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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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Abstract

Herbicide resistant (HR) rice is a novel approach to enhance selectivity and crop safety in rice cultivation. HR crops provide better suppleness in weed management and new solutions to difficult weed management problems. Studies on induced HR in Sri Lankan rice varieties are limited and further research are required to include HR rice in a cropping program. Mutational breeding with molecular methods play an important role in resistant development in modern agriculture. 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. Twenty five cultivated rice varieties were subjected to EMS treatment and after the mutation survived plants were exposed to glyphosate to check herbicide resistance. AFLP analysis was made on EMS mutated rice plants using 16 AFLP primer combinations. Seed-derived calli were obtained from glyphosate-susceptible rice variety and exposed to varying concentrations of EMS. Mutated calli were exposed to glyphosate and Tetrazolium test was applied to identify cell viability in calli. *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

The increasing world population at the rate of one billion people per year demands an additional rice production (100 million MT) required for every year (Hedge *et al*., 2013). In future, it is imperative that rice production continues to grow at least as rapidly as the population. In addition, to obtain a good yield in rice, farmers have to overcome several biotic and a biotic stresses. Weeds are a source of biotic stress in crop systems that decrease the yield, increase production costs, and contribute to income risk. (Frisvold *et al*., 2010) Therefore, weed control has been the most significant part of, cropping operations in ensuring the best quality harvest. Introduction of herbicide resistant (HR) rice is a novel approach to enhance selectivity and crop safety in rice cultivation. HR crops provide better suppleness in weed management and new solutions to difficult weed management problems (Duke et al., 2002) Farmers can thereby easily control weeds during the entire growing season and have more flexibility in choosing times for spraying. Herbicide resistant crops also facilitate low or no tillage cultural practices, which many consider to be more sustainable (Green, 2012) .Therefore, by

growing herbicide tolerant crops, higher doses of herbicides or even non-selective herbicides may become helpful to control the weeds in a single quick application (Rizwan *et al*., 2015).

In modern agriculture plant breeding is based on creating variation, selection, evaluation and multiplication of desired genotypes. To increase efficiency and make short cuts in each step, the plant breeders combine several techniques together. In so doing, plant breeders have the options to use in vitro culture for rapid multiplication, molecular methods to select specific genotypes and mutagenesis to enhance variation (Ahloowalia and Maluszynski, 2001). The use of induced mutations over the past five decades has played a major role in the development of smart crop varieties all over the world. The widespread use of induced mutants in plant breeding program across the globe has led to the official release of 3222 plant mutant varieties from 170 different plant species in more than 60 countries throughout the world (FAO, 2005).

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). Utilizing EMS mutation method, Sandhu *et al*., (2002) developed 21 Brazilian rice lines that were resistant to glufosinate. Haughn (1986) used EMS for the development of sulfonylurea resistant mutants of *Arabidopsis thaliana* and EMS were used to increase the probability of selecting resistant mutants in soybean (Sebastian *et al*., 1987; Sebastian *et al*., 1989) and tobacco (Chaleff *et al*., 1984).

Two new set of technologies, *in vitro* culture and molecular methods have created a new paradigm in the use of mutations in crop improvement (Ahloowalia and Maluszynski, 2001). Modern techniques including tissue or plant cell culture and transformation were used in past decades for the development of herbicide tolerant crops (Anderson and Georgeson,1989 ; Joel *et al*.,1995) Plant tissue culture represents the simplest of the biotechnologies available at present for crop improvement (Sudhakar *et al*., 2009). Application of molecular markers in plant breeding programs has established the need for information on variation on DNA sequences (Bhagwat *et al*. 1997). The identification, and analysis of mutants is based on the use of molecular techniques of DNA fingerprinting and mapping on PCR based markers. (Ahloowalia and Maluszynski .2001) Amplified Fragment Length Polymorphism (AFLP) is a DNA fingerprinting technique (Vos *et al*. 1995) which uses selective amplification of restriction fragments.

Transgenic rice cultivars resistant to broad-spectrum herbicides such as imidazolinone and glyphosate resistant varieties in the USA and Latin America have also been developed and released for commercial cultivation. Similarly, transgenic glyphosate-resistant rice will be released within a few years. Although transgenic HR rice varieties are developed, these varieties are not allowed to introduce to Sri Lanka due to the existing biosafety regulations. Thus, use of conventional breeding techniques including mutagenesis is of importance in developing HR rice. In Sri Lanka, so far, no attempts were made to develop HR rice including broad-spectrum herbicide resistant-rice to control weedy rice and other annual and perennial weeds. Therefore, it is important to develop glyphosate- resistant rice varieties with the aid of breeding techniques such as mutagenesis and the present study focused on inducing HR in rice by *in vivo* and *in vitro* EMS mutation.

