In vivo and *in vitro* application of a mutagen to induce herbicide resistance in Sri Lankan rice (*Oryza sativa* L.) varieties

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Summary Inducing herbicide resistance (HR) in cultivated rice is a novel approach to enhance selectivity and crop safety. Studies on induced HR in Sri Lankan rice varieties are limited and further research are required to include HR rice in a cropping program. Ethyl methyl sulfonate (EMS) is a chemical mutagen used for functional mutations in plants. The present study is an attempt to develop HR rice lines through conventional breeding methods using the chemical mutagen EMS. A detailed AFLP analysis was made to identify molecular markers for HR induced varieties. *In vivo* seed mutation induced HR in 14 varieties and *in vitro* mutated calli also exhibited resistance to glyphosate. E12M32 was found to be a specific AFLP marker for induced HR varieties.

Keywords Herbicide resistance, glyphosate, ethyl methyl sulfonate (EMS), seed culture, AFLP analysis.

INTRODUCTION

Effective weed control in any crop production system is a prerequisite if high yield and good quality are to be achieved. Introduction of herbicide resistant (HR) rice would improve efficiency of weed management thus rice growers throughout the world could benefit from HR cultivars. There is evidence that HR crops can bring significant benefits to farmers, consumers and the environment (Duke *et al*. 2002). Over the last two decades, mutational techniques have become one of the most important tools available to progressive rice-breeding programs (Sandhu *et al.* 2002) and most of the herbicide-tolerant mutants were developed through chemical mutagenesis followed by herbicide selection (Tan *et al.* 2005). Among the chemical mutagens, ethyl methyl Sulfonate (EMS) is a strong chemical mutagen which can make the chromosome structure different (Barro *et al.* 2001). Plant tissue culture represents the simplest of the biotechnologies available at present for crop improvement (Sudhakar *et al.* 2009) and mutagenesis techniques in tissue culture are commonly used in developing herbicide resistant (HR) rice varieties. Studies on induced herbicide resistance in Sri Lankan rice varieties are limited and the present study focused on inducing HR in rice by *in vivo* and *in vitro* EMS mutation.

MATERIALS AND METHODS

Materials Bg94-1, Bg250, Bg300, Bg304, Bg305, Bg352, Bg357, Bg359, Bg360, At362, Bw364, Ld365, Bg366, Bg369, Bg379-2, H4, Bg403, Bg450, Bg 454 inbred-developed rice varieties and five traditional varieties (KaluHeenati, Kurulu thuda, Suwadal, Rathhal, Madel, and Pachchaperumal) were collected from Rice Research Development Institute at Bathalagoda, Ambalanthota, Bombuwela and Labuduwa, Sri Lanka for the study.

Method 1 – Seed mutation Seeds from each variety were pre-soaked in distilled water for 24 h at room temperature and exposed to 4.5mmoll^{-1} EMS for 12 hours. The seeds were then washed with running tap water and allowed to leach the residual chemicals and let it to germinate. The germinated seedlings were transferred into mud pots and glyphosate (0.5 g a.e. L$^{-1)}$ was applied at two weeks after sawing (S_0 generation). Following the application of glyphosate, the dead plants were considered as susceptible to the herbicide and surviving plants with a substantial growth were considered as resistant to herbicide. For each rice variety, the number of resistant plants and percentage resistance was calculated. Plants with ≥50% resistance to glyphosate (0.5 g a.e. L$^{-1)}$ were arbitrarily considered as resistant varieties. The morphological and yield data were collected from the resistant varieties and seeds of self-pollinated S_0 generation of mutated plants were designated as S_1 seeds. Three panicles per S_1 plants were harvested from mutated resistant surviving plants and their resistance to glyphosate was evaluated using the same procedure (S_2).

Descriptive statistics were performed on the dataset. The mean and standard deviation was computed and ANOVA test were used to compare the mean. One-way-analysis of variance (ANOVA) was performed on agro-morphological characters. All statistical analyses were carried out using SAS Version 9.2 (SAS 2008).

Method 2 – AFLP molecular study AFLP analysis was carried out for the mutated seeds using 16 AFLP primer combinations**.** Genomic DNA was extracted from seven day old leaves using a PhytoSpin™ plant genomic DNA extraction kit (Ceygen Biotech). The protocol followed the traditional procedure described by Vos *et al.* (1995) with several modifications.

