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Natural and Induced Glufosinate-resistance in selected Rice (*Oryza sativa* L.) Varieties in Sri Lanka

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ABSTRACT

Purpose: *Herbicide-resistant rice varieties in combination with post-emergent broad-spectrum herbicides improve the effectiveness of weed management in rice fields. Herbicide-resistance can be acquired naturally or induced by classical or modern breeding techniques. In the present study, screening of Sri Lankan rice varieties to glufosinate was examined and attempts were made to develop glufosinate-resistant rice varieties with the help of the breeding technique, mutagenesis.*

Research Method: *Natural glufosinate-resistance was studied in seven (7) traditional and eighteen (18) inbred rice varieties under 0.27, 0.30 and 0.33 kg ha -1 glufosinate concentrations. Induced glufosinate-resistance in seed-derived calli of four glufosinate-susceptible rice varieties using Ethyl Methane Sulfonate was also observed and these were then treated with 0.2% (v/v) glufosinateto to determine induced glufosinate-resistance in seed-derived calli.*

Findings: *Two traditional varieties (Pachchaperumal, Ma Wee) and fifteen inbred varieties (At362, Bg250, Bg300, Bg352, Bg357, Bg358, Bg359, Bg360, Bg366, Bg369, Bg379/2, Bg403, Bg450, Bg454, and Bg94-1) were resistant to glufosinate. Hardly any distinct yield penalty in Bg94-1, Bg403, Bg454, Bg379-2 and Ma Wee was observed under all glufosinate concentrations. Visual symptoms and chlorophyll damages caused by glufosinate for all varieties recovered within 14 days of treatment. Higher glufosinate-resistance was observed in EMS-mutated calli derived from seeds of naturally glufosinate-susceptible varieties. Molecular (AFLP) analysis confirmed in vitromutation and theM31E10 primer combination was a potential AFLP marker to identify glufosinate-resistance in rice varieties.*

Research Limitation: *The study reports the results of screening natural and induced glufosinate-resistance in Sri Lankan rice. Inheritance of glufosinate-resistance is to be tested through several generations in Sri Lankan rice varieties.*

Original Value: *Study provides a screening method applicable to Sri Lankan Rice varieties in identification of herbicide-resistance to permitted concentrations of glufosinate.*

Keywords: *AFLP, EMS, glufosinate resistance, Oryza sativa, Seed culture*

INTRODUCTION

Effective weed management practices ensure high quality harvest in cropping systems including rice. Among the practices, post-emergent (PE) and broad spectrum herbicides (BSH) have become popular due to the capability of controlling hard-to-kill weeds and weed population that have evolved resistance to selective herbicides. In general, BSHs disrupt a unique and essential processes in plants such as, photosynthesis, pigment biosynthesis, mitosis or essential amino acid biosynthesis (Mulwa and Muwanza, 2006). Amino acid biosynthesis of plant is the main target process of the BSHs (Áy *et al*., 2012). Glufosinate ammonium is a pro-

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herbicide which is converted within the plant cell into the phyto-toxin, as Phosphinothricin (PT). As a structural analogue of glutamic acid, PT inhibits glutamine synthatase [GS (E.C.6.3.1.2.)] competitively and irreversibly (Senseman, 2007; Hensley, 2009). GS is an essential ammonia assimilation enzyme found in plants. Inhibition of GS causes a rapid, toxic accumulation of intercellular ammonia resulting metabolic disruption and inhibition of photosystem I and photosystem II in treated plants (Wendler, 1992; Wild *et al*., 1987; Logush*et al*., 1991; Senseman, 2007; Hensley, 2009). Over 40 monocotyledonous and more than 150 dicotyledonous species are sensitive to PT (Metz *et al*., 1998).

Particularly in rice fields, conspecific weed species with diverse dormancy patterns such as weedy rice (*Oryza sativa* f. *spontanea*) and cultivated rice (*Oryza sativa*) coexist throughout the cropping season. Due to the phylogenetic and morphological resemblance of weedy rice to cultivated rice, selectivity should be established based on the different ways BSHs act upon weeds and cultivated rice. Resistance of cultivated rice for BSHs is one of the eminent options available for establishing selectivity in rice fields. Herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type (WSSA, 1998) and resistance may be naturally occurring or can be effectively achieved by inducing resistance towards BSHs in cultivated rice *via* plant biotechnology techniques such as *in vitro* cell culture, mutagenesis, or genetic transformation followed by selection under herbicide pressure (Áy *et al*., 2012). 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 resistant (HR) mutants have been developed through chemical mutagenesis followed by herbicide selection (Tan *et al*., 2005).

With the advancement of HR technologies, several HR rice varieties such as Liberty Link® (glufosinate-resistant) (Gunther, 2011), Clearfield® (imidazolinone-resistant) (Gealy *et al*., 2003; Croughan, 2011) and Roundup ready® (glyphosate-resistant) (Monsanto, 2010) have been released for commercial cultivation. Among these, Liberty Link® (glufosinate-resistant) and Roundup ready® (glyphosate-resistant) rice have been developed through transgenic technology while Clearfield® (imidazolinone-resistant) rice has been developed *via* chemically induced seed mutagenesis and conventional breeding (Gealy *et al*., 2003; Tan *et al*., 2005).

Imidozolinone resistant plants such as maize (Newhouse *et al*., 1991), canola (Swanson *et al*., 1989), cotton (Subrmanian *et al*., 1990), soybean (Sebastian *et al*., 1989) and wheat (Pozniak *et al*., 2004) have been developed *via* seed, microspore, pollen and callus mutagenesis and somatic cell selections and somatic embryogenesis in rice from root tissue (Abe and Futsuhara, 1985), leaf tissue (Wernicke *et al*., 1981), inflorescence and caryopsis (Chen *et al*., 1985) and protoplast (Yamada *et al*., 1986) have been reported. In the present study, an integrated approach of *in vitro* cell culture and mutagenesis have been used to develop glufosinate-resistance in rice as a phenotypic trait in response to the functional mutation induced by Ethyl methane sulfonate (EMS).

