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RAPD analysis of herbicide-resistant Brasilian rice lines produced via mutagenesis

S.S. Sandhu[1], Cândido R. Bastos[2], Luiz Ernesto Azini[2], Augusto Tulmann Neto[3] and Carlos Colombo[4]

[1]Department of Biological Sciences, R.D. University, Jabalpur, India
[2]Centro de Graníferas, Instituto Agronômico, Campinas, SP, Brasil
[3]Centro de Energia Nuclear na Agricultura (CENA), Universidade de São Paulo, Caixa Postal 96, 13400-970 São Paulo, SP, Brasil
[4]Centro de Genética, Biologia Molecular e Fitoquímica, Instituto Agronômico, Caixa Postal 28, 13001-970 Campinas, SP, Brasil
Corresponding author: C. Colombo
E-mail: ccolombo@iac.br

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ABSTRACT. Over the last two decades, mutational techniques have become one of the most important tools available to progressive rice-breeding programs. In a mutation-breeding program initiated in 1999 at the Instituto Agronômico of Campinas, SP, Brazil, a rice line, IAC103, was selected for mutational studies with gamma radiation and ethyl methyl sulfonate mutagenesis, with the aim of developing a herbicide-resistant crop. After mutagenesis, surviving plants were exposed to glufosinate to check for herbicide resistance, which was examined up to the second generation. A detailed RAPD analysis was made of the resistant plants. Eighty Operon technology primers were tested and 10 were selected for a detailed study of RAPD markers that could tag herbicide resistance genes. Resistant and susceptible lines produced variation in the RAPD patterns and certain bands were found only in certain lines. These results suggest genetic ligation that will be confirmed through a genetic segregation study.

Key words: Rice, Mutagenesis, Gamma radiation, Glufosinate, Ethyl methyl sulfonate, EMS, Segregation, Herbicide resistance

INTRODUCTION

Herbicide treatment of crops allows economically viable weed control and also provides cost-effective increases in the productivity of agricultural crops. Most of the herbicides currently in use combine good effectiveness with suitable production costs, are nontoxigenic and are rapidly biodegraded, hence they are "eco-friendly". But some lack selectivity, therefore limiting their use to preemergence applications in the field. Breeding herbicide resistance into the crop is a new means to confer selectivity and enhance crop safety and production (Guttieri et al., 1992; Boutsalis and Powles, 1995; Hervieu and Vancheret, 1996).

Herbicides generally affect processes that are unique to plants, e.g., photosynthesis or amino acid biosynthesis. These processes are shared by both weeds and crops. Therefore, developing herbicide-resistant crops is very difficult and a challenge to scientists, because every year a number of new herbicides are discovered. Generally two approaches to engineering herbicide resistance are used. In the first of these the target molecules in the cell are either rendered insensitive or are over-produced. In the second approach a metabolic pathway that degrades or detoxifies the herbicide is introduced into the plant (Tsaftaris, 1996).

Phosphinothrin (PPT), commercially known as glufosinate or Round Up®, is an irreversible inhibitor of glutamine synthetase in plants and bacteria. Bialaphos, produced by *Streptomyces hygroscopicus*, consists of PPT and two alanine residues. When these residues are removed by peptidases the herbicidal component, PPT, is released. To prevent self-inhibition of growth, bialaphos-producing strains of *S. hygroscopicus* produce an acetyltransferase that inactivates PPT by acetylation. The bar gene that encodes acetylase has been introduced into many crops, including rice (Rathore et al., 1993; Jiang et al., 2000), to make transgenic herbicide-resistant crops. But this resistance can also be achieved by classical genetics and selection for over expression of glutamine synthetase, to make stable resistant lines instead of a plant modified by genetic engineering.

We report the development of herbicide resistance by mutation and selection, using RAPD markers, for "Round Up" herbicide resistance in Brazilian rice cultivars.

MATERIAL AND METHODS

Rice varieties

One rice line very susceptible to glufosinate, IAC 103, and 20 resistant lines (218-1,3-7; 219-1,3,5-10; 222-1 to 222-3; 197-1 to 197-2 and 165-3) were selected for analysis and designated as R1 to R21. These lines are continuously maintained in the greenhouses and research fields of the Instituto Agronômico of Campinas (IAC), Campinas, SP, Brazil, where they are periodically checked for herbicide resistance.

Glufosinate application in the greenhouse

The 21 rice lines were grown to the third or fourth leaf stage in the greenhouse and were tested for their response to glufosinate by spraying with a 1.0% (v/v) solution plus 0.1% (v/v) Tween 20. The actual concentration of PPT used was 480 mg/l. Resistant and susceptible plants were scored on the 15th day after treatment.

DNA isolation and amplification

For DNA isolation the methods of Sandhu et al. (2002) were followed. Fresh leaves (≈2 g) were ground in liquid nitrogen and 300-400 mg of ground tissue transferred to polypropylene centrifuge tubes containing 15 ml of pH 8.0 extraction buffer (0.1 M Tris-HCl, 1.25 M NaCl, 0.02 M EDTA), 2% alkyltrimethylammonium bromide and 1% β-mercapto-ethanol. The mixture was slowly stirred for 90 min at 65ºC and an equal volume of 24:1 chloroform: isoamylalcohol added twice. The mixture was centrifuged at 10,000 *g* for 10 min and the supernatant transferred to a clean plastic tube containing 100 µl of a 10 mg/ml RNAse solution and incubated at 37ºC for 30 min, after which DNA pellets were obtained by adding 0.8 volumes of isopropanol. After washing with 70% ethanol, the DNA pellets were vacuum dried and dissolved in 200 µl of pH 8.0 TE buffer (10 mM Tris-HCl, 1 mM EDTA) and the quality and concentration of DNA fragments evaluated by electrophoresis in 0.8% agarose gels. This process was repeated for each of the 21 rice lines.

