Tolerance to aryloxy-phenoxy-propionate (APP) as a model for Lazarroz FL rice in vitro gamma irradiation variability selection

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Abstract

In vitro gamma ray ($^{\text{60}}$Co) mutagenesis is a powerful tool to achieve variability in commercial rice lines used by farmers, such as Lazarroz FL. We previously reported the optimized in vitro gamma mutagenesis system for Lazarroz FL Indica callus. As a continuation, in the present study, we targeted the ACC2 gene mutagenesis that provides tolerance to aryloxy-phenoxy-propionate (APP) fluazifop-P-butyl as a model to show the system's potential to create variability while providing a solution for weed management. The DL50 of fluazifop-P-butyl was calculated in calli as DL50 = 6.93 mg/L (0.425 mg/L - 15.743 mg/L, $R^2 = 0.402, 1000n$) and regenerated *vitroplants* at an LD50 of 3.771 mg/L ($R^2 = 1, 290n$). We used 5 mg/L fluazifop-P-butyl as a selection agent and the second round of selection of 10 mg/L (3000 *vitroplants*) resulted in one survivor plant when using calli as a starting material. The putative tolerant plant also tolerated 150 mg/L in the greenhouse. The ACC2 gene was sequenced, and a heteroecious mutation, T2222I/T2222M, was discovered that may be linked to tolerance. We improved the in vitro system by using seeds as a gamma irradiation starting point instead of embryogenic calli, followed by calli induction, regeneration, and exposure to the selection agent. The modification allowed higher gamma doses with an LD50 of 350 Gy and one to thirty-one putative tolerant plants. The in vitro model showed that gamma-ray mutants from recalcitrant indica rice materials are possible, and the use of selection agents such APP can help create variability useful for breeding a more resilient rice.

1. Introduction

Rice (*Oryza sativa* L.) is a crucial crop responsible for 20% of calories of half the human population and is facing constraints of yield related to biotic factors, such as pathogens, and abiotic stresses, such as salinity, drought, heat or cold [1–2]. Breeding can introduce desired traits based on genetic variability related to heat, salt, soil acidification stress, and plague tolerance to the few cultivars preferred by farmers in specific geographic areas [3–6]. Rice-induced genetic variability obtained by seed irradiation is well known and has been used since it was proven in 1928 on vegetables; however, it is slow and requires land, resources, and human capabilities to find desired traits [7–10]. Plant tissue culture provides a platform to produce gamma mutations from primitive embryogenic cells [11–13]. We previously reported a system to create variability using in vitro gamma radiation at an optimized dose of 60 Gy in embryogenic calli of Lazarroz indica rice [14]. The proposed model required a method to narrow the in vitro selection while correlating with desired mutations. In this paper, we suggest resistance to stressors such as the herbicide aryloxy-phenoxy-propionate (APP) to regenerate complete plants from tolerant mutated cells as a model for such challenges. The herbicide is commonly used to control weeds in rice by acting on the ACC2 enzyme, which is related to fatty acid synthesis, while mutations in the carboxyl transferase domain of ACC2 correlate with APP tolerance, as described below [15–21].

The herbicides acting on the ACC2 enzyme include at least two main groups, aryloxy-phenoxy-propionates (APP) and cyclohexanediones [15]. In grasses, there are two types of ACCases, cytosolic and plastidic. The second ACC2 is affected by APPs in rice. The APPs include clodinafop-propargyl, cyhalofop-butyl, fenoxapropethyl, metamifop, diclofop-meth fenthiaprop, quizalofop-ethyl, haloxyfop-R-methyl,
and fluazifop-P-butyl. Fluazifop is a molecule of 327.25 g mol\(^{-1}\) that can control grasses at a dose of 210 g ai Ha\(^{-1}\) and 420 g ai Ha\(^{-1}\) preemergent with low residual effects [15–16]. Specific mutations in ACC2 enzymes correlate with APP tolerance, as described below.