Molecular screening of mutations has been developed recently after the improvement of efficiency of chemical mutagens (Brockman *et al*., 1984; Serrat *et al*., 2014). Molecular markers such as SSR, AFLP, RAPD and RFLP have been employed to confirm chemically induced mutations at callus level and plant levels based on the information of variations on DNA sequences (Bhagwat *et al.,* 1997). Among these marker systems, AFLP is considered to be a highly informative DNA fingerprinting method, which can be used to assess a large number of markers without prior sequence knowledge (Rajkumar *et al*., 2011). Even though, a detailed RAPD study has been conducted on mutated, glufosinate resistant and susceptible Brazilian rice lines (Sandhu *et al*., 2002), such studies,

to identify mutagenic response of seed-derived rice calli to EMS and evaluate their HR have not yet been reported in Sri Lanka (Ekanayake *et al*., 2016). In this study AFLP analysis was performed to analyze the variations in fragment sizes after the chemically induced mutation.

In Sri Lanka, attempts have been made to develop resistance to glyphosate in rice (Ekanayake *et al*., 2015b). Previous studies have shown that there is a wider variation of resistance to glyphosate in traditional and inbred rice varieties in Sri Lanka (Weerakoon *et al*., 2013; Ekanayake *et al*., 2014). Mutagenesis studies with EMS have produced stable glyphosate-resistant Sri Lankan rice varieties (up to the fifth generation) (Ekanayake *et al*., unpublished). Therefore, it is important to screen for natural glufosinate resistance in Sri Lankan rice varieties and attempt to develop glufosinate-resistant rice varieties with the help of the breeding technique, mutagenesis.

MATERIALS AND METHODS

Seed Material

Twenty five rice varieties including seven traditional varieties (003136 - *Pachchaperumal*, 003656 - *Kuruluthuda*, 002196 - *Rathal*, 003471 - *Kaluheenati*, 005486 - *RathSuwadal*, 004054 - *Handiran*, and 003618 - *Ma Wee*) and eighteen inbred lines (Bg352, Bg360, Bg359, Ld365, Bg366, Bg357, Bg94-1, Bg369, Bg379-2, Bg450, Bg403, Bg250, Bg454, Bg358, Bg300, Bg304, Bg305, At362) collected from Rice Research and Development Institute (RRDI), Sri Lanka at Batalagoda, Ambalantota and Labuduwa were used for the study. These lines were maintained in a plant house at the Open University of Sri Lanka, located in low country wet zone of Western province, with an average temperature of 28-32^{o}C and 65-70% relative humidity. The basis of selecting the traditional and inbred rice varieties in this experiment was mainly built on the contribution of traditional rice in the development of inbred varieties and resulting progenies which potentially possess traits including herbicide resistance in donor rice varieties (Dwivedi *et al*., 2018) and on the availability and most commonly cultivated varieties in Sri Lanka.

Evaluation of naturally existing HR resistance among rice varieties

The selected seeds were pre-soaked overnight and allowed to germinate. One week old seedlings were planted in pots filled with puddle soil (5.5 kg per pot) and excess plantlets were thinned out after one week (Ekanayake *et al*., 2015a). Fertilizer application and crop practices were performed according to the recommendations of the Department of Agriculture, Sri Lanka. Three different concentrations of glufosinate as 0.27 kg ha^{-1}, 0.30 kg ha^{-1} and 0.33 kg ha^{-1}(Davis *et al*., 2009) were applied at 3-4 leaf stage (Ellis *et al*., 2003). Complete Randomized Design (CRD) was followed with thirty (30) replicates for each treatment and non-treated plants were served as control.

The total number of plants and the number of surviving plants after glufosinate application were counted for each variety and percentage (%) of resistance was calculated as below. The diagnostic characters of herbicide vulnerability was assessed by the number of scorched leaves (>=50% of scorches leaves) and number recovered pant after herbicide treatment.

$$\text{Percentage resistance (\%)} = \frac{\text{Number of resistant seedlings in a variety}}{\text{Total number of seedlings grown in the same variety}} \times 100\%$$

(Neve and Powles, 2005; Weerakoon *et al*., 2013)

Varieties with ≥ 50% resistance to glufosinate treatment were considered as glufosinate-resistant (Neve and Powles, 2005; Weerakoon *et al*., 2013). Variables such as plant height, number of leaves, and number of tillers were observed in every two weeks after sawing and, the yield parameters were also obtained.

Determination of the Recovery of Visual Quality and Chlorophyll content

A hand-held chlorophyll content meter (Model

SPAD-502, Minolta, Japan) was used to assess the chlorophyll status and to correlate it with the visual quality in treated and non-treated rice varieties (Chapman and Baretto, 1997). Visual colour rating was performed using the leaf colour chart issued by the International Rice Research Institute (IRRI). Three mature leaves were selected per pot and three SPAD measurements were taken per plant (Rodriguze and Miller, 2000). SPAD readings and visual colour ratings were taken in 2 days interval before and after glufosinate treatment for a total of six sampling dates. Sampling was done at the same time of the day (around 10.00 am - 11.30 am) under the light intensity of 833- 1228 µ mol m^{-2} S^{-1} and subjecting to the temperature range of 28-32^{o}C and 65-70% relative humidity.

Spectrometric Analysis of Leaf Chlorophyll content in glufosinate treated Rice varieties

Leaves of all glufosinate-treated and non-treated rice varieties were excised from the plants, avoiding mechanical injuries. Fresh, clean leaf samples (10 mg) were then cut into small pieces and preserved in cold distilled water to prevent excess loss of moisture (Krishnan *et al*., 1996). Three replicates were prepared from three different plants of the same variety.

Rice leaf tissues were incubated in 2 ml of 80% buffered acetone (pH = 7.8) in dark, with a temperature of 4^{0}C and tubes were shaken occasionally to accelerate pigment extraction (Krishnan *et al*., 1996). Then the extracted liquid was filtered using Whatman No.1 filter papers and leaf pieces were removed and the volume was made up to 2 ml and transferred to 3 ml sealed quartz-glass cuvettes with 1 cm path length.