PCR conditions

PCR was carried out in a 25-µl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 10 ng template DNA, 1.0 µM primer, 100 µM of each dNTP and 1 unit of Taq polymerase. DNA was amplified in a Primus 96 Plus thermocycler (MWG-Biotech, Germany) at 96ºC for 4 min followed by 45 cycles of 1 min at 94ºC, 1 min at 35ºC, 1.3 min at 72ºC and a final stage of 7 min at 72ºC. The mixtures were maintained at 4ºC prior to analysis. For electrophoretic analysis 2.5 µl of 0.5% 1:2:1 bromophenol:blue:glycerol buffer was added and the amplification products loaded onto a 1.5% agarose gel in 1X TAE electrophoresis buffer. The gels were stained with ethidium bromide and photographed and analyzed using a Pharmacia Biotech gel documentation system. About 80 primers (Operon Technology, USA), OPF 1-20, OPJ 1-20, OPG 1-20 and OPK 1-20, were tested for polymorphism and differentiation in the 21 rice cultivar lines.

Germination test of mature S_1 seeds

Seeds of self-pollinated S-0 mutated plants were designated as S_1 seeds. In the first experiment, herbicide resistance was examined by germination of S_1 seeds on RT medium (Murashige and Skoog, 1962) containing herbicide, RT medium-MS salts and eight vitamins, 20 g/l Phytagel (Sigma), pH 5.8. The actual concentrations of PPT used were 5 and 10 mg/l (Figure 12A-C). Germinated seeds were transferred to greenhouse (Figure 13A,B) and tested further for response to Round Up in a 1.0% (v/v) aqueous solution (Figure 13C).

Mutational studies on rice lines

About 5,000 seeds of Brazilian rice line IAC-103 (*Oryza sativa* var. Indica) were treated in 12 sets of mutagenic exposures (Table 1). These seeds were designated as the S-0 generation. Chemical mutagenesis was provoked by using ethyl methyl sulfonate (EMS; Sigma, USA), either alone or with gamma irradiation. Gamma radiation was applied with a Gammacell-220 machine (USA) at CENA, University of São Paulo, SP, Brasil. The doses used were 200, 250 and 300 Krad/h with a ^{60}Co source, with or without exposure to the chemical mutagen, EMS, applied at two dosages: 0.5 ml/l or 1.5 ml/l.

Standard methodology, as described by Camargo et al. (1996), Tisseli et al. (1996) and Tulmann et al. (2001) for gamma radiation and by Zhu et al. (1995) for EMS mutagenesis, was followed. After treatment all the seeds were germinated in a greenhouse and the percent survival was noted (Table 1). Surviving plants were transplanted to the IAC Rice Field, in Pindamonhangaba, SP, Brasil.

Table 1. Percent survival after mutagenesis.

Set No.	Mutagenesis sets		No. of seeds treated	No. of surviving plants	Survival in the field (%)
	Gamma radiation*	EMS in ml/l			
1	0	0.0	5000	4800	96.00
2	200	0.0	5000	3922	78.44
3	250	0.0	5000	3496	69.92
4	300	0.0	5000	3000	60.00
5	0	0.5	5000	3525	70.50
6	0	1.5	5000	3000	60.00
7	200	0.5	5000	3400	68.00
8	200	1.5	5000	880	17.60
9	250	0.5	5000	2544	50.88
10	250	1.5	5000	1701	34.02
11	300	0.5	5000	1140	22.80
12	300	1.5	5000	2147	42.94

*Gamma radiation exposure unit in Krad/h. EMS = ethyl methyl sulfonate.

Three panicles per plant were harvested from these mutated plants and the seeds were designated as the S_1 generation. Approximately 5,000 seeds from different sets of mutations were germinated in a greenhouse and then transplanted to the field, giving a total of 320 lines. They were exposed to the herbicide at the 4-5 leaf stage. The first application of glufosinate was 1.5 l/ha followed by a second application of 2 l/ha, 15 days after the first treatment. A third round was applied seven days after the second application. Surviving plants were grown until they produced seed, and three panicles per plant were harvested from each of them. These seeds were designated as the S_2 generation. The S_2 generation seeds were then germinated, subjected to herbicide resistance tests, and then planted in the rice field for later DNA analysis.

Data analysis

Only clearly amplified fragments were analyzed. Scores of 1 (present) or 0 (absent) were used to form a matrix. Simple matching coefficients (Sokal and Michener, 1958) were obtained to perform cluster (UPGMA) and principal coordinate analyses (PcoA). The variation set was represented by PcoA scores (99% total variation) based on RAPD markers. NTSYS-pc software (Rohlf, 1993) was used for the calculations.

