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Natural Herbicide Resistance (HR) to Broad-spectrum Herbicide, Glyphosate among Traditional and Inbred-cultivated Rice (*Oryza sativa* L.) Varieties in Sri Lanka

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Abstract: Weeds along with insect pests and plant diseases are sources of biotic stress in crop systems. Weeds are responsible for serious problems in rice worldwide affecting growth and causing a considerable reduction in quality and quantity in yield. High concentrations of pre-emergent-broad-spectrum systemic herbicide, Glyphosate is prevalently applied to control rice weeds which intern causes severe damages to cultivated rice varieties, susceptible to Glyphosate. However, there may be rice varieties with natural Herbicide Resistance (HR) which are so far, has not been evaluated. In this study Six traditional and eighteen developed-cultivated rice varieties (Bg, Bw, At and Ld series developed by Rice Research Development Institute, Sri Lanka) were used to screen their natural HR. RCBD with five replicates and three blocks in each treatment-combination was used as the experimental design. As observations, time taken-to seed germination, time taken to flowering; plant height and number of leaves at 12-weeks after sawing, leaf-length, breadth, panicle-length, number of seeds/panicle of resistant plants and controls were recorded. Plants with $\geq$40% resistance were considered as resistant to Glyphosate. Ten inbred-cultivated rice varieties (Bg250, Bg94-1, Bg304, Bg359, Bg406, Bg379-2, Bg366, Bg300, Bw364, At362) and three traditional rice varieties ("Kalu Heenati", "Sudu Heenati", "Pachchaperumal") were naturally resistant to 0.25 g L^{-1} Glyphosate concentration and when increased the concentration (0.5 g L^{-1}) resistance was reduced. This study showed the usefulness of modern statistical method, classification and regression tree analysis (CART) in exploring and visualizing the patterns reflected by a large number of rice varieties (larger experimental database) on herbicide resistance in future.

Key words: Herbicide resistance, glyphosate, *Oryza sativa*, Sri Lanka

INTRODUCTION

Sri Lanka is considered as a country of self-sufficient in rice in past history. However, at present rice cultivation has faced a number of challengers and among those the emergence of rice weeds play an important role since it has been reported that yield loss due to the infestation of rice fields with 30-40% yield reduction. Therefore, the weed control is an essential component of profitable crop production and weeds can be controlled by mechanically, chemically or manually. Though the use of herbicide has been increased in the recent years, there are a number of weed species which are beyond the chemical control. Mainly three categories of herbicides are recommended for rice. The 'Pre-plant herbicides' which are applied before sawing the crop, 'pre-emergence herbicide' before emergence of weed and'post-emergence herbicide' which are applied after emerging the weeds. These herbicides are either broad-spectrum (non-selective) or narrow-spectrum (selective). In order to control rice weeds in the paddy fields of Sri Lanka, it was recommended to apply pre-plant broad-spectrum herbicides such as Glyphosate (round-up) and Paraquat (Navaratne *et al*., 2007) (paraquat is no longer applied and totally banned in Sri Lanka). Glyposate targets both monocotyledons and dicotyledonous weed which will probably permit less herbicide use in terms of amount and number of application. But these herbicides cause damages to the cultivated rice as well (Labrada, 2007; Davis *et al*., 2009). It inhibits 5-enolpyruvylshikimate-3-phosphate synthesis, an enzyme involved in the shikimic acid pathway of plants (Della-Cioppa *et al*., 1986). Glyphosate can cause a significant damage to rice yield with a reduction of yield up to 80% (Davis *et al*., 2009).

Therefore, screening for varieties with natural HR among traditional and inbred rice varieties cultivated in Sri Lanka is useful to incorporate them in breeding programmes to breed new HR varieties. This is a novel measure used to increase selectivity and enhance crop safety and production. HR rice has the potential to

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improve the efficiency of weed management practices and it contributes to reduce rice production costs, increase productivity of rice and reduce usage of herbicides and thereby having less harmful effects to the environment. Since there is an inadequacy of research efforts on natural HR among rice varieties in Sri Lanka, the present study was carried out to screen the presence of HR to pre-emergence broad-spectrum systemic herbicide, Glyphosate in traditionally-cultivated and inbred-cultivated (Bg, At, Bw and Ld series) rice (inbred) varieties (*Oryza sativa* L.).