Materials and Methods

Materials

Fourteen inbred-developed rice varieties and five traditional varieties were collected from Rice Research and Development Institute at Bathalagoda and regional rice research stations Ambalanthota, Bombuwela and Labuduwa, Sri Lanka.

Table 1: List of rice varieties used in the study

RRDI /Bathalagoda	**Bg 94-1,Bg250, Bg300, Bg304, Bg305, Bg352, Bg357, Bg359, Bg360, Bg366, Bg369, Bg379-2, H4, Bg403, Bg450, Bg 454**
Rice research station /Ambalanthota	At 362
Rice research station /Bombuwela	Bw364
Rice research station /Labuduwa	At365
Traditional rice varieties'	KaluHeenati, Kuruluthuda, Suwadal, Rathhal, Madel, Pachchaperumal

Method 1 - Seed mutation

Seeds from each variety were pre-soaked in distilled water for 24 h at room temperature and exposed to 4.5 $mmoll^{-1}$ 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/l) 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 $\geq$ 50% resistance to glyphosate (0.5 g/l) 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 on the mutated calli using 16 AFLP primer combinations**.** Genomic DNA was extracted from mutated calli using PhytoSpin™ Plant Genomic DNA extraction kit (Ceygen Biotech). AFLP analysis was performed as described by [29] with minor modifications.

One µg of DNA from each sample was digested with 5 units of *Eco*R1, 5 units of *Mse*1 enzymes.The digested samples were incubated at 37 ºC for 3.5 h. To the double digested DNA, *Eco*R1 adaptor (10 $pmol\mu l^{-1}$) ,*Mse*1 adapter(10 $pmol\mu l^{-1}$) 5U of T4 DNA ligase were added and the tubes were incubated overnight (~16) at 37 ºC. After the ligation of adapters, 5 µl of digested/ligated DNA were pre-amplified in 25 µl reaction containing 2 µl each of pre-amplification primers (8 pmol/µl), 0.2 mMdNTPs, 1X PCR buffer (Genscript, USA), and 1 unit of *Taq* DNA polymerase (Genscript, USA) (5 units/µl). PCR amplification was performed using the following cycle conditions. Denaturation at 94ºC for 30 s, annealing at 56 ºC for 60 s, extension at 72 ºC for 60 s and cycles were repeated for 30 times. The pre-amplification product was diluted 20 x with sterile distilled water and used as a template for selective amplification. The selective amplification reaction was conducted in a final volume of 20 µl containing 2 µl of diluted pre-amplification PCR products, 0.6 µl of *Eco*R1 fluorescent (Fam,Pet,Vic,Ned) labeled primer (8 pmol/µl), 0.6 µl of *Mse*1 primer (8 pmol/µl), 0.2 mMdNTP, 1X PCR buffer (Genscript, USA), and 1 unit of *Taq*DNA polymerase. The PCR amplification was carried out as follows: Denaturation at 94 ºC for 30 s, annealing at 65 ºC for 30 s. in the first cycle and then the annealing temperature was reduced by 0.7ºC per cycle for the next 12 cycles. Then the cycles were repeated 23 more times with the annealing temperature of 56ºC. Extension was at 72 ºC for 60 s for all cycles.

A fluorescent labeled DNA size strand LIZ 600 with known fragments size was mixed with each 2.5µl amplified PCR products and 5µl of deionized formamide loading solution and 2.5µl of ultra-pure water. Then the samples were loaded in 96 well plates (Axygen Biosciences, USA). Samples were denatured at 95 ^{o}C for 2 min., chilled immediately placing

plate on ice. The denatured amplified products were separated through capillary electrophoresis Mega BACE 1000 automated DNA sequencer (GE Healthcare Life Sciences, USA). AFLP fragment analysis was performed with Genemapper® 4.1 software.