Dual beam recording UV visible spectrophotometer was used to measure absorption of the extract at the wavelength of 663 nm and 645 nm (Zhang *et al*., 2009). The concentrations of chlorophyll a, chlorophyll b, and total chlorophyll were then calculated (Arnon *et al*., 1949).

$$\text{Chlorophyll a} = 12.27A_{663} - 2.59A_{645} \quad (2)$$

$$\text{Chlorophyll b} = 22.9A_{645} - 4.67A_{663} \quad (3)$$

$$\text{Total chlorophyll} = 20.31A_{645} + 8.05A_{663} \quad (4)$$

Callus Induction

This is based on the findings of the previous experiment carried out on the evaluation of naturally existing herbicide resistance among rice varieties selected for the present study (Bg304, *Kaluheenati*, *Kuruluthuda* and *Handiran* showed susceptibility to Glufosinate and Bg454, *Ma Wee* and *Pachchaperumal* were resistance to glufosinate). Glufosinate-susceptible rice varieties were used to produce callus and glufosinate-resistant rice varieties served as the reference/control. Mature, undamaged seeds were selected by visual observations and were de-husked manually. Surface sterilization of seeds were achieved by soaking seeds in 70% ethanol for 2 minutes and, washed with autoclaved distilled water to remove ethanol traces. Subsequently, seeds were soaked in 10% Sodium Dodecyl Benzene Sulphonate (Teepol) and distilled water for 5 minutes (Ekanayake *et al*., 2016). After washing the seeds with autoclaved distilled water for four times, seeds were dipped in 20% commercial Clorox (0.78% Sodium hypochlorite) with an addition of three drops of Tween 20 as a wetting agent for 10 minutes (Libin *et al.*, 2012). Then seeds were rinsed three times with sterilized distilled water and soaked in sterilized distilled water for 15-20 minutes (Chowdhury *et al.*, 2012) and, cultured on solid MS media supplemented with Myo Inositol, Kinetin, BAP and different 2,4-D concentrations. Cultures were maintained at 25^{o}C ± 2^{o}C in complete dark.

Chemical Mutagenesis

Scutellum-derived calli were selected for the chemical mutagenesis with EMS and discarded the calli derived from the radical and shoot of the albino plant. Selected calli with approximately 1-2 mm in diameter (10 calli) were transferred into sterilized 50 ml conical flasks containing 30 ml of liquid MS media without 2,4-D, but supplemented with 125 g l^{-1} ascorbic acid and 125 g l^{-1} citric acid to prevent oxidation of calli during mutagenesis treatment (Serrat *et al*., 2014). Then calli were immediately mutagenized by adding 0.2% (v/v) EMS

(Sigma-Aldrich, Madrid, Spain) (Ekanayake *et al*., 2016). EMS solution was filter-sterilized before use (Fang and Traore, 2011). No EMS was added to the control flasks and there were three replicates for each treatment. Conical flasks were covered with aluminium foil to avoid light and were placed on an orbital shaker (150 rpm) for two hours. Subsequently, incubated calli were rinsed 10 times with 2,4-D free MS liquid medium and transferred to new sterilized conical flasks containing MS liquid medium with 2,4-D and were shaken (120 rpm) for two additional days (28 ± 2°C, in dark) (Serrat *et al*., 2014; Ekanayake *et al*., 2016). The treated and non-treated calli were sub-cultured on MS solid medium supplemented with NAA and Kinetin. Initial screening of surviving calli after EMS treatment was carried out based on the colour changing from creamy white (alive) to brown colour (dead) (Ekanayake *et al*., 2016). Viability of calli in which brown colour was not observed and was confirmed by 1% 2,3,5-Triphenyltetrazolium (TTC) viability assay (Towill *et al*., 1975; Ekanayake *et al*., 2016). Survival percentage of calli was calculated using the following equation.

$$Calli\ survival\ percentage = \frac{[Number\ of\ viable\ calli]}{[Number\ of\ treated\ calli]} \times 100$$

(Ekanayake *et al*., 2016)

Evaluation of glufosinate-resistance in mutated calli

After ten days of EMS treatment, twenty viable calli were sub-cultured and treated with 0.2% (v/v) glufosinate solution (Ekanayake *et al*., 2016). Then characteristics were compared with untreated calli and 1% TTC viability assay after seven days (Towill *et al*., 1975; Ekanayake *et al*., 2016) was performed to confirm the viability of calli and surviving percentages were calculated.

AFLP analysis

Genomic DNA was extracted from mutated and non-mutated calli using DNeasy®Plant Mini Kit (QIAGEN®). 3 µl from each sample was run on 1% agarose gel to verify the presence of DNA. AFLP analysis was performed by following the protocol described by Vos *et al*., (1995) with the following minor modifications.

One µg of each DNA sample was digested with 5 units of *Eco*R1 and 5 units of *Mse*1enzymes. The digested DNA samples were incubated at 37°C for 3½ hours. *Eco*R1adaptor (10 pmol µl-1), *Mse*1 adaptor (10 pmol µl-1), 5 units of T4 DNA ligase were added to the double digested DNA samples and were incubated overnight (~16 hours) at 37°C (Rajkumar *et al*., 2011). Subsequently, 5 µl of each digested/ ligated DNA samples were pre-amplified in 25 µl reaction volume containing 2 µl of each pre-amplification primers (8 pmol µl-1), dNTPs (0.2 mM), 1X PCR buffer (Genscript, USA) and 1 unit of *Taq* DNA polymerase (5 units µl-1) (Genscript, USA) (Ekanayake *et al*., 2016). PCR amplification was performed with the following temperature profile: 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 60 s, extension at 72°C for 60 s (Rajkumar *et al*., 2011; Ekanayake *et al*., 2016).

Pre-amplified product was diluted 20 times and used as templates of selective amplification and eight primer combinations were used. Final volume of 25 µl was prepared by adding 5 µl diluted pre-amplified PCR products, 0.6 µl of *Eco*R1 fluorescent (FAM, NED, VIC, PET) labeled primer (8 pmol µl-1), 0.6 µl of *Mse*1 primer (8 pmol µl-1), 0.2 mMdNTP, 1X PCR buffer (Genscript, USA), and 1 unit of *Taq* DNA polymerase (Genscript, USA). PCR amplification was performed by subjecting the samples to following temperature profile: denaturation at 94°C for 30 s, annealing at 65°C for 30 s, in the first cycle and then the annealing temperature was reduced by 0.7 °C per cycle for the next 12 cycles. Cycles were repeated 23 times with the annealing temperature at 56°C and extension at 72°C for 60s.

