important for the characterization of the variety. Specifically, one synonymous SNP on the sixth glutamic acid triplet (GAA/GAG) gives clues of a putative origin of the germplasm ancestors from the Southeast Asia region because of its unique presence and matches with three cultivars of the region: the Pakistan cultivar NARC 17958 (GenBank KP827660.1), the Indonesia cultivar Pandak Kembang (GenBank MZ198248) and the Vietnam cultivar "Lua Khau Ky" isolate GBVN15800 (GenBank KR073275.1). We also detected two synonymous biallelic mutations at glycine 82 and 150 codifying triplets (GGC/GGA; GGT/GGC), which helped characterize and further identify the material. None of the mutations suggested biological importance since the open reading frame remains unaltered.

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Figure 1. DNA markers used to identify the MATK and Rubisco genes in the local rice variety. Note in green the synonymous SNP (C/T) and the biallelic synonymous mutations A/G and G/T (circled). None of the mutations had biological importance but helped in the genetic characterization of the cultivar.

Rice tissue culture

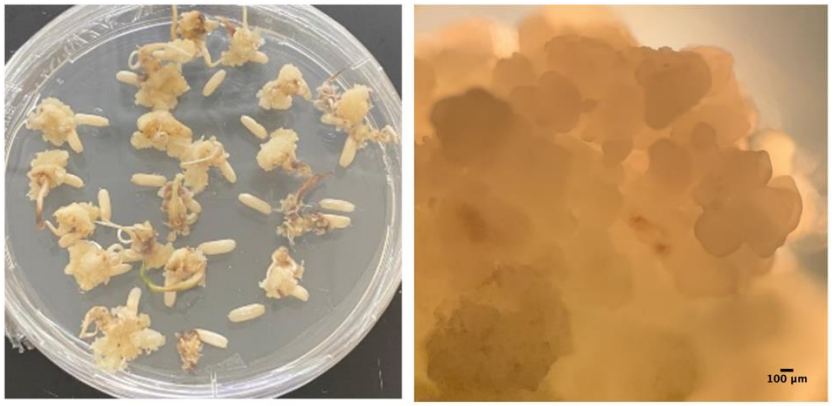

Figure 2. Calli induction on MS medium with 2 mg/L 2,4-D after 30 days. (A) The induction in Petri dishes and (B) the compact and friable calli as observed under the stereoscope.

Table 1. Rice callus induction and oxidation rate from different induction treatments

Treatment	Embryogenic calli (%)	Oxidation rate (%)
1 mg BAP + 1 mg de 2,4-D	12.77 d	0.66 c
1 mg BAP + 2 mg de 2,4-D	16.77 c	0.21 c
2 mg 2,4-D + 0,25 mg de TDZ	23.00 b	0.66 c
2 mg 2,4-D	71.44 a	3.55 a
2,5 mg 2,4-D	21.44 b	2.00 b
1 mg BAP + 1 mg 2,4-D	12.77 d	0.66 c

[1] All treatments had 30 replicates (n=30). Letters represent a significant differences ($p \leq 0.05$).

The best callus induction was achieved with 2 mg/L 2,4-D alone. The callus induction step with a 2 mg/L 2,4-D concentration was initially not expected because of previous local cultivar reports. Local cultivars CR5272 and CR1113 had a positive response at 2.5 mg/L 2.4 D but a low performance at 2 mg/L 2,4-D, while CR-201, CR-1707, CR-1821, CR-8334, and CR-8341 had recalcitrant and unpredictable *in vitro* behaviors[16,19]. Similarly, better callus induction occurred with higher concentrations of 2,4-D (from 2.5 mg to 3 mg/L) with other cultivars, such as MR220, GNY-53, and JP-5 [22,23]. In our case, the positive induction at 2 mg/L 2,4-D contrasted with higher oxidation and represented a challenge for the next steps of regeneration and our final irradiation goal, which also triggers oxidation.