RESULTS AND DISCUSSION

Screening of primers

About 80 primers were tested on the 21 rice lines and among these, 13 were selected as suitable on the basis of good DNA amplification, with at least three sharp electrophoretic bands. Among these 13 primers, three, namely OPG18, OPG19 and OPF5, gave 100% polymorphism in all the 21

rice lines. The other 10 primers: OPK12, OPK19, OPF17, OPF19, OPJ7, OPJ9, OPJ16, OPJ17, OPG8 and OPG17 gave clear variations in the electrophoretic profile of the RAPD analysis of the 21 rice lines, and were thus considered to be suitable as RAPD markers for herbicide resistance in these lines.

RAPD analysis

In the comparison of resistant and susceptible lines, several DNA fragments produced variation in the RAPD patterns. In line R1 (IAC-103), $K19_{1050}$ (Figure 1) was found to be a strong marker for the susceptible line, but another marker, $F17_{1300}$ (Figure 2) was also found in this line, which corresponded to a marker of the resistant line, R19 (197-1). The variation in the R4 (218-4) line was the missing $G17_{900}$ (Figure 3) band, which was found in the other lines. Another marker band not found in R4 was $G17_{500}$, which corresponds to R19 (197-1), $G17_{700}$ corresponded to R5 (218-5) and $G17_{1300}$ corresponded to R19 (197-1) and R20 (197-2); they could be treated as markers of resistance in these lines. The R5 (218-5) line varied at $G17_{700}$, which also corresponds to R4 (218-4).

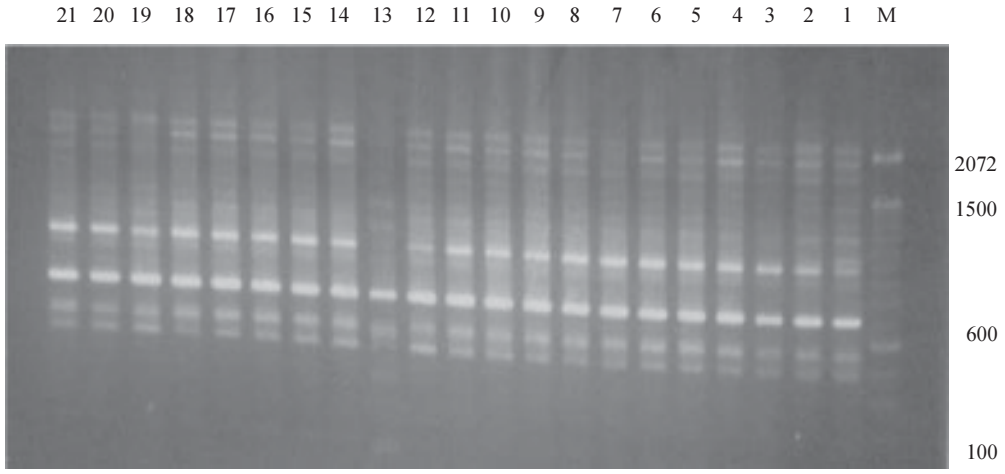

Figure 1. Ethidium bromide-stained electrophoretic profile of RAPD markers $OPK19_{350}$, $OPK19_{2800}$ and $OPK19_{3000}$ in rice line R13, in comparison with 20 other rice lines. M = size marker.

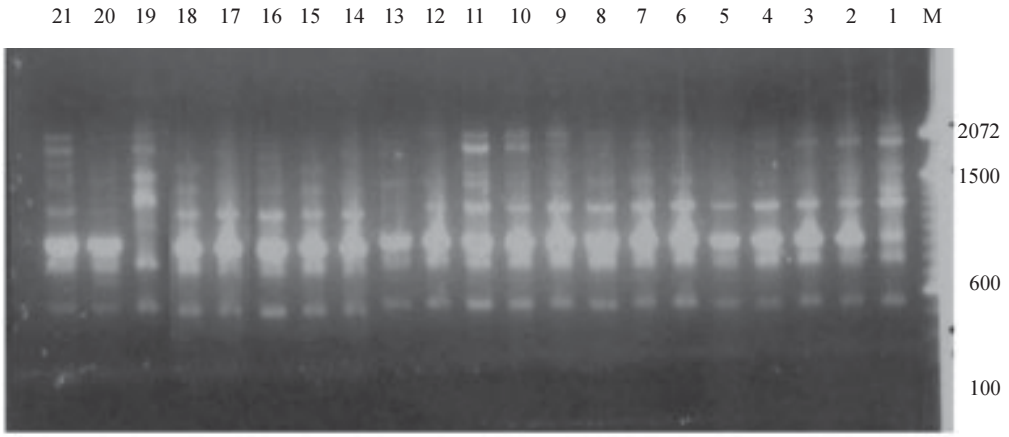

Figure 2. Ethidium bromide-stained electrophoretic profile of RAPD markers $OPF17_{550}$ and $OPF17_{600}$ in rice line R13, in comparison with 20 other rice lines. M is the 100-base pair DNA marker.