A fluorescent labeled DNA size strand, LIZ 600 with known fragment size was mixed with 2.5 µl of each amplified PCR product, 5µl de-ionized formamide loading solution and 2.5

µl of ultra-pure water. Then the samples were loaded in well plates (Axygen Biosciences, USA) following the denaturing at 94°C for 2 min, and then chilled immediately by placing the plate on ice. 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. (Sequences of primers and adaptors used for AFLP analysis is given in Table 01.

Statistical analysis

Principal coordinate analysis (PCoA) was performed between the glufosinate resistant varieties based on their agro-morphological parameters using Gower General similarity coefficient (Gower and Legendre 1986) to find out possible relationships. The resulted first and the second axis (PCoA1 and PCoA2) from PCoA were plotted using MVSP software (Kovach, 1998). Descriptive statistics such as mean, standard deviation and standard error were calculated using SPSS PC Ver. 20 and the comparison of treatment was carried out using one-way analysis of variance and mean comparison was carried out using test of least significant difference (LSD).

RESULTS AND DISCUSSION

Evaluation of naturally existing herbicide resistance among rice varieties

The results obtained from screening for glufosinate resistant varieties revealed that some of the selected traditional rice varieties and inbred lines possess the ability to tolerate the detrimental effects of glufosinate (Figure 01). According to Figure 01, the lowest concentration of glufosinate, *i.e*. 0.27 kg ha^{-1} was found to cause minimal damage to selected rice varieties, and only two cultivars (*Rathal*–13% and Bg305–17%) were susceptible to 0.27 kg ha^{-1}glufosinate concentration. Most of the varieties (At362-90%, Bg250-83%, Bg300-96%, Bg352-100%, Bg357-53%, Bg358-53%, Bg359-100%, Bg360-96%, Bg366-73% , Bg369-83% , Bg379/2-93%, Bg403-100% , Bg450-57% , Bg454-97% , Bg94/1 –73% , *Ma Wee*-100%, *Pachchaperumal*-3%) were able to survive under the application of 0.30 kg ha^{-1}glufosinate concentration and only six cultivars (*Ma Wee* –77%, Bg366 -57%, Bg379/2 –53%, Bg403 –73%, Bg454 –77%, Bg94/1–57%) were able to survive at 0.33 kg ha^{-1}glufosinate concentration (Figure 01).

Table 01: Sequences of the adaptors, pre-amplification primers and selective amplification primers used for AFLP analysis

Adaptors and Primers	Sequence
Adaptors	
*Mse*1 adaptor	5'– GAC GAT GAG TCC TGA AG -3'
	3'– TAC TCA GGA CTC AT – 5'
*Eco*R1 adaptor	5'– CTG CTA GAC TGC GTA CC – 3'
	3'– CTG ACG CAT GGT TAA – 5'
Pre-amplification primers	
EOOS primer	5'– GAC TGC GTA ACC AAT TC – 3'
MOOS primer	5'– GAT GAG TCC TGA GTA A – 3'
Selective amplification primers	
*Eco*R1 primer	5'– GAC TGC GTA CCA ATT CNN – 3'
*Mse*1 primer	5'– GAT GAC TCC TGA GTA AAA N – 3'
	N – selective nucleotide

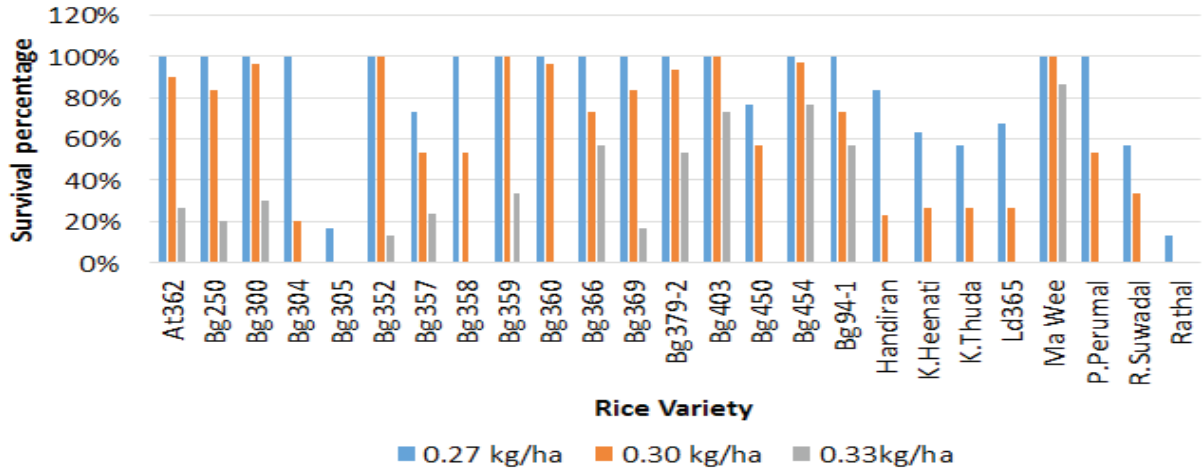

Figure 01: Resistance of rice varieties exposed to glufosinate at 0.27 kg ha^{-1}, 0.30 kg ha^{-1} and 0.33 kg ha^{-1} concentrations.

(Survival percentage ≥50% - Resistant, and Survival percentage <50% – Susceptible)

Among the glufosinate-resistant varieties at 0.30 kg ha $^{-1}$and 0.33 kg ha $^{-1}$, only three red grain rice varieties *(Pachchaperumal, Ma Wee*, and At362) are included indicating that most of the cultivated traditional rice varieties, except *Pachchaperumal* and *Ma Wee* do not possess the ability to resist glufosinate similar to inbred rice varieties. Sri Lankans admire red grain rice such as *Handiran*, *Kuruluthud*a, *Kaluheenati*, *Rathal* and *Rathsuwadal* due to their high nutritive qualities. Therefore, it is important to note that such varieties need to be developed as BSH resistant varieties in future.

On the other hand according to the results of the study, relatively high survival percentage towards glufosinate was reported by inbred rice varieties which possess many valuable attributes other than glufosinate-resistance and they can be incorporated in rice breeding programs where diseases like brown plant hopper (Bph), rice gall midge (GM), rice blast (BL) and bacterial blight (BB) are common.