Table 2. Rice callus regeneration, sprouting and oxidation rate of different treatments

Induction Treatment	Regeneration Treatment	Regeneration (%)	Sprouting (%)	Oxidation (%)
2 mg 2,4-D	0.5 mg ANA + 3 mg BAP	69.04 a	7.14 ab	9.52 d
	0.5 mg ANA + 0.5 mg TDZ	38.09 c	2.38 b	61.90 a
	0.5 mg ANA + 0.5 mg Kinetina	47.61 b	9.52 a	23.80 c
	0.5 mg ANA + 0.5 mg BAP	28.57 d	2.38 b	54.76 b
1 mg BAP + 2 mg 2,4-D	0.5 mg ANA + 3 mg BAP	28.29 b	0 a	58.43 b
	0.5 mg ANA + 0,25 mg TDZ	58.82 a	0 a	100 a
	0.5 mg ANA + 0.5 mg Kinetina	9.22 c	0 a	56.81 b

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	0.5 mg ANA + 0.5 mg BAP	12.82 c	0 a	44.26 c
1 mg BAP + 1 mg 2,4-D	0.5 mg de ANA + 3 mg BAP	58.45 ab	16.38 a	18. 69 bc
	0.5 mg de ANA + 0.5 mg TDZ	61.75 a	10.71 ab	27.93 a
	0.5 mg de ANA + 0.5 mg Kinetin	56.31 ab	15.92 a	20.01 ab
	0.5 mg de ANA + 0.5 mg BAP	49.88 b	4.16 b	11.66 c
	0.5 mg de ANA + 3 mg BAP	58.45 ab	16.38 a	18. 69 bc
2,5 mg 2,4-D	0.5 mg ANA + 3 mg BAP	34.64 b	0 b	9.20 c
	0.5 mg ANA + 0.5 mg TDZ	51.41 a	9.61 a	22.96 a
	0.5 mg ANA + 0.5 mg Kinetin	43.62 ab	0 b	15.73 b
	0.5 mg ANA + 0.5 mg BAP	18.00 c	0 b	15.19 b
2 mg 2,4-D + 0,25 mg TDZ	0.5 mg ANA + 3 mg BAP	77.27 a	2.27 a	96.59 a
	0.5 mg ANA + 0.5 mg TDZ	50.25 b	0 a	86.36 b
	0.5 mg ANA + 0.5 mg Kinetin	73.86 a	3.40 a	82.95 b
	0.5 mg ANA + 0.5 mg BAP	44.29 b	0 a	72.81 c

[1] All treatments had 6 replicates (n=6). Letters represent significant differences ($p \leq 0.05$).

The best regeneration rate of approximately 70% resulted from 0.5 mg of NAA+ 3 mg of 6-BAP, with sprouting of 7.14% and oxidation of only 9.52% (Table 2). Other regeneration medium recipes also resulted in regeneration but with higher oxidation and were consequently useless for our next step, the gamma radiation mutagenesis.

The lethal dose of the embryogenic calli was 110 Gy, while the 20% lethal dose was 64 Gy, resulting in a 0 to 120 gray gradient exposure, with 200 calli per exposure (Table 3).

Table 3. Probit gamma ray lethal dose calculation on the rice embryogenic calli[1]

Lethal gamma rays dose model	Dose (Gy)	Lower limit (Gy)	Upper limit (Gy)
LD10	41,145	34,552	46,708
LD20	64,799	60,083	69,20
LD25	73.785	69,388	78,139
LD30	81,855	77,507	86,403
LD40	96,429	91,649	101,85
LD50	110,050	104,435	116,720

[1] All treatments had 10 replicates with 20 calli each (n=20), $p \leq 0.05$. Data were compiled at 30 days post-radiation.

The best radiation/regeneration ratio was achieved at 60 Gy with 83% regeneration, allowing a balance between gamma radiation at lethal dose 20 and regeneration (Table 4). The increased regeneration achieved with 40 Gy and 60 Gy versus the control is a hormetic behavior previously reported in our lab[13].

Table 4. Radiation dose influence on regeneration, sprouting and oxidation rates[1]

Radiation dose	Regeneration	Sprouting	Oxidation
80 Gy	29.76 d	0 c	15.47 a
60 Gy	83.85 a	0 c	3.57 c
40 Gy	75.00 b	3.57 b	7.14 bc
0 Gy	69.04 c	7.14 a	9.52 b

[1] All treatments had 12 replicates (n=20), $p \leq 0.05$. Data were compiled at 15 days post-radiation.