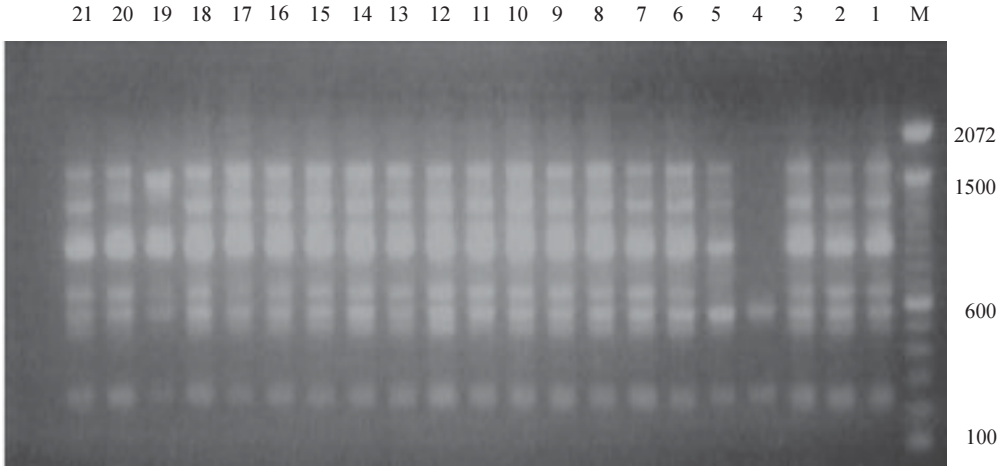

Figure 3. Ethidium bromide-stained electrophoretic profile of RAPD markers $OPG17_{350}$ and $OPG17_{2800}$ in rice line R13, in comparison with 20 other rice lines. M = size marker.

The largest variation in the resistant and susceptible lines was observed in the most susceptible lines, R1(IAC-103) and R13 (219-8). Seven fragments, $OPJ7_{350}$ (Figure 4), $OPJ17_{2600}$ (Figure 5), $OPK12_{550}$, $OPK12_{600}$, $OPK12_{900}$, $OPK12_{1100}$ (Figure 6) and $OPK19_{400}$, were present in R13 and were not present in the susceptible line or in the other 19 resistant lines. At the same time, the following bands were missing in R13: $K12_{650}$, $K12_{700}$, $K12_{850}$, $K12_{1000}$, $K19_{950}$, $K19_{1700}$, $K19_{1900}$, $K19_{2300}$, $OPJ7_{2800}$, $OPJ7_{3000}$, $OPJ9_{700}$, $OPJ9_{800}$, $OPJ9_{1000}$ (Figure 7), $OPJ16_{300}$, $OPJ16_{1200}$ (Figure 8), $OPF17_{1200}$, $OPF17_{1200}$, $OPF19_{600}$ (Figure 9) and $OPF19_{900}$.

Some variation was also observed in R14, as the $OPJ17_{1600}$ and $OPJ17_{2400}$ bands were absent. R19 exhibited variation as it had the $OPF17_{1300}$ band, which corresponded to susceptible line R1. $OPG8_{1800}$ (Figure 10) was present in R19, which corresponds to the resistance marker in R20. $OPF17_{700}$ was found to be a separating marker for R20.

The following DNA bands were absent in R19: $OPK12_{850}$, $OPK19_{1900}$, $OPG17_{500}$, $OPG17_{1300}$, while $OPG8_{1800}$ and $OPF17_{1300}$ were present. $OPG8_{1800}$ and $OPF17_{700}$ were present in the R20 line,

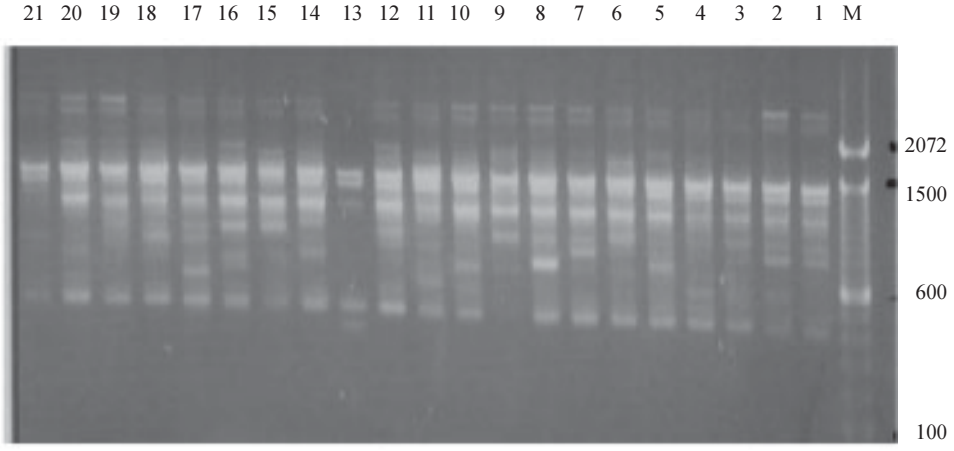

Figure 4. Ethidium bromide-stained electrophoretic profile of RAPD markers $OPJ7_{550}$, $OPJ7_{600}$, $OPJ7_{900}$ and absence of $J7_{650}$, $J7_{850}$, $J7_{1000}$ in rice line R13, in comparison with other 20 rice lines. M = size marker.