The enzyme acetyl-CoA carboxylase 2 ACC2 (EC 6.4.1.2, UniProt: A2Y2U1) is located on chromosome 5 at amino acids 14,067,726 – 14,079,652 and is delivered to the plastid. Mutations in the carboxyl transferase domain of ACC2 between amino acids 1,781-2,078 and 2,027 – 2,096 are related to APPS tolerance, such as Ile1781-Leu, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn, Ile-2041-Val, Asp-2078-Gly, Cys-2088-Arg, and Gly-2096-Ala [16–19]. Mutations such as A2004 V provide wheat with tolerance to 10 g ai Ha\(^{-1}\) of quizalofop [16, 17]. Similarly, *Oryza japonica* ACCase 2 (*LOC_Os05g22940*), mutations on I1781 V, C2088R, and W2027C, also provide tolerance to APPs [21]. Finally, an indica rice with a mutation tolerant to APPs with the mutation I1781 L has been available in the USA market since 2018 [22].

This paper aims to improve our previous system and obtain more predictable traits that correlate with specific mutations. Here, we present a nonreported mutation, T2222I/T2222M, that may be linked to tolerance to APPs and present an improvement of our earlier *in vitro* system by using seeds instead of calli during gamma irradiation. We also show aryloxy-phenoxy-propionate (APP) fluazifop-P-butyl tolerance as a model to demonstrate the system's potential for incorporating features that correlate with mutations. The obtained mutant can be incorporated into the breeding program to look for functional variability.

2. Results

2.1 Fluazifop-P-butyl toxicity to embryogenic calli

The toxicity of fluazifop-P-butyl to Lazarroz FL embryogenic calli was calculated based on calli browning to be LD50 = 6.93 mg \(\text{L}^{-1}\) R2 = 0.402 (Table 1). A higher brown callus rate of 79% resulted in over 10 mg \(\text{L}^{-1}\) of the selection APP agent corresponding with the calculated LD75 = 17.8 mg (Table 1). We decided to test regenerated *vitroplants* from calli instead of directly exposed calli to fluazifop-P-butyl because of the absence of regeneration of nonbrowned surviving calli (139n) and the variability of the results (Table 2).
Table 1
Nonirradiated Lazarroz FL calli toxicity to fluazifop-P-butyl

<table>
<thead>
<tr>
<th>Treatment 1</th>
<th>Total</th>
<th>Non brown</th>
<th>Brown</th>
<th>Browning rate (%) 2</th>
<th>LD_{50}^1</th>
<th>LD_{75}^1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(mg L$^{-1}$)</td>
<td>(mg L$^{-1}$)</td>
</tr>
<tr>
<td>0 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>200</td>
<td>120</td>
<td>80</td>
<td>40</td>
<td>6.93</td>
<td>17.8</td>
</tr>
<tr>
<td>1 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>200</td>
<td>97</td>
<td>103</td>
<td>48</td>
<td>(0.42–15.74)</td>
<td>(4.1–35.2)</td>
</tr>
<tr>
<td>10 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>200</td>
<td>42</td>
<td>158</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>200</td>
<td>0</td>
<td>200</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Probit statistical analysis resulted in R$^2 = 0.402, y = -0.42 + 0.6x$

2 Brown or necrotic/total calli

2.2 Vitroplant toxicity to fluazifop-P-butyl

The Fluazifop-P-butyl toxicity to Lazarroz FL vitroplants regenerated from calli was calculated based on necrosis of plants at 21 days and resulted in LD$_{50} = 3.771$ mg·L$^{-1}$ and LD$_{75} = 4.287$ mg·L$^{-1}$, R$^2 = 1$ (Table 2, Fig. 1A, 1B, 1C).
Table 2
Nonirradiated Lazarroz FL rice *vitroplant* toxicity to fluazifop-P-butyl

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total</th>
<th>Alive</th>
<th>Necrotic</th>
<th>Mortality Rate (%)</th>
<th>LD$_{50}^1$ (mg L$^{-1}$)</th>
<th>LD$_{75}^1$ (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>55</td>
<td>55</td>
<td>0</td>
<td>0</td>
<td>3.771</td>
<td>4.287</td>
</tr>
<tr>
<td>1 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>40</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>71</td>
<td>36</td>
<td>35*</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>58</td>
<td>0</td>
<td>58*</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>88</td>
<td>0</td>
<td>88*</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>57</td>
<td>0</td>
<td>57*</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mg L$^{-1}$ Fluazifop-P-butyl</td>
<td>70</td>
<td>0</td>
<td>70*</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Probit statistical analysis resulted in $R^2 = 1, y=-4.59 + 7.6x$

* Necrotic *vitroplants* were subcultured into fresh medium to verify mortality

The necrotic plants were subcultured into fresh medium for 21 days to verify mortality, resulting in null sprouting, regeneration, or green spots but remaining necrotic (Fig. 1D).