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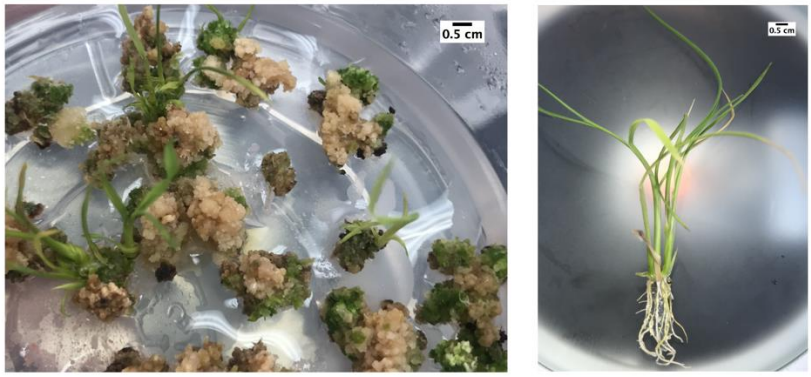


(A.) (B.)

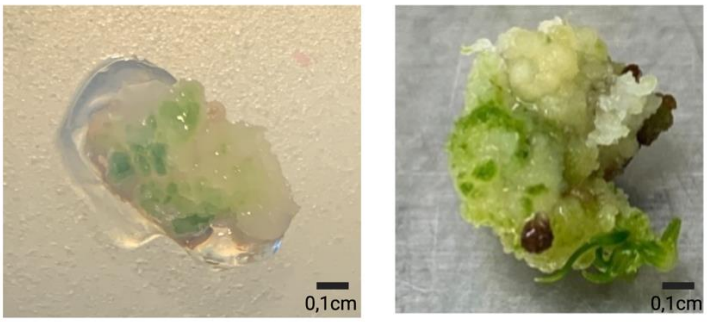
Figure 3. Regeneration of 60 Gy irradiated calli after (A) 45 days and (B) 60 days.

Gamma-irradiated calli regenerated plants after 45 days in induction medium and fully *in vitro* plants at 60 days post-irradiation.

Table 6. Immersion regeneration, sprouting and oxidation rates[1]

Immersion time per 8 hrs	Regeneration	Sprouting	Oxidation
60 seconds	100.00 a	25.00 a	60.00 a
120 seconds	97.56 a	31.71 a	97.56 a

[1] All treatments had 12 replicates (n=20), $p \leq 0.05$. Data were compiled at 15 days post-radiation.



(A.) (B.)

Figure 4. Regeneration in MS medium with 0.5 mg ANA + 3 mg BAP of an induced callus with 2 mg/L of 2,4-D after 15 days of induction. Regeneration in (A) semisolid medium and (B) RITA®.

The potential of a temporary immersion system for regenerating difficult materials was also validated for the well-known recalcitrant cultivars CR1821 and CR1113 (Table 7, Fig. 5).

Table 7. Immersion regeneration, sprouting and oxidation rates for the controls[1]

Cultivar	Immersion time per 12 hrs	Regeneration	Oxidation
CR5272	60 seconds	100.00 a	60.00
CR5272	120 seconds	100.00 a	60.00
CR1821	60 seconds	55.00 b	50.00
CR1821	120 seconds	60.00 b	50.00
CR1113	60 seconds	45.00 b	60.00
CR1113	120 seconds	50.00 b	60.00

[1] All treatments had 12 replicates (n=20), $p \leq 0.05$. Data were compiled at 15 days post-radiation.