Method 3 – Callus mutation A glyphosate susceptible rice variety, Bg250, was selected for the study (Weerakoon *et al.* 2013) and glyphosate resistant variety, Pachchaperumal, was taken as the reference/control. Mature rice seeds were de-husked and surface disinfected after soaking for 1 minute in 70% ethanol and subsequently seeds were washed with distilled water and sodium dodecyl benzene sulphonate (Teepol) for 1 minute. Bleach-sterilization was carried out for 20 minutes in 50% commercial bleach. Following three rinses with sterilized water, seeds were cultured (10 seeds per Petri dish) in solid MS medium for callus induction (Murashige and Skoog 1962). Cultures were incubated in a culture room maintaining at 25°C ± 2°C in complete dark. For the mutation process three weeks old callus masses were transferred into Falcon™ 50 mL tubes which containing 30 mL, liquid MS media without 2, 4-D.

Subsequently, the tubes were immediately mutagenised by adding different concentrations of EMS (0.0, 0.1%, 0.2%, 0.3%and 0.4% v/v) (Sigma-Aldrich). Each treatment consists of 10 calli and there were three replicates for each treatment. Culture tubes were covered with aluminium foil and placed on an orbital shaker (150 rpm) for two hours. The incubated calli were rinsed 10 times with MS liquid medium and transferred to new tubes containing MS liquid media with 2,4-D and shaken (120 rpm) for two more days (28°C ± 2°C, in darkness). The treated calli were subcultured on to a basal media of MS containing NAA and kinetin. Initial screening of viable/non-viable calli was carried out based on the changing from creamy white colour to brown. The browning calli were considered as susceptible to EMS and white calli with substantial growth in the subculture media were selected as resistant to EMS. To confirm viability, a tetrazolium chloride (1% TTC) test was conducted (Towill *et al*. 1975). Data were arbitrarily collected on callus and they were expressed as percentage response against total treated callus. Sub-cultured media was treated with 0.02 g a.e. L^{-1} glyphosate solution after ten days and observed the characteristics of the callus compared to untreated callus. Regenerated plantlets were also exposed to glyphosate and evaluated for their HR.

RESULTS AND DISCUSSION

Results indicated that fourteen varieties increased their resistance to glyphosate (Bg94-1, Bg352, Bg359, Bg360, At362, Bw364, Ld365, Bg366, Bg379-2, Bg403, Bg454, Kalu heenati, Pachcha perumal and Madel) in the first generation-S_0 (Figure 1). The pair-wise statistical analysis of variance of agro-morphological characters clearly showed significant differences ($P \leq 0.05$) between control and treated plants. The percentage of resistance in S_1 was almost similar in all the varieties except Bg454 and 'Madel'. In contrast, there were statistically significant differences ($P \leq 0.05$) between the EMS-mediated-mutated rice plants with non-mutated rice plants related to agro-morphological and yield characters such as plant height, number of panicles per plant, seeds per panicle and 1000 grain weight. However, differences in number of leaves per plant and number of tillers per plant were not statistically significant when comparing mutated rice plants with non-mutated rice plants.

AFLP analysis of EMS mutated rice lines indicated variations in several fragments. Out of sixteen primer combinations, five primer sets (E10M31, E10M33, E11M32, E12M32, and E12M33) indicated the possibility to be used in differentiating HR-varieties. Among those the 78 bp fragment of E11+M13 primer (TGT AAA ACG ACG GCC AGT GAC TGC GTA CCA ATT CAC and M32 primer (GAT GAG TCC TGA GTA AAA) was identified as a specific marker for the resistant lines. That marker was common to all the HR induced varieties after mutation. Cluster analysis based on UPGMA for the mutated rice varieties indicated that control and mutated lines are genetically different to each other. The varieties which increased their resistance after the mutation appeared more or less in the same cluster. The varieties with induced HR after mutation clustered in separate groups (Figure 2).