### 2.3. Gamma radiation mutagenesis and plant regeneration

We decided to use 5 mg L$^{-1}$ as the fluazifop-P-butyl dose to select putative tolerant mutants to avoid false-positives. We selected 100 putative resistant lines out of 8000 *vitroplants* regrown from embryogenic calli irradiated at 60 Gy ($^{60}$Co) that survived when exposed to 5 mg L$^{-1}$ fluazifop-P-butyl stress (Fig. 2). The plants grew normally and went through a second selection round to validate their tolerance. The second round of selection consisted of a higher dose of 10 mg-L fluazifop-P-butyl with the survival of only one putative tolerant plant out of 100 (Fig. 2).

### 2.4. Molecular markers used for the identification of the rice cultivars

We sequenced and published exon 32 of the acetyl-CoA carboxylase 2 gene (*ACCase2*) MZ558337 and compared it with nonirradiated Lazarroz FL plants to validate perfect matches with our control. The control sequence perfectly matched our accession, and the reference genes of *Oryza sativa indica* at chromosome five demonstrated the identity and absence of mutations as expected.
The sequence of exon 32 of the acetyl-CoA carboxylase 2 gene (ACCase2) of the fluazifop-P-butyl tolerant *vitroplant*, showed one mutation in the expected domain related to tolerance. The mutation may have resulted in a change in the amino acid T2222I/T2222M (Fig. 3). The plant remained tolerant in a greenhouse at a dose of 150 mg/L.

We also sequenced the *matK* and *rbcL* genes of the mutants that matched almost perfectly with the control reported sequences MZ558335 and MZ558334, respectively. We noted that a biallelic G/T in *matK* may change the amino acid isoleucine into another nonpolar amino acid or methionine. We also noted a biallelic *rbcL* GT/AG mutation that could change the amino acid tyrosine into another uncharged polar amino acid serine. The results are consistent with our mutation in the ACC2 gene of 1 per 1000 bp (Fig. 4).

Our results were positive, but we still had room for improvement since the number of putative mutants was low. We tried a different approach that allowed us to increase the gamma dose and, consequently, the mutation rate. We used seeds instead of calli for gamma exposure and kept the same tissue culture methods as previously reported [14]. The system improved in terms of having a larger window for optimization that allows for using a range of gamma rays from 50 to 350 Gy with 31 tolerant *vitroplants* after the two rounds of fluazifop-P-butyl selection at 5 and 10 mg L\(^{-1}\) (Fig. 5).

### 3. Discussion

In previous experiences, gamma radiation allowed the development of salt and drought tolerance in cultivar CR-5272 [9]. Nevertheless, such a trait was useless for farmers and breeders who no longer use that variety for planting or breeding programs. It was also not a good trait for optimization because salt tolerance can be a multigenic trait. We investigated Lazarroz and developed its protocols for gamma radiation variability [14]. Lazarroz is a commercial cultivar used and preferred by farmers, representing 45% of planted rice in Costa Rica [23]. The present work resulted in an optimized *in vitro* gamma-ray (60Co) on the ACC2 mutagenesis system based on APPs selection from a recalcitrant Costa Rican Lazarroz FL cultivar. This trait will help breeding programs to manage weeds.