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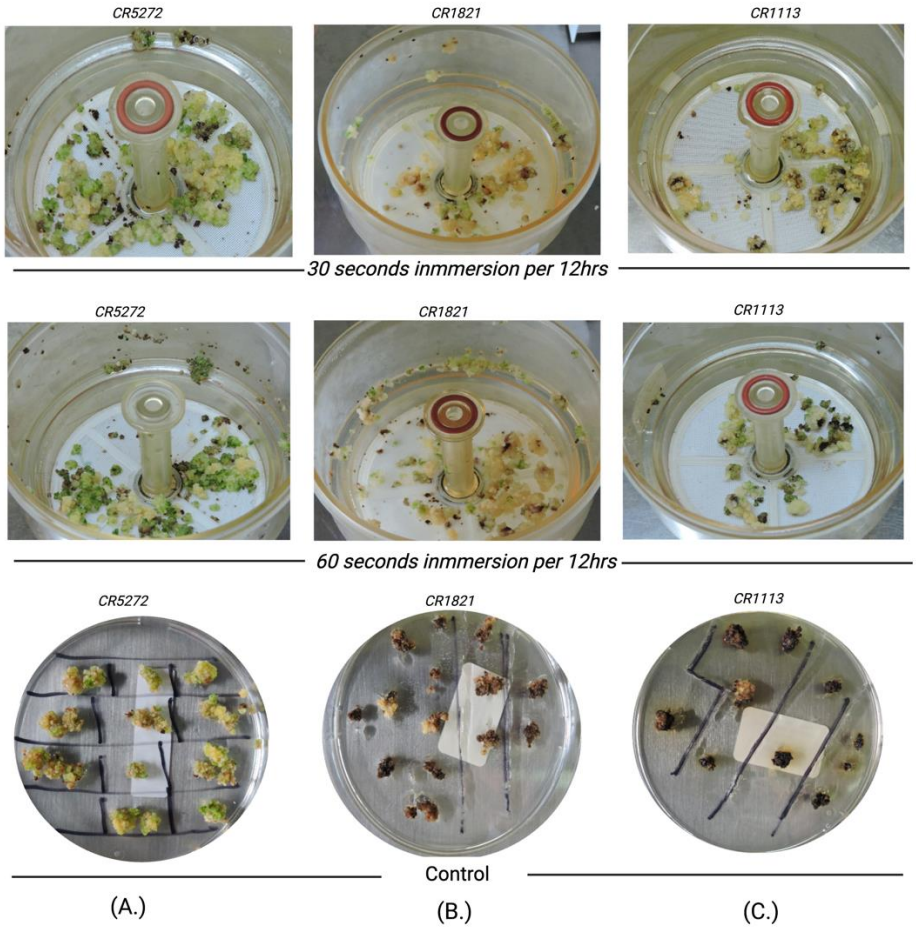

Figure 5. Regeneration of cultivars CR5272 and recalcitrant CR1821 and CR1113 in MS medium with 0.5 mg ANA + 3 mg BAP from calli induced with 2 mg/L of 2,4-D. (A) CR5272 regeneration in SIT with immersion for 30 seconds per 12 hours, immersion for 60 seconds per 12 hours and the semisolid medium control; (B) CR1821 regeneration in SIT with immersion for 30 seconds per 12 hours, immersion for 60 seconds per 12 hours and the semisolid medium control; (C) CR1113 regeneration in SIT with immersion for 30 seconds per 12 hours, immersion for 60 seconds per 12 hours and the semisolid medium control. The absence of regeneration of the recalcitrant cultivars CR1821 and CR1113 was observed in the semisolid media.

3. Discussion

The present work optimized *in vitro* gamma ray (60Co) mutagenesis in an embryogenic callus of a recalcitrant Costa Rican cultivar. The cultivar seems to be related to Southeast Asian rice cultivars based on its Rubisco sequence pattern. The best callus induction was observed on MS with 2,4-D and regeneration with MS with 0.5 mg ANA + 3 mg BAP. This result is similar to that obtained by other authors on Southeast Asian cultivars such as Malaysia MR219, where 2,4-D is critical and performs the best as an inducer at 2 mg/L[20,21]. Regeneration was improved using a temporal immersion system (RITA®) with either 60 or 120 seconds of immersion every 8 hours, which achieved a predictable and more homogeneous regeneration response. The radiation dose at which to start mutagenesis was proposed as a lethal dose 20 at 60 Gy, as regeneration was not affected but remained as high as 80%. At 80 Gy, the regeneration fell to 30%, consistent with the oxidative damage provoked by radiation and corresponding to the lethal dose 30 of the embryogenic calli. We believe 60 Gy is an excellent condition to start mutagenesis, considering

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that in other *in vitro* plants, such as pineapple, potato, and banana, a 5-40 Gy dose of gamma irradiation was shown to be sufficient to produce variability[24]. The calculated radiation dose of the cultivar at 60 Gy was the same as the LD50 for CR5272[13].