Determination of the recovery of visual quality and chlorophyll content in glufosinate-resistant varieties

Glufosinate injuries were identified as rapid chlorosis [Figure 2(B)] of treated leaves followed by wilting, necrosis [Figure 02 (C)] and ultimate death of susceptible plants. Similar symptoms have been reported for different rice varieties (Davis *et al.,* 2009; Ellis *et al.,* 2003; Hensley, 2009) and for wheat (Deeds *et al.,* 2009). In addition, brown colour lesions [Figure 2(A)] were also observed on leaves, and browning of leaf tips [Figure 2(D)] commonly occurred on all varieties.

The injuries were significantly higher after one week from herbicide application. Severe chlorosis was observed in rice leaves depending on the susceptibility of the varieties within 3-6 days after herbicide treatment. Within two weeks after herbicide application, the observable symptoms were disappeared even in the varieties which were exposed to highest concentration of glufosinate. Since glufosinate is not readily translocated within the plant (Steckel *et al*., 1997), resistant varieties were able to produce new symptom-free leaves. Ellis *et al*., (2003) and Davis *et al*.,(2009) also reported similar observations. According to the visual rating results, SPAD readings, and chlorophyll concentration values, all the resistant varieties were able to recover from chlorosis within two weeks after treatment. Irrespective of the herbicide treatment, chlorophyll content of glufosinate-resistant Bg357, Bg450, and Bg403 showed no significant difference ($p > 0.05$), compared to the control plants. In general, recovering ability of chlorophyll b ($p > 0.05$) in glufosinate-resistant rice varieties was higher than chlorophyll a ($p \leq 0.05$).

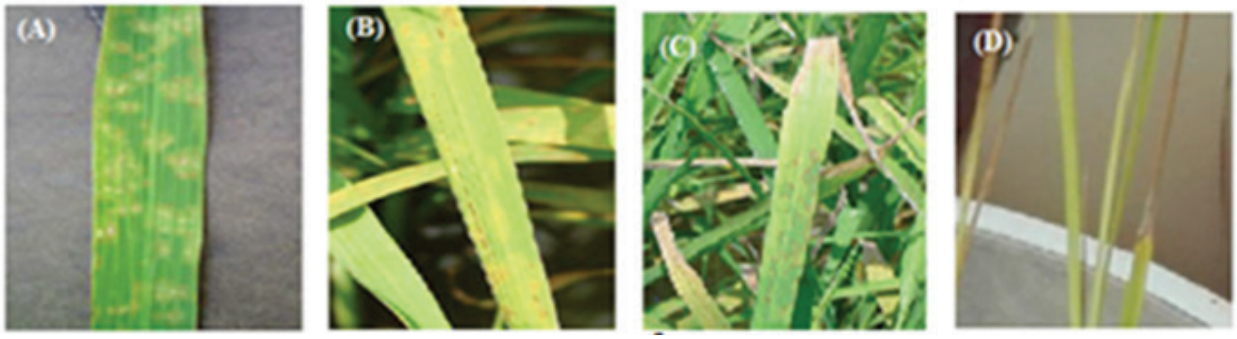

Figure 02: Visual Symptoms due to glufosinate (1WAT) (A) Brown lesions; (B) Chlorosis; (C) Necrotic areas; (D) Browning of leaf tips

Evaluation of glufosinate impact on agro-morphological characters of resistant plants

The comparison of growth and yield parameters of treated plants after eight weeks of glufosinate application revealed that there was a considerable reduction in growth parameters but relatively less reduction in yield parameters of *Oryza sativa* var. *indica* cultivated in Sri Lanka (Table 2). Similar findings were reported by Davis *et al*., (2009) and Ellis *et al*., (2003). However, Ellis *et al.,* (2003) has reported 5% canopy height reduction from glufosinate and 50% reduction by glyphosate when applied at 3-4 leaf stage, According to Davis *et al*.,(2009), plant height reduction was 10–15% but in the present study, canopy height reduction of resistant varieties due to glufosinate application at 3-4 leaf stage was 22.15–35.42% (Table 02).

Results of ANOVA revealed that there was a significant retardation of growth parameters ($p \leq 0.05$) at 0.33 kg ha^{-1} in all six glufosinate resistant varieties as well as in the varieties which were tolerant at 0.30 kg ha^{-1}. Plant height of Bg379-2 showed no significant difference ($p > 0.05$) at 0.30 kg ha^{-1}. There were no significant differences for leaf blade width of Bg366 and Leaf length of Bg352 at 0.30 kg $ha^{-1}$of glufosinate application. Comparatively, the control plants and the plants at 0.30 kg ha^{-1}glufosinate, number of leaves per plant, and number of tillers per plant were not statistically significant.

Analysis of the variance of yield parameters indicated no significant difference for number of spicklets per panicle at both concentrations except for Bg454. Almost all varieties indicated

significant differences ($p \leq 0.05$) for flag leaf length, flag leaf width at both concentrations which mean glufosinate causes reduction in flag leaf quality even when applied at 3-4 leaf stage of the plant. Varieties such as Bg360, Bg357, Bg369, Bg379-2, Bg450, Bg403, Bg250,

Bg454, and *Ma wee* reported insignificant differences for hundred seed weight character at 0.30 kg ha^{-1}. Similar results reported by all tolerant varieties at 0.33 kg ha^{-1} except Bg366. Significant yield reduction was observed for Bg352, Bg359, Bg94-1, Bg358, Bg300, and At362 at 0.30 kg ha^{-1}. All tolerant varieties at 0.33 kg ha^{-1} except Bg366 have indicated insignificant difference in yield. This ability of tolerating high glufosinate dosage indicated their suitability in plant breeding programs, breeding for glufosinate resistance.

The calculated Gower General Coefficient (GGC) (Figure 03) based on agro-morphological characters showed that GGC values of glufosinate-resistance varied from 0.732 to 0.937. Plants of Bg403 at 0.30 kg ha^{-1} and at 0.33 kg ha^{-1} showed the highest GGC value (0.937). The Dendrogram resulted from UPGMA (Figure 3) indicated that glufosinate-resistant varieties are grouped into four clusters at the GGC coefficient of 0.816. First cluster includes two varieties whereas the second cluster consists of three varieties. Third cluster further subdivides into two sub-clusters 3-a and 3-b and the fourth cluster contains four varieties.