The selected tissue and fluazifop-P-butyl dose were vital in optimizing the mutation system. We were aware that selection on calli reduces time, but we preferred using regenerated *vitroplants* for optimization because of the following. We used calli browning to calculate mortality, but the interval was not as accurate as *vitroplants*. The LD50 on calli resulted in 6.93 mg L\(^{-1}\) R2 = 0.402, 1000 n, compared with a more precise LD50 of 3.771 mg for regenerated *vitroplants*. We believe browning triggered by radiation and tissue culture can have an oxidative background noise that masks the accurate lethal dose [24, 25]. Our second issue was that calli regeneration was problematic, having nonbrown calli unable to regenerate after irradiation and stress selection; 139 calli that survived at 1 mg (97n) and 10 mg exposition (42n) did not regenerate (Table 1).

For the first attempt we used 60 Gy irradiation on calli and direct selection with 5 mg L\(^{-1}\) fluazifop-P-butyl but had no regeneration after 21 d (500 n, data not presented). Direct calli selection is an option with a
recovery time after irradiation and a low selection agent dose to allow regeneration. Other authors used such rationale in rice but with the CRISPR/Cas9 technique at a maximum amount of 2 µM of a similar molecule haloxyfop-R-methyl (0.654 mg L\(^{-1}\)) for calli selection and further validation in a greenhouse of 150 mg L\(^{-1}\) [21, 26]. In maize (Zea mays L.) and other grass, such as Paspalum vaginatum, selection was possible on calli with a different molecule acting on ACC2 at 10 µM of sethoxydim [27–29].

In our case, a recovery step was preferred on regeneration medium for 21 days when having green calli and subsequent selection with fluazifop-P-butyl at 5 mg L\(^{-1}\). The rationale is that investing 21 days in this previous step guaranteed predictable regeneration. The putative mutants had a second selection round that helped us better select stable mutants linked to tolerance. We considered a mutation stable when micropropagation resulted in 100% clonal offspring with tolerance to a higher concentration of the herbicide, 10 mg L\(^{-1}\). We had 100 mutants, but only one had clonal offspring resistance. The plant had one mutation in the expected domain that has never been reported to be linked with APPs tolerance, T2222I/T2222M. It seems that such a putative mutation can potentially be a real linked mutation because of the nature of the mutation and the dose used. Threonine's uncharged polar nature differs from the hydrophobic nature of isoleucine or methionine. The tolerance at 150 mg/L is the dose of selection used by other authors linked to ACCase mutations [21]. New mutations are being discovered, such as I1879S, P1927Y and W2097G, very near our site of mutation [30]. However, we still cannot fully validate such a hypothesis because tolerance could also result from another detoxication mechanism, and we have not yet studied detoxication mediated by cytochrome P450, glutathione S-transferase, reduced absorption or reduced xylem or phloem translocation [31, 32].

The proposed system of using APP tolerance could allow the selection of small genomic mutations of approximately 1 per 1000 bp, which seems to be valid and a starting point for working with cultivars that farmers are using. We foresee using whole genome sequencing and analysis of the offspring of our mutant to accurately understand the mutation percentage and potential to create variability for the breeding program.

Our finding of using seeds instead of calli, which improved our method from 1 putative plant tolerant to APPs to 31 plants, could help create variability for even more difficult tissue culture cultivars for rice and other plants. The tissue was consistently damaged as the gamma dose increased, having brown nonembryogenic calli, which is common in stressed indica rice cells [33]. Having a window between 50 and 350 Gy also allows flexibility in the kind of mutations resulting from the DNA damage response (DDR) as well as the desired or required dose for a given trait [34].

4. Materials And Methods

4.1. Fluazifop-P-butyl toxicity to embryogenic calli

Embryogenic calli induction was achieved as previously described starting with seeds followed by disinfection with 4% (v/v) NaOCl and water [14]. The calli induction media was composed of mineral
salts and vitamins as described by Murashige and Skoog (MS), with 20 gL\(^{-1}\) sucrose, 0.1 gL\(^{-1}\) hydrolyzed casein and 2.5 mgL\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D). The pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl, and 5.4 gL\(^{-1}\) Gelzan\(^\circledR\) was added as a gelling agent.