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/petri dish) in solid MS medium for callus induction (Murashige and Skoog, 1962). Cultures were incubated in a culture room maintaining at 25^0 C ± 2^0 C in complete dark. For the mutation process three weeks old callus masses were transferred into Falcon™ 50ml tubes which containing 30ml, 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 aluminum 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 (28^0C ± 2^0C, in darkness).The treated calli were sub-cultured 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 color 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/l 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

The results obtained from the screening the glyphosate resistant of EMS mutated seeds revealed that some of the selected traditional rice varieties and inbred lines 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 (Fig.1). The resistant percentage of S_1 generation was almost similar in all the varieties except Bg454 and "Madel".

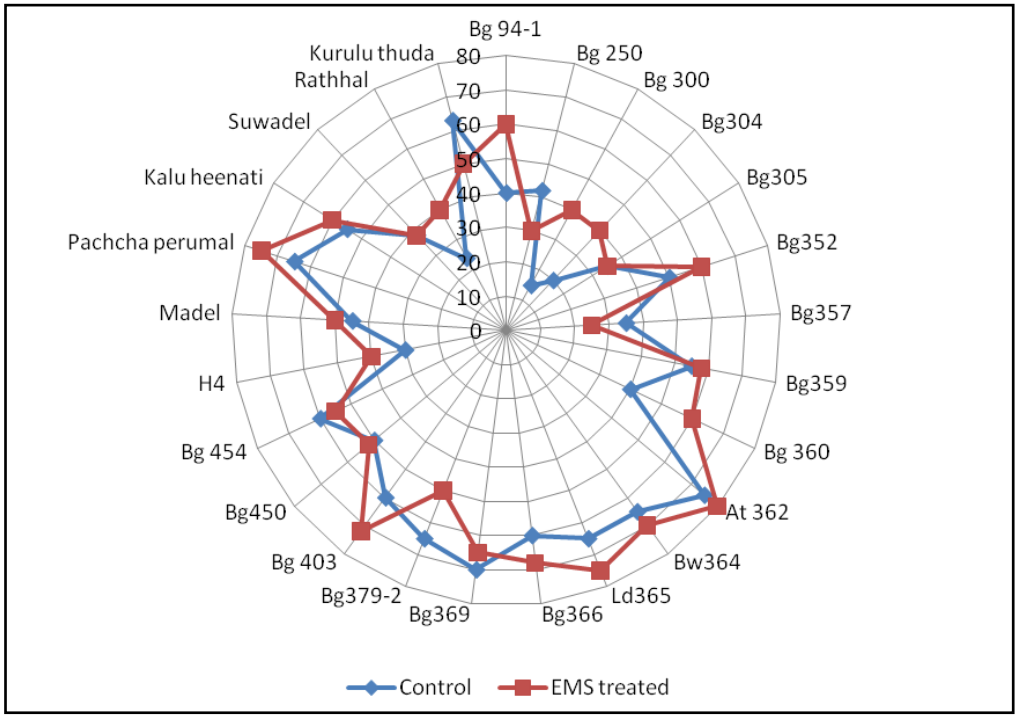

Figure 1: Resistant percentage (No. of survived plants) of S_0 rice varieties compare to control (0.5 g/l glyphosate concentration)

Analysis of the variance (data not given) indicated 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. In relation to plant height most of the mutated lines showed a lower mean value compare to controls. Previous studies reported that the induction of ionizing mutation was effective in reducing plant height in rice genotypes (Martins *et al*.,2005) and the use of EMS, in different rice mutant generations leads to an increment of the trait plant height (Siddiqui and Singh 2010).How ever in the present study EMS mutated rice lines were further subjected to the glyphosate and the studies conducted by Ellis *et al*., (2003) and Kurtz and Street (2003) confirm that the glyphosate applied to rice resulted in reduced rice plant height. In general, cereal breeding programs tend to select short plants, because tall plants without thick stems usually have low yield potential and are more susceptible to lodging (Ni *et al*., 2000) There for the resulted dwarf lines will be important in future HR rice breeding programs.

The other agro morphological traits, number of leaves/plant and number of tillers/plant were not statistically significant when comparing mutated rice lines with non-mutated. Number of panicles/plant, Number of seeds/panicle and 1000 grain weight were indicating significant different in mutated lines. Although most of the varieties resulted reduction in final yield the loss was lower than the loss occur due to the weeds. In the development of EMS induced rice variety Nagina22 Mohapatra *et al.*,2014 also reported that in addition to the mutations in developmental pathways relevant to growth, maturity, plant architecture and yield, mutations influencing high temperature ,drought tolerance and herbicide tolerance possessed by Nagina22 are of special importance. These three are highly complex traits and need immediate attention to meet the challenges of climate change.