Table 02: **Percentage difference of agro-morphological parameters of glufosinate resistant plants (% difference of glufosinate-resistant rice plants at 0.27 kg ha^{-1} were not shown as they are more or less similar to control plants)**

Parameter	Percentage difference at 0.30 kg ha^{-1} treatment (%)	Percentage difference at 0.33 kg ha^{-1} treatment (%)
Vegetative phase		
Plant height (± 0.5 cm)	22.15	35.42
Number of leaves per plant	16.37	30.38
Leaf blade length (± 0.5 cm)	21.47	25.81
Leaf blade width (± 0.5 cm)	33.36	37.22
Number of tillers per plant	08.92	16.87
Reproductive phase		
Heading date	29.43	36.26
Flag leaf length (± 0.5 cm)	28.91	24.40
Flag leaf width (± 0.5 cm)	40.27	41.44
Number of reproducible tillers	00.94	02.74
Number of spicklets	01.43	00.88
Hundred seed weight (± 0.005 g)	01.02	00.94
Seed length (± 0.5 cm)	00.54	00.41
Seed width (± 0.5 cm)	05.28	05.66
Yield per plant	00.87	00.22

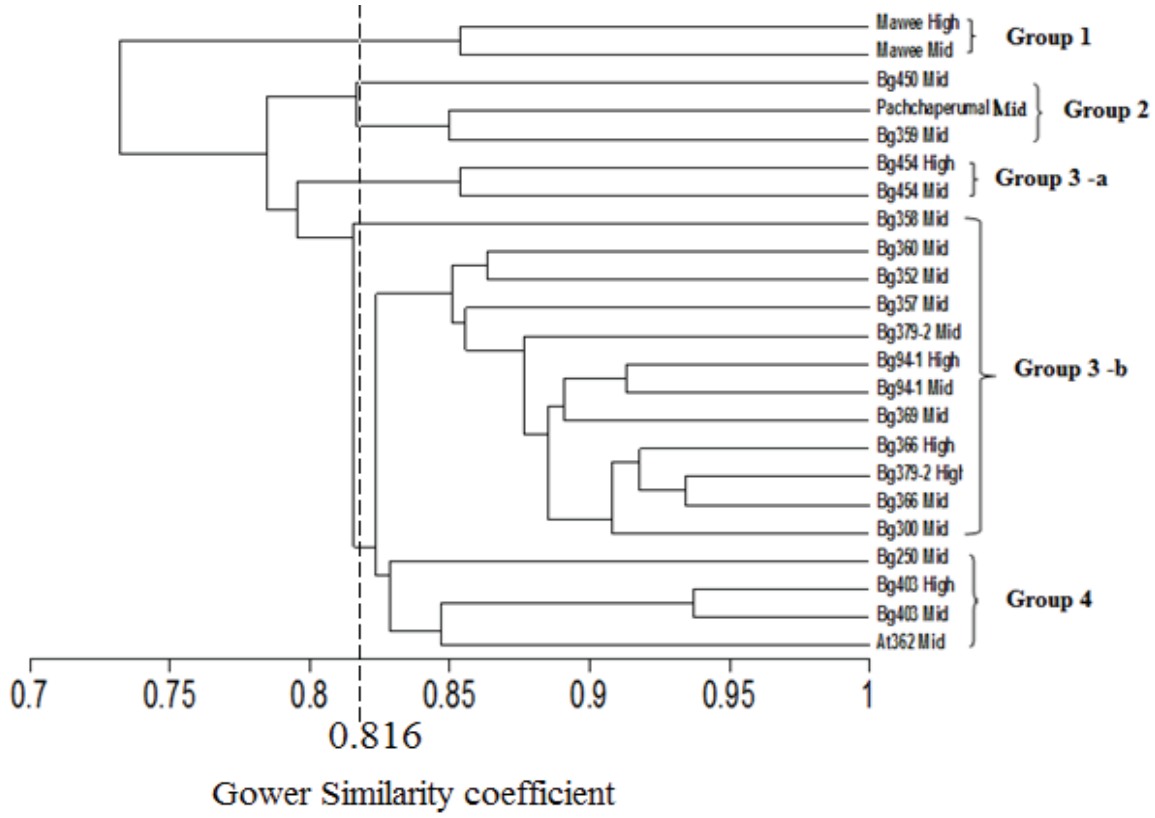

Figure 03: **UPGMA dendogram based on Gower General Similarity Coefficient of glufosinate resistant rice varieties (glufosinate resistant rice varieties at 0.27 kg ha^{-1} were not considered for the cluster analysis as they are more or less similar to control plants)**

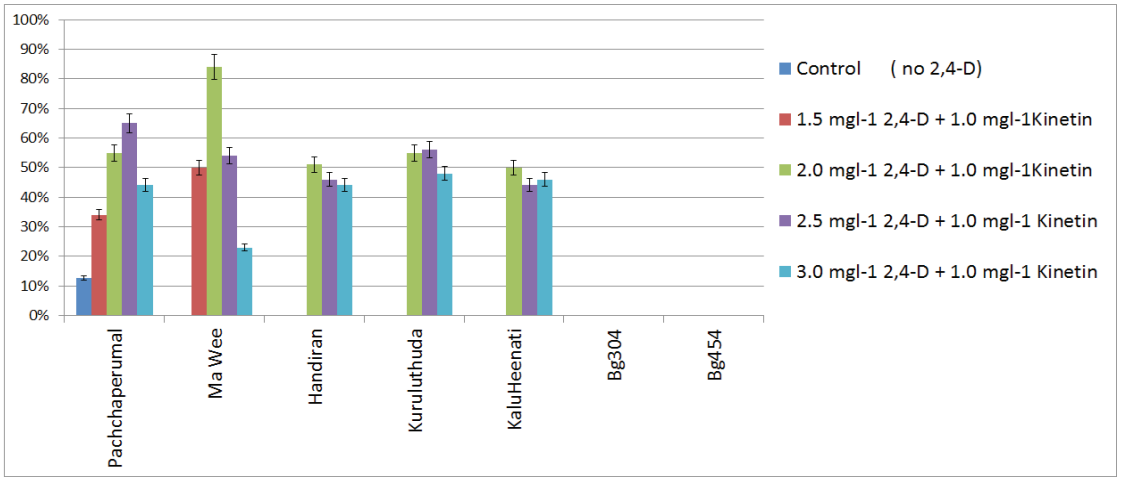

Figure 04: Callus induction under different 2,4-D and Kinetin concentrations

As indicated by UPGMA Dendrogram (Figure 4) group 3-b contains nine glufosinate-resistant varieties and their life span is limited to 3 ½ months except Bg379-2 (4 - 4 ½ months). Most of these varieties are resistant to GM, BL, BB, Bph and have high yield potential with recovering ability of chlorophyll and which can be used in sustainable cultivation.