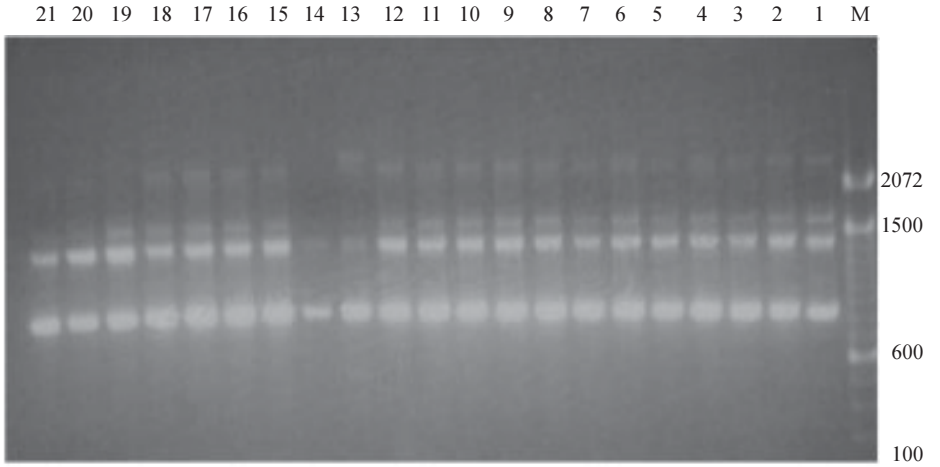

Figure 5. Ethidium bromide-stained electrophoretic profile of RAPD markers $OPJ17_{350}$, $OPJ17_{2800}$ and $OPJ17_{3000}$ in rice line R13, in comparison with 20 other rice lines. M = size marker.

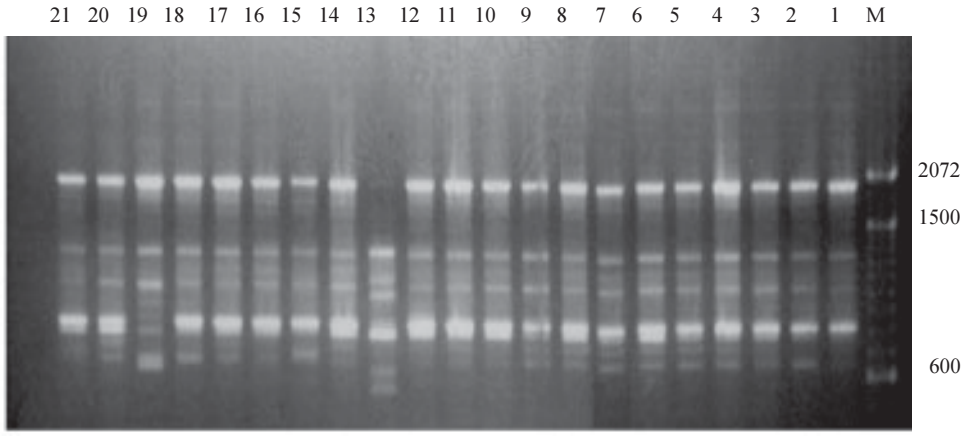

Figure 6. Ethidium bromide-stained electrophoretic profile of RAPD markers $OPK12_{550}$, $OPK12_{600}$ and $OPK12_{900}$ and absence of $K12_{650}$, $K12_{850}$ and $K12_{1000}$ in rice line R13, in comparison with 20 other rice lines. M is the DNA marker (100-bp DNA ladder).

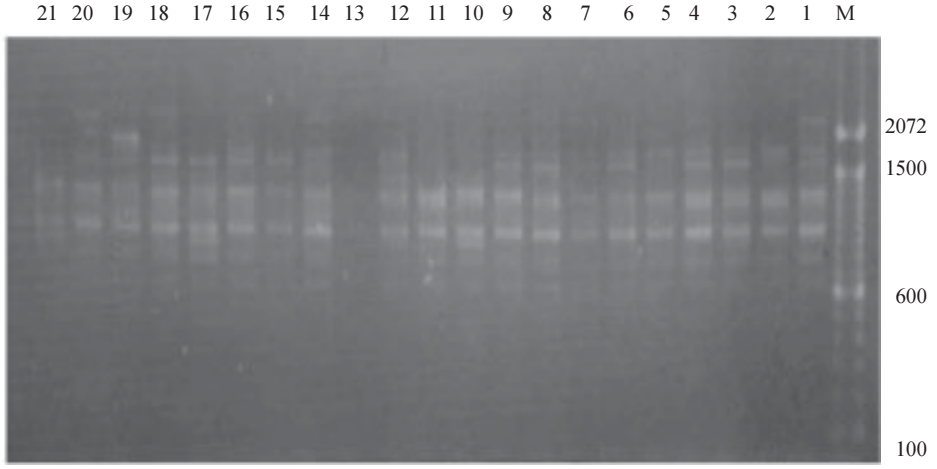

Figure 7. Ethidium bromide-stained electrophoretic profile of RAPD markers $OPJ9_{350}$, $OPJ9_{2800}$ and $OPJ9_{3000}$ in rice line R13, in comparison with 20 other rice lines. M = size marker.