In vivo application of EMS revealed that the viability of seed-derived callus decreased when the EMS concentration was increased. At 0.2% v/v EMS concentration, 60% of the calli survived and the same concentration was selected for further studies. One week after EMS treatment, no noticeable differences were detected in calli between control and treatments. During 7–10 days after mutation, 0.3 and 0.4 of treated calli showed some degree of browning. Subsequent to the application of glyphosate, EMS-mutated calli indicate a positive response for TTC test. Sixty percent (60%) of the calli turned into red colour following the staining process. The control treatment which was not mutated with EMS signified negative results and the colour of the calli were not changed in staining. It indicated that glyphosate resistance has developed

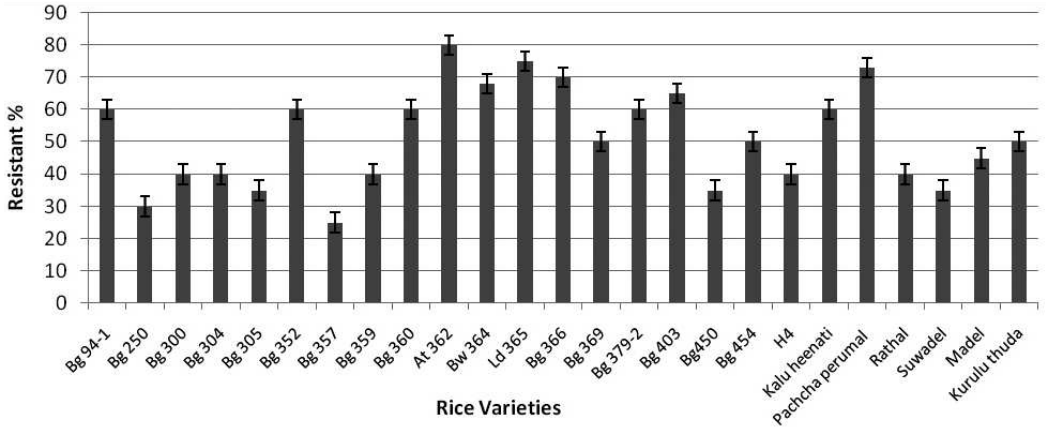

Figure 1. Resistant percentage (No. of survived plants) of S_0 rice varieties 0.5 g a.e. L^{-1} glyphosate concentration.