Callus Induction

Among the seven rice varieties the highest callus induction percentage was recorded by

Pachchaperumal (Figure 04 and 05). Calli developed from the scutellar region of the seeds were selected for mutational study and were visible 4-8 days after culturing (Figure 06).

Relatively high callus induction was observed when the culture media was supplemented with $2mgl^{-1}$2,4-D and $0.1mgl^{-1}$BAP (Figure 4 and 5). Therefore, that combination of growth regulators can be used for the callus induction from *Kaluheenati, Kurulthuda,Handiran, Ma Wee* and *Pachchaperumal.*

Auxin (2,4-D) is considered to be the most favorable plant growth regulator for callus induction and callus proliferation (Upadhya *et al*., 2015). In order to optimize the best suited 2,4-D concentration to induce somatic embryogenesis from rice, MS media was supplemented with different concentrations of 2,4-D along with constant concentrations of Kinetin and BAP. Results indicated that 2 mgl^{-1} 2,4-D with $0.1mgl^{-1}$ Kinetin or BAP was the most appropriate combination for callus induction. This finding agrees with the previous studies documented by Revathi *et al.,* (2011), Alam *et al.,* (2012), Dahanayake and Ranawake, (2012), Mannan *et al.,*(2013), Dissanayaka and Dahanayaka, (2014), and Upadhya *et al.,* (2015).

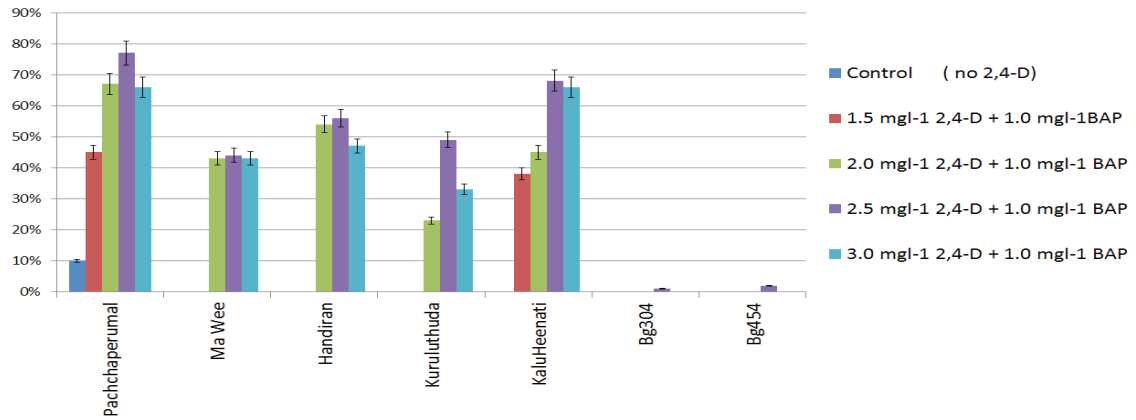

Figure 05: Callus induction under different 2,4-D and BAP concentrations

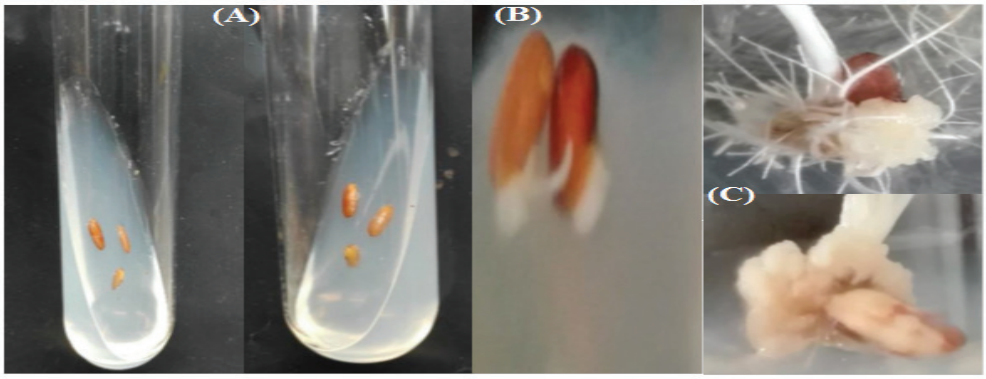

Figure 06: **Different stages of callus induction in cultured rice seeds. (A) Initial stage; (B) 8th day after inoculation; (C) 12th day after inoculation**

First week after EMS treatment, a noticeable difference was observed in the color of certain treated calli compared to that of the viable calli after the treatment and the calculated callus survival percentage values revealed that all three varieties were able to survive under EMS treatment (Figure 7). According to Ekanayake *et al*., (2016), 50% survival of calli was recorded when treated with 0.2% EMS concentration. Results of the 1% TTC assay (Figure 8) have confirmed the inferences of visual evaluation of EMS treated calli.

Evaluation of glufosinate-resistance in mutated calli

Based on the findings of natural glufosinate-resistance, *Ma Wee* and *Pachchaperumal* were selected as references to evaluate induced glufosinate-resistance in mutated calli. A remarkable change in the resistivity towards glufosinate was noted after the mutation of earlier susceptible rice varieties (Figure 9). The non-mutated control calli showed negative results. Same results have been reported by Ekanayake*et al*., (2016) for glyphosate. TTC viability assay verified the viability of calli after glufosinate treatment.