Optimization of embryogenic calli of a recalcitrant cultivar is essential for the following reasons. First, the alternative immersion method allowed our material to fully generate homogeneous and predictable *in vitro* plants after irradiation and was demonstrated to work for our cultivar and other recalcitrant rice lines. An immersion system is a little-explored tool for gamma radiation mutagenesis with great potential. The constant liquid and airflow can dilute oxidation compounds and facilitate more homogeneous exposure to nutrients. We validated the results with recalcitrant cultivars CR1821 and CR1113, which showed regeneration rates above 40%, which contrasted with null regeneration in the conventional semisolid method. Second, we can develop desired mutations in a cultivar that farmers are already using, which consequently leads to faster breeding and adoption of a derived improved cultivar. Rice traits associated with specific genes are well known, which paves the way for producing novel cultivars based on mutation of desired or required conditions, such as biotic and abiotic stress tolerance[10].

Plant cultivars' nonhomogeneous *in vitro* behavior is not fully understood, but recent discoveries provide insights into the genetic basis of sucrose metabolism and oxidation. External phytohormones seem to trigger rice sucrose metabolism required for regeneration. The system appears to rely on the expression of endogenous cytokinin, auxin, and ABA signaling genes: ORR1, PIN-formed 1, and *late embryogenesis-abundant 1*[26]. The expression of OsSRO1c, a regulator of oxidative stress, seems to be vital for avoiding callus browning in indica cultivars [27].

Tissue culture and gamma radiation produce oxidation via reactive oxygen species (ROS), which are usually contained in chloroplasts, peroxisomes, and mitochondria. ROS include superoxide O_2^-, hydroxyl OH radicals, H_2O_2 and HO_2. Tissue culture and gamma rays trigger ROS, consequently damaging the DNA by oxidation of the molecule into 8-oxo-7-hydroxyguanosine (8-oxo-dG) and further transversions of C/G and T/A[25]. SIT seems to help overcome oxidation while allowing the tissue to recover.

4. Materials and Methods

4.1. Embryogenic callus production

For initial assays, the palea and lemma of rice caryopsis of a commercial indica cultivar were removed with No. 80 grit sandpaper. The obtained seeds were surface-sterilized as previously reported[13] through two incubations in 4% (v/v) NaOCl for 10 min each with constant agitation, using 10 mL of disinfectant solution for every gram of seeds. After the first and final incubation, the seeds were washed six to seven times with distilled sterilized water and were cultured in media composed of mineral salts and vitamins as described by Murashige and Skoog (MS, 1962), with 20 g. L^{-1} sucrose and 0.1 g. L^{-1} hydrolyzed casein. Treatments consisted of supplementing the basal medium with one of the following combinations of plant growth regulators: 2.5 mg. L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) was used as a control, a second treatment with a lowered concentration of 2.0 mg. L^{-1} 2,4-D, a third treatment consisting of 1.0 mg. L^{-1} 2,4-D + 1 mg. L^{-1} 6-benzylaminopurine (6-BAP), a fourth treatment consisting of 2.0 mg. L^{-1} 2,4-D + 1 mg. L^{-1} 6-BAP, and a fifth treatment consisting of 2.0 mg. L^{-1} 2,4-D + 0,25 mg. L^{-1} 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea (thidiazuron, TDZ). After adding plant growth regulators, the media volumes were adjusted as required, the pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl, and 5.4 g. L^{-1} Gelzan ® was added as a gelling agent. All previously mentioned chemicals were supplied by Phytotechnology Laboratories® (Shawnee Mission, Kansas, USA). After sterilization in an autoclave (1.2 ATM.cm^{-2} and 121 °C) for 30 min, 20 mL of medium was dispensed on 94x16 mm vented polystyrene Petri dishes (Greiner Bio-One, Fisher-Scientific, Waltham, Massachusetts, USA) in a laminar flow chamber. For each treatment, at least 900 seeds were cultured after surface sterilization. Light parameters for all treatments were an intensity of 72 µmol·s-1·m-2, a photoperiod of 16 h and a temperature of 26 ± 2 °C. Regeneration, sprouting, and oxidation were recorded as response variables and

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analyzed in a completely randomized design with a generalized linear model with a Poisson distribution and logit link function. Post hoc analysis consisted of a Honest Significant Difference test to establish differences between the means.