Molecular markers provide valuable information which can be used in a number of ways in any crop improvement program. In the present study AFLP technique has selected since it offers several advantages over the other molecular makers. Out of sixteen primer combinations used in the study, 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. Further the selected E11M32 marker can be possibly incorporated in HR rice breeding programs to identify resistance even at the seedling stage. Cluster analysis based on UPGMA for the mutated rice varieties indicated that control and mutated lines are genetically different to each other. All the mutated lines were clustered in cluster I while the control lines were clustered in cluster II (Figure 2).

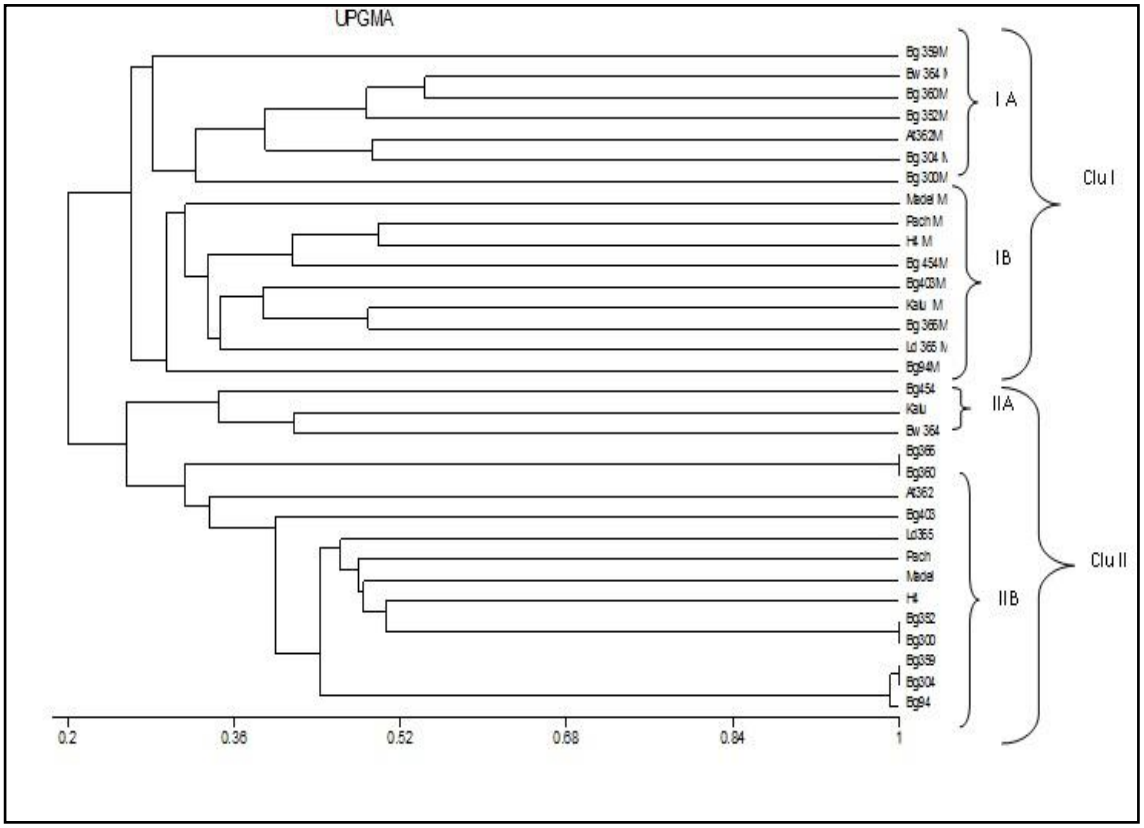

Figure 2. The dendrogram showing genetic diversity among rice varieties (M denote –S_1 varieties) (based on Jaccard's similarity coefficient).

Tissue culture methods and mutagenesis techniques could significantly shorten the breeding process and overcome some substantial agronomic and environmental problems. In most cases, the embryo-derived rice *callus* regeneration is only generated from a few cells. Thus, the regenerated *M1*plantlets from mutant *calli* could be screened directly without waiting for a self-pollinated *M2* population (Serrat *et al.*, 2004). In current study *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 0.4of 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 color following the staining process. The control treatment which was not mutated with EMS signified negative results and the color of the calli were not changed in staining. It indicated that glyphosate resistance has developed 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.

Conclusion

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.

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