To determine the median lethal dose (LD50) of fluazifop-P-butyl, 200 calli per treatment were subcultured into 0, 1, 10, and 100 mgL\(^{-1}\) fluazifop-P-butyl in basal regeneration medium supplemented with 0.5 mg L\(^{-1}\) NAA + 3 mg L\(^{-1}\) 6-BA. All previously mentioned chemicals were supplied by Phytotechnology Laboratories\(^\circledR\) (Shawnee Mission, KS, USA). An autoclave (1.2 ATM. cm\(^{-2}\) and 121°C for 30 min) was used for the sterilizing medium and further dispensed on 94 x 16 mm vented polystyrene Petri dishes (Greiner Bio-One, Fisher Scientific, Waltham, MA, USA) in a laminar flow chamber. Calli browning rates (brown or necrotic/total calli) were recorded as response variables and analyzed in a completely randomized design with a generalized linear model with a Poisson distribution and logit link function. Lethal doses were calculated using probit analysis on IBM SPSS version 27 \[35\] based on calli browning rates.

### 4.2. Fluazifop-P-butyl toxicity to vitroplants

The determination of the toxicity of fluazifop-P-butyl for *vitroplants* was calculated based on regenerated *vitroplants* from embryogenic calli as described next. Embryogenic calli obtained as described in 4.1 were transferred to regeneration medium as described by Sudhakar et al. \[36\] and were constituted by MS mineral salts and vitamins, 20 gL\(^{-1}\) sucrose and 0.3 gL\(^{-1}\) hydrolyzed casein supplemented with 0.5 mg L\(^{-1}\) NAA + 3 mg L\(^{-1}\) 6-BA. To determine the median lethal dose (LD50), the 42-day regenerated *vitroplants* were subcultured into regenerated plants containing 0, 1, 4, 5, 10, 25 and 100 mg L\(^{-1}\) fluazifop-P-butyl. After adding the growth regulators NAA and BA, the pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl, and 5.4 g L\(^{-1}\) Gelzan\(^\circledR\) (Phytotechnology Laboratories\(^\circledR\), Shawnee Mission, KS, USA) was added as a gelling agent. All previously mentioned reagents were supplied by Phytotechnology Laboratories\(^\circledR\) (Shawnee Mission, KS, USA). After properly dissolving the gelling agent, 60 mL of media was dispensed on 475 mL polypropylene WNA Deli Containers supplied by Phytotechnology Laboratories\(^\circledR\) (Shawnee Mission, KS, USA), and afterward, media was sterilized at 1.2 ATM.cm\(^{-2}\) and 121°C for 30 min. Necrotic plants were considered dead after transplantsing into fresh medium with no fluazifop-P-butyl for 100 days and remained necrotic. Lethal doses were calculated using probit analysis on IBM SPSS version 27 \[35\] based on calli browning rates.

### 4.3. Gamma irradiation of embryogenic calli

Embryogenic calli irradiation was achieved at 60 Gy with an Ob-Servo Ignis type gamma irradiator with 24 cobalt 60 source pencils (Institute of Isotopes Co, Ltd., Budapest, Hungary). We tested 1000 embryogenic calli exposed to 60 Gy and transferred them to basal regeneration medium supplemented with 0.5 mg L\(^{-1}\) NAA + 3 mg L\(^{-1}\) 6-BA. A total of 8000 regenerated *vitroplants* were exposed to the same medium containing 5 mg L\(^{-1}\) fluazifop-P-butyl for 21 days. The putative tolerant *vitroplants* had 21 days of recovery in medium with no selection agent, followed by another round of selection of a higher dose of
10 mg-L fluazifop-P-butyl. The tolerant plant was taken to the greenhouse for acclimatization, grown until the 3–5 leaf stage and exposed to 150 ml/L spray fluazifop-P-butyl to validate tolerance, similar to Liu et al. [21].

4.4. Gamma irradiation of seeds

Seed irradiation was achieved at 0 to 500 Gy with an Ob-Servo Ignis type gamma irradiator as described above. We tested 100 seeds per dose, followed by callus induction and regeneration as described previously. Selection of the putative mutants was performed as described in 4.3.