4.2. Molecular markers

A NucleoSpinTM Tissue Kit from Macherey-Nagel (Germany) was used for DNA extraction, and Thermo Fisher K1071 was used for the subsequent PCR following the recommendations of the manufacturer. The primers used in this study are described as follows: for Rubisco, rbcLaf 5'ATGTCACCACAAACAGAGACTAAAGC3' and rbcLar 5'GTAAAATCAAGTCCACCRCG-3' or rbcLaf 5'ATGTCACCACAAACAGAGACTAAAGC3' and rbcLr590 5'AGTCCACCGCGTAGACATTCAT-3'; for MatK, matkxf 5'TAATTTACGATCAATTCATTC-3' and matkr 5'ACAAGAAAGTCGAAGTAT-3'. The thermocycling program was 95 °C for 5 minutes, 40 cycles at 95 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min, and a final cycle of 72 °C for 7 minutes.

4.3. Gamma Radiation

Embryogenic callus radiation was achieved with a CoS 44HH-N Ob-Servo Ignis with 24 cobalt 60 sources (Institute of Isotopes Co, Ltd., Budapest, Hungary) with gradient exposure from 0 to 120 Gy, ten repetitions, and 20 embryogenic calli per exposure. IBM SPSS version 27 was used to calculate the lethal dose for the statistical probit analysis.

5. Conclusions

This section is not mandatory but can be added to the manuscript if the discussion is unusually long or complex.

6. Patents

NA.

Supplementary Materials:

Table S1. Lethal doses based on the probit model

Probability	Estimate	95% Confidence limits for Doses	
		Lower Limit	Upper Limit
0,01	-15,03	-28,403	-4,387
0,02	-0,373	-11,855	8,821
0,03	8,926	-1,383	17,23
0,04	15,922	6,474	23,575
0,05	21,612	12,848	28,754
0,06	26,455	18,259	33,176
0,07	30,702	22,989	37,068
0,08	34,504	27,212	40.565
0,09	37,962	31,04	43,757
0,1	41,145	34,552	46,708
0,15	54,325	48,927	59,09
0,2	64,799	60,083	69,2
0,25	73,785	69,388	78,139
0,3	81,855	77,507	86,403
0,35	89,333	84,838	94,254

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0,4	96,429	91,649	101,85
0,45	103,29	98,132	109,305
0.5	110,05	104,44	116,72
0.55	116,81	110,68	124,193
0,6	123,67	116,98	131,831
0,65	130,77	123,46	139,761
0,7	138,25	130,25	148,148
0,75	146,32	137,56	157,225
0,8	155,3	145,67	167,356
0,85	165,78	155,1	179,188
0,9	178,96	166,95	194,101
0,91	182,14	169,81	197,706
0,92	185,6	172,91	201,624
0,93	189,4	176,32	205,934
0,94	193,65	180,13	210,748
0,95	198,49	184,47	216,241
0,96	204,18	189,56	222,696
0,97	211,18	195,83	230,635
0,98	220,47	204,15	241,193
0,99	235,13	217,26	257,841

Author Contributions: Conceptualization, A.H.-S., J.P.-C., R.F.-Z, R.R.-V, and A.G.-A; writing—original draft preparation, A.H.-S, J.P.-C., and A.G.-A.; writing—review and editing, A.H.-S, J.P.-C., A.G.-A, and A.A.-E; visualization, A.H.-S and J.P.-C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: In this section, please provide details regarding where data supporting the reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to the suggested Data Availability Statements in the section "MDPI Research Data Policies" at https://www.mdpi.com/ethics. You might choose to exclude this statement if the study did not report any data.

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Conflicts of Interest: "The authors declare no conflicts of interest."

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