AFLP molecular study

Comparison of AFLP patterns from eight primer combinations (M31E10, M31E11, M31E12, M31E13, M32E10, M32E11, M32E12, and M32E13) indicated a variation of fragment sizes in calli of reference rice variety (*Ma Wee*), non-mutated and mutated calli-DNA (*Kuruluthuda*, *Kaluheenati*, *Handiran*). Further analysis of AFLP data indicated similar fragment sizes for mutated calli of the susceptible varieties *Handiran*, *Kuruluthuda*and *Kaluheenati* and the reference resistant *Ma wee* calli from M31E10 indicating that there is a possibility of inducing glufosinate resistance in susceptible rice varieties using EMS. Since these fragments were not observed in non-mutated calli, M31E10 could be useful as a potential AFLP marker to identify glufosinate resistant rice varieties.

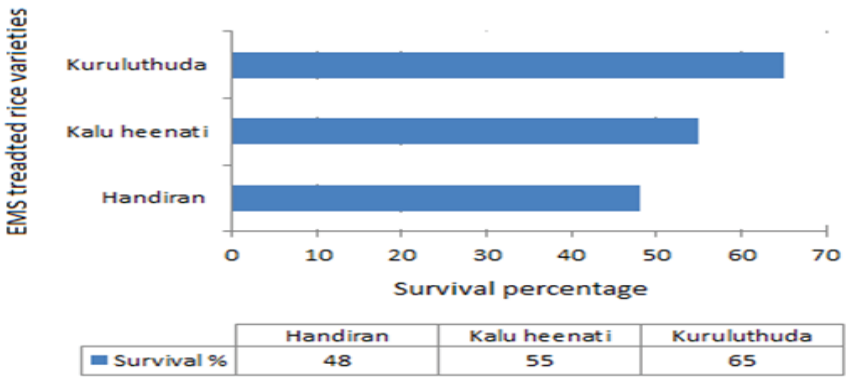

Figure 07: **Survival percentages of mutated calli with 0.2% EMS after one week from the treatment**

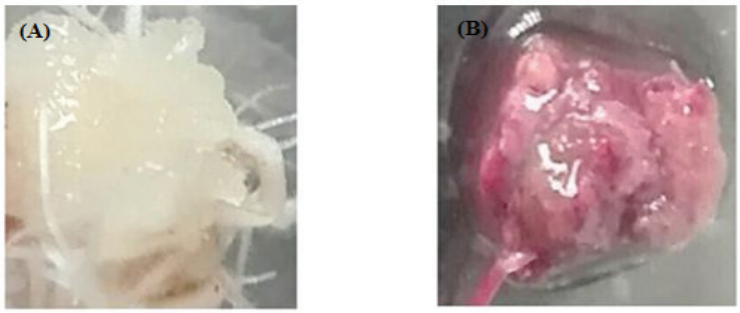

Figure 08: **2,3,5-Triphenyltetrazolium (TTC) staining. (A)Viable callus before staining; (B) Viable callus after staining**

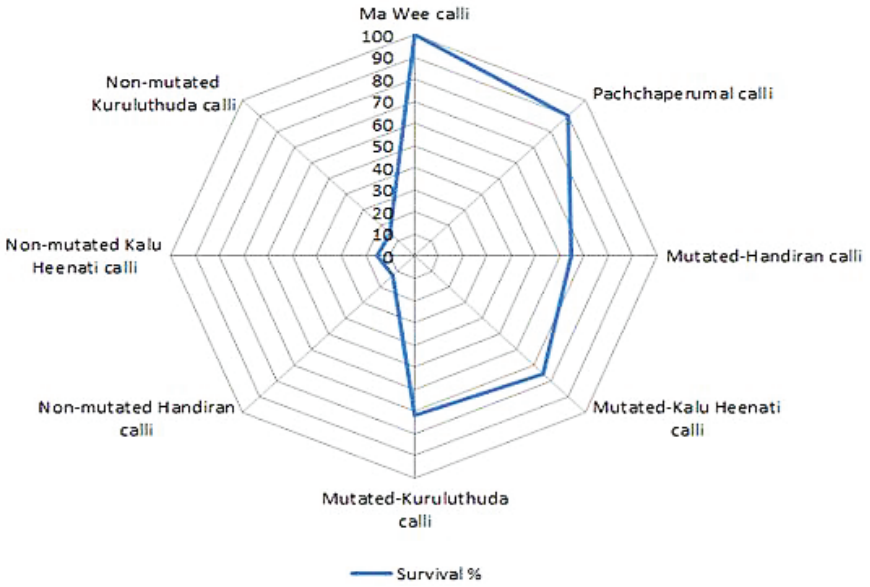

Figure 09: **Survival percentages of mutated and non-mutated calli under 0.2% (V/V) glufosinate application.**

DNA-based markers are not tissue specific and can be detected at any developmental phase of an organism (Ganie *et al*., 2015). Therefore, AFLP marker M31E10 can be potentially used to identify glufosinate-resistant rice varieties. There were hardly-any records on the induced glufosinate-resistance in Sri Lankan rice varieties using classical breeding techniques and this study reveals the possibilities to induce glufosinate-resistance *via in vitro* mutagenesis in rice varieties.

CONCLUSIONS

Present study revealed two traditional rice varieties (*Pachchaperumal*and *Ma wee*) and fifteen inbred ricevarieties (At362, Bg250, Bg300, Bg352, Bg357, Bg358, Bg359, Bg360, Bg366, Bg369, Bg379/2, Bg403, Bg450, Bg454, and Bg94-1) were naturally resistant to glufosinate. Higher percentage of natural glufosinate-resistance was resulted from 0.27 kg ha^{-1} and 0.30 kg ha^{-1}. However, the natural resistance was low at 0.33 kg ha^{-1}glufosinate concentration. Glufosinate application with all three glufosinate concentrations at 3-4 leaf stage did not create a yield penalty in Bg94-1, Bg403, Bg454, Bg379-2 and *Ma Wee*. Visual symptoms including chlorophyll pigment damages caused by glufosinate at low, mid and high concentrations for all varieties faded within 14 days after the treatment.

Glufosinate-resistance could be induced by *in vitro* callus mutation using EMS. The EMS mutated rice varieties are potential candidates in rice breeding programs to breed glufosinate-

resistant varieties. The primer combination, M31E10 is useful as a potential AFLP marker to identify glufosinate-resistance in rice varieties.

Further studies are required to assess the induced glufosinate-resistance in Sri Lankan rice varieties focusing the aspects such as regeneration of plants from seed-derived calli and confirmation of the stability of induced mutations and glufosinate-resistance in regenerated plants from calli for several generations.

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