MATERIALS AND METHODS

Materials: Twenty four rice varieties with germination percentage of ≥85% were selected for the study. Six traditionally cultivated varieties ("Kalu heenati", "Sudu heenati", "suwadal", "Suduru samba", "Pachchaperumal" and "Murungakayan") and eighteen inbred-developed rice varieties (Bg94-1, Bg250, Bg300, Bg304, Bg305, Bg352, Bg357, Bg358, Bg359, Bg360, Bg366, Bg379-2, Bg403, Bg406, Ld365, At362, At308, Bw364) were collected from Rice Research and Development Institutes at Bathalagoda, Ambalanthota, Bombuwela and Labuduwa, Sri Lanka.

Methodology: Seeds of the twenty four rice varieties were surface sterilized and placed in moist chamber for germination (Fig. 1). The germinated rice seedlings were immersed in Glyposate solution with two different concentrations, 0.25 and 0.5 g L^{-1} for 4 days.

Randomized Complete Block Design (RCBD) was used in each treatment and there were five replicates used for each treatment and three blocks in each treatment combination (Fig. 2). A control treatment was carried out without Glyposate. All seedlings were subsequently transferred to soil medium and plant growth observations were taken for ten weeks. Dead plants were considered as susceptible to the herbicide and surviving plants with a substantial growth were considered as resistant to the herbicide (Fig. 3).

For each rice variety, number of resistant plants and % resistance were calculated. The plants with% resistance of ≥40% were considered as resistant to Glyposate. The percentage (%) of resistance was calculated using the following equation:

$$\text{Resistance percentage} = \frac{\text{Number of resistance seedlings}}{\text{Total number of seedlings}} \times 100$$

A plant which did not grow but remained green was considered as tolerant to the herbicide and percentage of tolerance was calculated using the following equation:

$$\text{Tolerance percentage} = \frac{\text{Number of tolerant seedlings}}{\text{Total number of seedlings}} \times 100$$

Statistical analyses: The datasets were subjected to descriptive statistics and whenever possible Analysis of Variance (ANOVA) were also performed. In addition, mean separation test (least significant difference-LSD) was also performed on the dataset. The Regression Tree

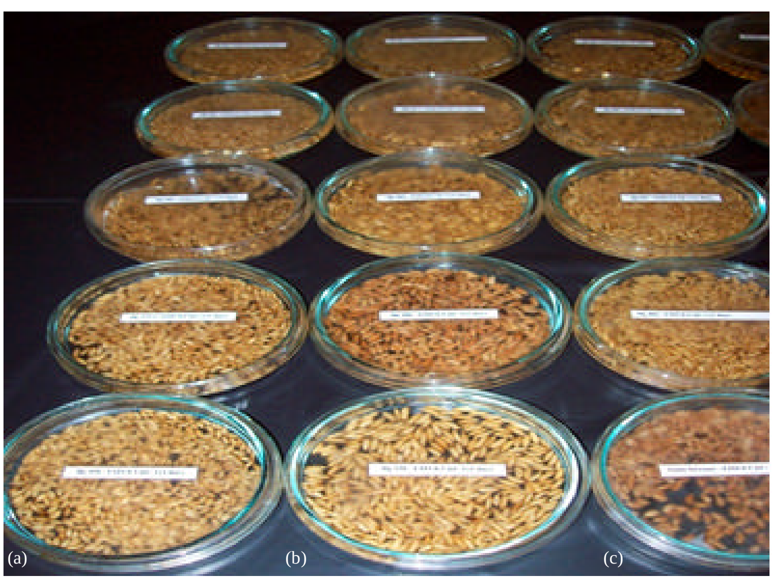

Fig. 1(a-c): Germination of rice varieties were done in distilled water, (a) Control, (b) -0.25 g L^{-1} glyphosate solution and (c) -0.5 g L^{-1} glyphosate solution