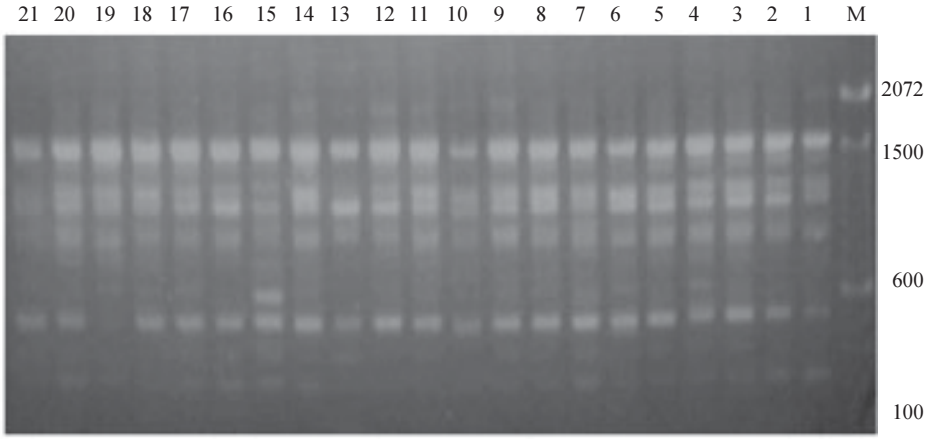

Figure 8. Ethidium bromide-stained electrophoretic profile of RAPD markers $OPJ16_{550}$, $OPJ16_{600}$ and $OPJ16_{900}$ and absence of $OPJ16_{650}$, $OPJ16_{850}$, $OPJ16_{1000}$ in rice line R13, in comparison with 20 other rice lines. M = size marker.

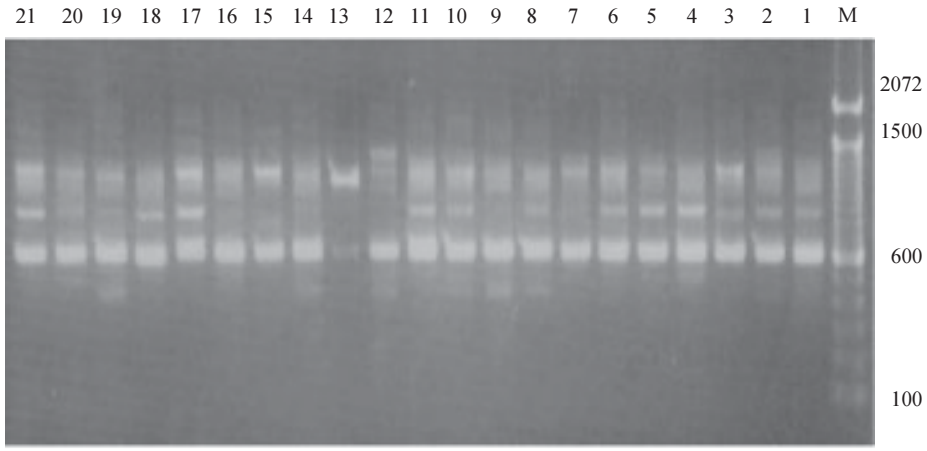

Figure 9. Ethidium bromide-stained electrophoretic profile of RAPD marker $OPF19_{350}$ in rice line R19, in comparison with 20 other rice lines. M = size marker.

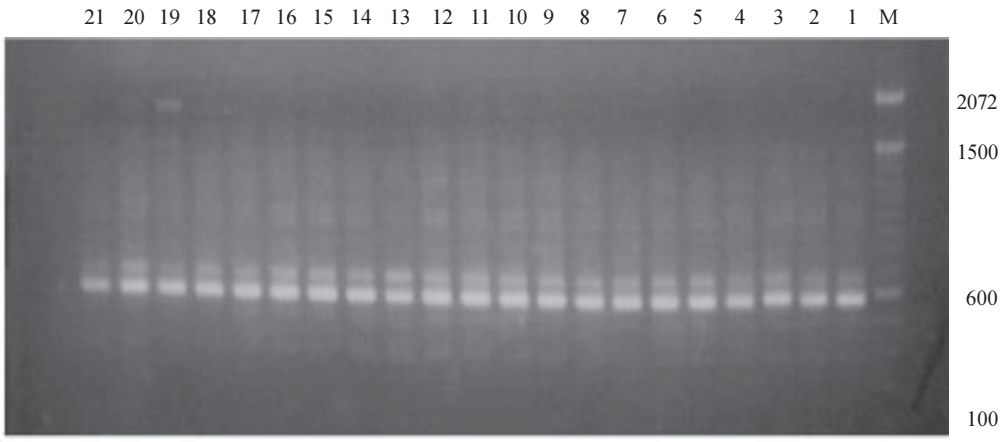

Figure 10. Ethidium bromide-stained electrophoretic profile of RAPD marker $OPG8_{1800}$ in rice line R19, in comparison with 20 other rice lines. M = size marker.

whereas $OPG17_{1300}$ was absent in R20. The R15 line showed variation, as $OPJ16_{600}$ was present.

The Jaccard coefficient results (Table 2 and Figure 11) also indicated that line R13 has the highest variability among the 21 lines.

Molecular markers are a more stable and informative alternative to isoenzymes. According to Cohen et al. (1991) and Colombo et al. (2000), these markers are more efficient

Table 2. Jaccard coefficients showing variation among 21 rice lines based on 96 RAPD bands.