4.5. Molecular markers

A NucleoSpin™ Tissue Kit Macherey-Nagel (Düren, Germany) was used for DNA extraction from 1 mg of nonirradiated lyophilized leaf tissue of Lazarroz FL. Thermo Fisher K1071 (Vilnius, Lithuania) was used for the subsequent PCR following the recommendations of the manufacturer. The primers used in this study are as follows: for 1844 bp amplification of ACC2 Exon 32, ACC2-DF 5'-GGATCATTTGGCCCAAGGGA-3' and ACC2-DR 5'-AGGGCTTGGCAAATCTGAGCT-3'; for 1465 bp amplification of ACC2 Exon 32, ACC2-F 5'-GTGCTCGAATTGGCATAGCAG-3' and ACC2-R 5'-CGTGATTCTTCCCCAGTCCACA-3'. The PCR master mix consisted of a mixture (50 µL) containing 1X Dream Taq Master mix Thermo Fisher (Vilnius, Lithuania), 20 µM of each primer, and 5 µL of DNA (50 ng/µL). The thermocycling program was 95°C for 5 min, 40 cycles at 95°C for 45 s, 55°C for 100 s and 72°C for 1 min, and a final cycle of 72°C for 7 min.

PCR and sequencing of the matK and rbcL genes of the mutants were performed as reported for the control sequences MZ558335 and MZ558334 [14].

5. Conclusions

In our previous work [14], we suggested the potential of gamma radiation to trigger innovation of rice lines used by farmers. Here, we proved the model with a specific trait based on chemical selection and showed the potential way to start searching for other mutations of interest on desired traits for commercially used varieties.

Declarations


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Informed Consent Statement: Not applicable.

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

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Figures
Figure 1

Regenerated calli of *vitroplants* of the Lazarroz FL cultivar on MS medium with 3 mg L\(^{-1}\) BA under fluazifop-P-butyl stress after 21 days of culture. **A)** 5 mg L\(^{-1}\) fluazifop-P-butyl, **B)** 4 mg L\(^{-1}\) fluazifop-P-butyl, **C)** 1 mg L\(^{-1}\) fluazifop-P-butyl, **D)** necrotic *vitroplants* subcultured in fresh medium for 21 days to verify mortality after exposure to 5 mg L\(^{-1}\) and 4 mg L\(^{-1}\) fluazifop-P-butyl stress, as shown in (A) and (B). Note that all remained necrotic and had no sprouting or green spots.

Figure 2

Tolerant *vitroplant* of the Lazarroz FL cultivar obtained by gamma mutagenesis on calli at 60 Gy on MS medium with two rounds of selections, the first at 5 mg L\(^{-1}\) of fluazifop-P-butyl stress after 21 days of culture, 21 days of recovery with no stress agent, and another round of 21 days of 10 mg L\(^{-1}\) of fluazifop-P-butyl stress. Note that nontolerant plants became necrotic at 5 mg L\(^{-1}\) fluazifop-P-butyl stress after 21 days of culture, while only 1 out of 100 putative tolerant *vitroplants* remained green during the second round of selection at 10 mg L\(^{-1}\) fluazifop-P-butyl stress.

Figure 3

DNA markers used to identify exon 32 of the *ACC2* gene in the nonirradiated Lazarroz FL rice variety. Note in (A) the sequence of exon 32 is shown in green, and the locations of the primers used for PCR and sequencing are shown in black and blue boxes. In the red box is the site corresponding to the biallelic mutation. **B)** Sequencing electropherogram and corresponding amino acid mutation putative changes.

Figure 4

DNA markers (A) *matK* and (B) *rbcL* genes of the mutants in comparison with the control reported sequences MZ558335 and MZ558334. Note in green the biallelic mutations G/T in *matK* resulting in isoleucine change to methionine and GT/AG coding for tyrosine that may change into serine in a mutation in *rbcL*.

Figure 5
Gamma radiation optimization using Lazzaroz FL seeds as a starting material and further calli induction, regeneration, and selection in two rounds of fluazifop-P-butyl selection at 5 and 10 mg L\(^{-1}\). Note the optimization window of gamma exposure ranges from 50 to 350 Gy, allowing predictable calli induction and regeneration, as well as putative tolerant *vitroplants*. 