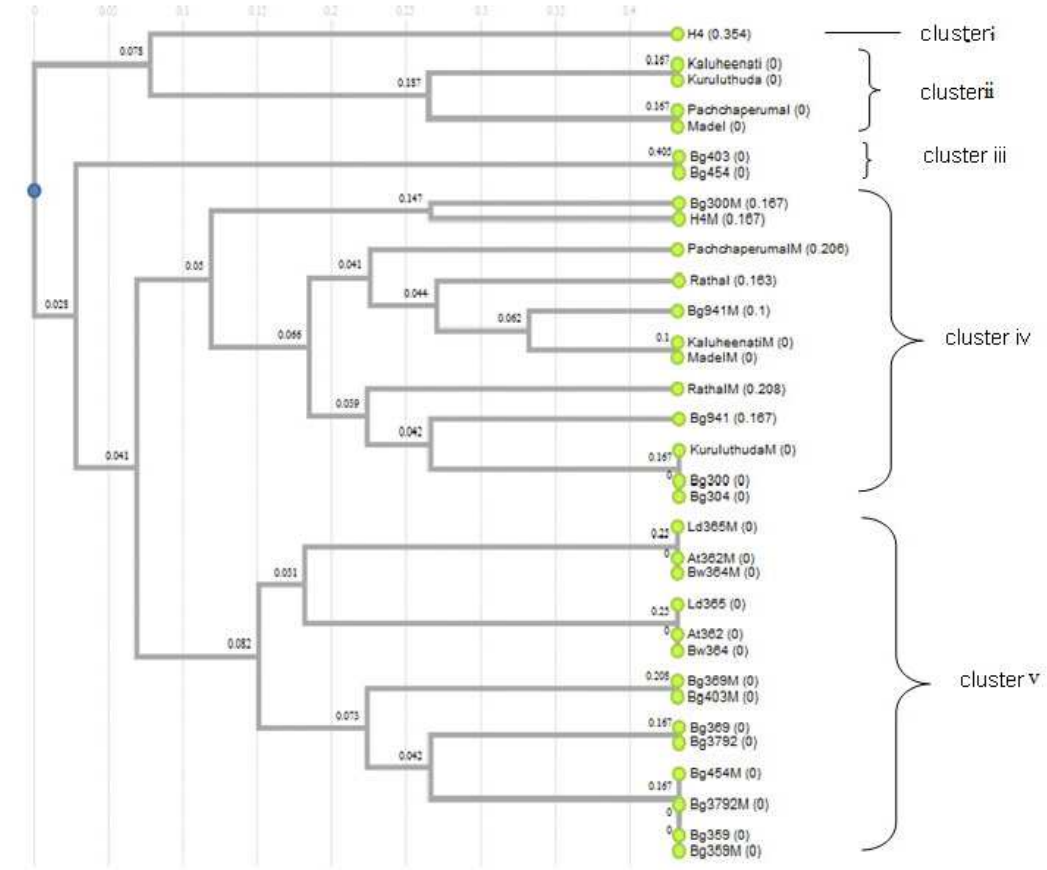

Figure 2. The dendrogram showing genetic diversity among rice varieties (M denote – S_0 varieties) (based on Jaccard's similarity coefficient).

in the EMS-mutated callus compared to the control. Callus derived from Pachcaperumal seeds also showed positive results to TTC test without EMS mutation. The regenerated plants of mutated calli also showed resistance to glyphosate.

The technique of induced mutations has over the past 50 years played a major role in the development of superior crop varieties. The present study revealed, EMS application *in vivo* and *in vitro* was successful in inducing HR in certain Sri Lankan rice varieties and induced HR rice varieties have a higher potential in rice breeding programs. Furthermore, the identified AFLP markers may be potentially useful for differentiating HR rice varieties in future.

ACKNOWLEDGMENTS

The research grant provided by the National Research Council, Sri Lanka (Grant No. NRC 12-037) is greatly appreciated and the proper guidance of Mrs A.S.K. Abeysekara, Senior Research Officer, RRDI, and Bathalagoda, Sri Lanka is also greatly acknowledged.

REFERENCES

Barrio, F., Fernandez-Escobar, J., De La Vega M. and Martin, A. (2001). Doubled haploid lines of *Brassica carinata* with modified erucic acid content through mutagenesis by EMS treatment of isolated microspores. *Plant Breeding* 120 (3), 262-4.

Duke, S.O., Scheffler, B.E., Dayan, F.E. and Dyer, W.E. (2002). Genetic engineering crops for improved weed management traits. Proceedings of ACS Symposium Series No. 829, pp. 52-66. American Chemical Society, Washington, DC.

Gealy, D.R., Mitten, D.H. and Rutger, J.N. (2003). Gene flow between red rice (*Oryza sativa*) and herbicide-resistant rice (*O. sativa*): implications for weed management. *Weed Technology* 17, 627-45.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologic Plantarum* 15, 473-97

Sandhu, S.S., Bastos, C.R., Azini, L.E, Neto, A.T and Colombo, C. (2002). RAPD analysis of herbicide-resistant Brazilian rice lines produced via Mutagenesis. *Genetics and Molecular Research* 1, 359-70.

Sudhakar, P., SaradaMani, N. and Ramana, T. (2009). Long-term maintenance of callus cultures from immature embryo of *Sorghum bicolor*. *World Journal of Agricultural Sciences* 5 (4), 415-21.

Smith, J.H., White, W.T. and Brown, B.R. (1994). Yield response of wheat to herbicides. Proceedings of the First International Control Congress, p. 310. Weed Science Society of Victoria, Melbourne.

Tan, S., Evans, R.R., Dahmer, M.L., Singh, B.K. and Shaner, D.L. (2005). Imidazolinone-tolerant crops: history, current status and future. *Pest Management Science* 61, 246-57.

Towill, L.E. and Mazur, P. (1975). Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. *Canadian Journal of Botany* 53 (11), 1097-102.

Vos, P., Hogers, R., Bleeker, M., Vandelee, T., Hornes, M., Fritjers, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (2001). AFLP: a new technique for DNA finger printing. *Nucleic Acid Research* 23, 4407-14.