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16	R17	R18	R19	R20	R21
R1	1.00																				
R2	0.93	1.00																			
R3	0.90	0.97	1.00																		
R4	0.81	0.86	0.88	1.00																	
R5	0.85	0.92	0.94	0.90	1.00																
R6	0.87	0.95	0.97	0.88	0.94	1.00															
R7	0.88	0.96	0.96	0.85	0.93	0.95	1.00														
R8	0.89	0.96	0.97	0.86	0.95	0.95	0.93	1.00													
R9	0.87	0.95	0.96	0.84	0.91	0.93	0.95	0.96	1.00												
R10	0.85	0.89	0.91	0.84	0.92	0.91	0.91	0.91	0.89	1.00											
R11	0.85	0.88	0.89	0.86	0.91	0.92	0.89	0.87	0.86	0.93	1.00										
R12	0.84	0.92	0.92	0.86	0.89	0.92	0.93	0.92	0.93	0.89	0.86	1.00									
R13	0.57	0.61	0.62	0.56	0.62	0.62	0.64	0.60	0.61	0.63	0.62	0.61	1.00								
R14	0.85	0.89	0.88	0.82	0.86	0.88	0.89	0.91	0.88	0.86	0.85	0.87	0.57	1.00							
R15	0.87	0.93	0.92	0.81	0.87	0.92	0.93	0.90	0.91	0.86	0.87	0.91	0.60	0.89	1.00						
R16	0.90	0.97	0.97	0.86	0.94	0.95	0.97	0.97	0.95	0.88	0.87	0.91	0.61	0.91	0.93	1.00					
R17	0.89	0.96	0.95	0.87	0.89	0.92	0.93	0.92	0.91	0.91	0.90	0.87	0.60	0.89	0.92	0.95	1.00				
R18	0.92	0.97	0.97	0.88	0.92	0.95	0.96	0.95	0.93	0.88	0.87	0.89	0.61	0.91	0.92	0.97	0.96	1.00			
R19	0.85	0.84	0.83	0.77	0.78	0.81	0.82	0.83	0.82	0.82	0.81	0.78	0.56	0.79	0.81	0.83	0.83	0.83	1.00		
R20	0.84	0.88	0.89	0.91	0.90	0.89	0.88	0.88	0.86	0.88	0.87	0.87	0.60	0.88	0.86	0.89	0.89	0.91	0.82	1.00	
R21	0.85	0.89	0.89	0.83	0.89	0.89	0.89	0.87	0.86	0.93	0.89	0.84	0.62	0.83	0.87	0.89	0.92	0.89	0.80	0.88	1.00

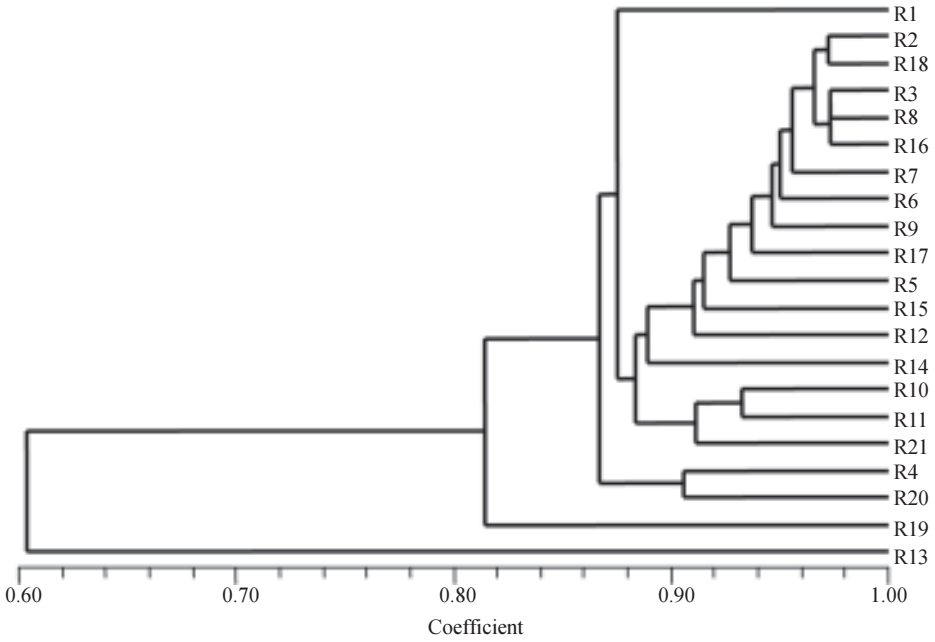

Figure 11. Dendrogram based on UPGMA model calculated from genetic distance among 20 (R2-R21) rice lines, employing 96 RAPD markers.

for examining the genetic diversity of collections. Molecular markers have been used to study the genetic diversity of various species (Williams et al., 1993).

Germination test of mature seeds from the S_1 and S_2 generations

Glufosinate resistance was first demonstrated by the germination of S_1 seeds in 5 mg/l and 10 mg/l glufosinate (Figure 12A-C). The plants were then transferred to a greenhouse (Figure 13A,B) and were found to be resistant to a 1.0% (v/v) aqueous solution of glufosinate. The resistance was also similar in the S_2 generation in RT medium as well as in the greenhouse. This

Figure 12. Seed germination of the S_1 generation on RT medium containing the herbicide glufosinate. (A) Germination in 5 mg/l glufosinate, (B) germination in 10 mg/l glufosinate, (C) S2 generation seeds on RT medium containing 10 mg/l glufosinate (left hand sets of tubes are non-mutated and right hand sets are mutated in all cases).

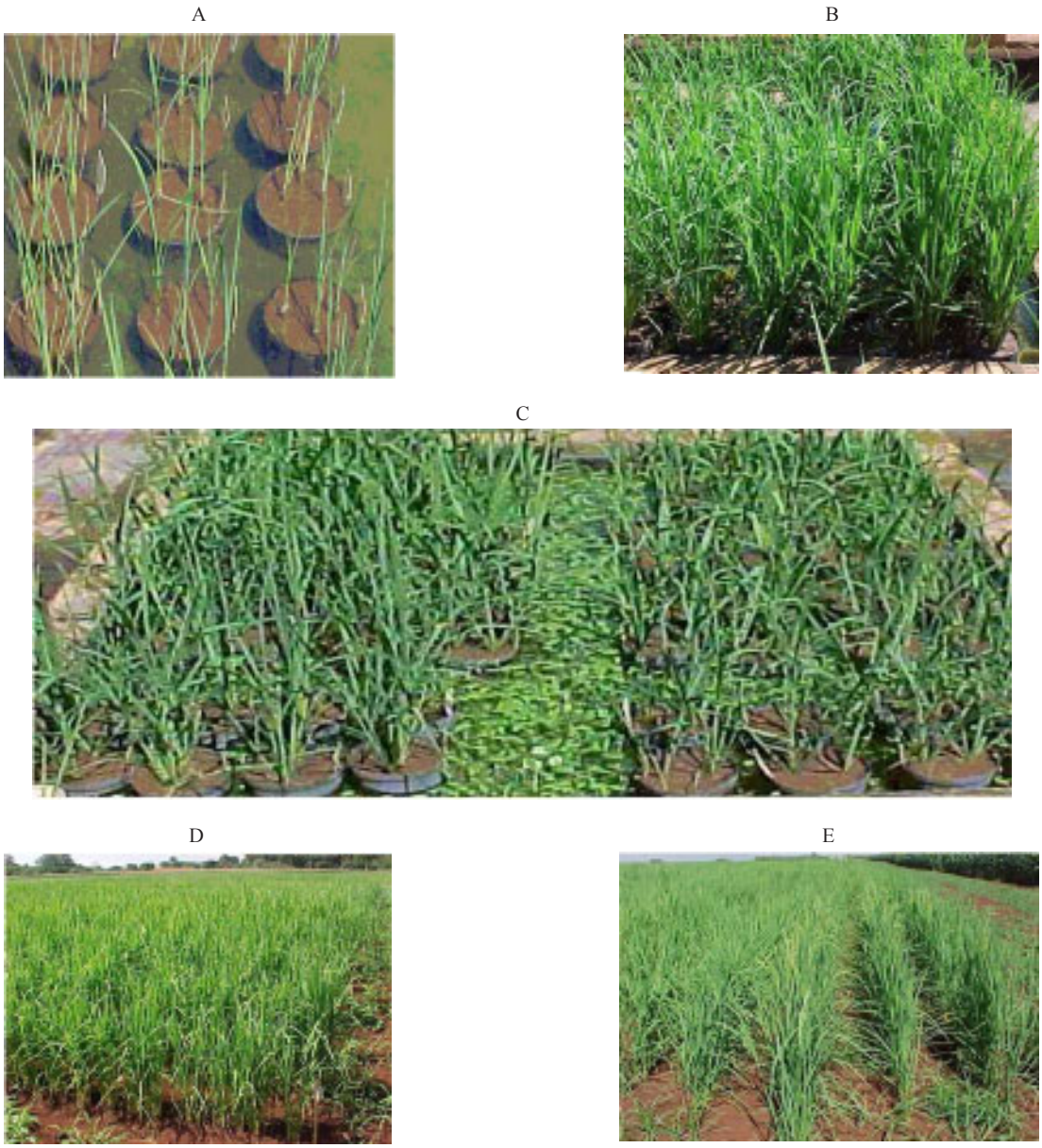

Figure 13. Greenhouse and field tests of herbicide (glufosinate = Round Up) resistance in the S_1 and S_2 generations. (A) Plantlets regenerated from tissue culture media and transferred to the greenhouse, (B) S_1 generation plants in the greenhouse after herbicide application, (C) S_2 generation plants in the greenhouse, (D) S_1 generation plants in the field, (E) S_2 generation plants in the field.

showed that herbicide resistance is transferred to the second generation. The first and second generation plants were also resistant in the field evaluation at the IAC rice field station (Figure 13D,E).

Over the last few decades mutational techniques have become among the most important tools available to progressive plant-breeding programs. Mutation and subsequent selection, with further breeding, has given stable and dominant rice lines with desired crop characters. Although we have not cloned the *bar* gene for PPT herbicide resistance in the rice, we were able to use classical genetics, mutation and selection on 20 rice lines to develop resistance

against glufosinate. This resistance may be due to an over expression of glutamine synthetase, which would nullify the effect of PPT in the plants (Old and Primrose, 1998).

Availability of a codominant RAPD marker for herbicide "PPT" resistance will be extremely useful for homologous resistance gene-pyramiding studies in these 21 rice lines to breed for herbicide resistance in Brazilian rice lines.

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