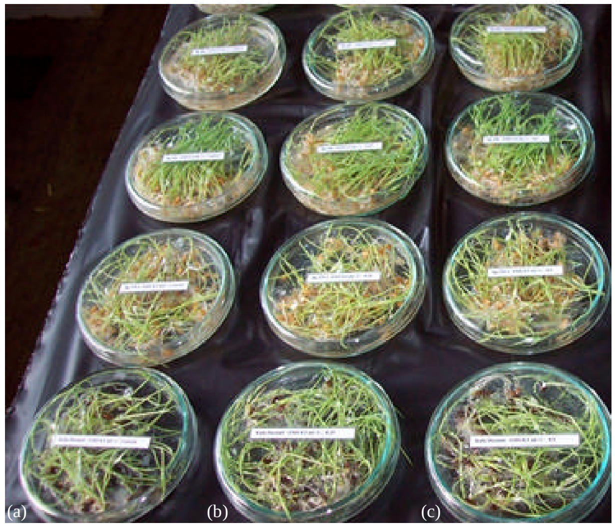

Fig. 2(a-c): Rice seedlings were immersed in two concentrations of glyphosate with a control for 4 days

Fig. 3: Traditional, developed (cultivated) rice varieties were grown after treating with glyphosate treatment

(RT) modeling is an exploratory technique based on uncovering structure in data (Clark and Pregibon, 1992).

Trees explain variation of a single response variable by one or more explanatory variables. The response variable is usually either categorical or numerical and explanatory variable can be categorical and/or numeric. The tree is constructed by recursively splitting the data, defined by a simple rule based on single explanatory variable. At each split the data are partitioned into two mutually exclusive groups, each of which is as homogenous as possible.

The splitting procedure is then applied to each group separately. The objective is to partition the response variable into homogenous groups but also to keep the tree reasonably small. The size of a tree equals the number of final groups. Each group is typically characterized by either the distribution or mean value of the response variable, group size and the values of the explanatory variables that define it. The way that explanatory variables are used to form splits depends on their type. For categorical explanatory variables with two levels, only one split is possible, with each level defining a group. For numeric explanatory variables, a split is defined by a value less than and greater than, some chosen values. Trees are represented graphically, with root node which represents the undivided data, at the top and the branches and leaves (each leaf represents one of the final groups).

The Classification and Regression Tree Analysis (CART) highlights the optimal tree based on the lowest cross-validated relative error, the overall goal being to create a tree with the greatest predictive accuracy for future samples (Breiman *et al*., 1984; Steinberg and Colla, 1995; Quinn and Keough, 2002). All partitions resulted by all variables are compared with the reduction in heterogeneity that they provide. In RTs the heterogeneity in a group is measured by computing mean squared error. According to Breiman *et al*. (1984) relative mean squared errors (R(d)) is defined for a group of observed values y as:

$$R(d) = \frac{1}{N}\sum_{n}(y_n - \bar{y})^2$$

where, $\bar{y}$ is the mean value across all observations y_i.

Each partition of RT generates a left:

$$R(d)_L = \frac{1}{N}\sum_{n}(y_n - \bar{y})^2$$

and right:

$$R(d)_R = \frac{1}{N}\sum_{n}(y_n - \bar{y})^2$$

MSE values where subscript L and R indicates an assignment of number of samples in branches in a partition. The partition that minimizes the change in mean square error:

$$\Delta R(d) = R(d)\text{-}R(d)_L\text{-}R(d)_R$$

is the partition to be selected. The repetitive partitioning of a large database produces a tree with a very large number of terminal nodes. For such databases, there is a possibility to loose generality of the predictive ability of such large trees, because of over-fitting the data in the model. To avoid such over-fitting, a tree has to be pruned to be useful for prediction. The RT methodology has variations regarding tree pruning (Bell, 1999). One common method to access tree fitting is by using developed tree to predict a new set of data. In this process, deviance is replaced by sum squared prediction error and the best sub tree in the sense of minimizing prediction error can be determined. However, holding a subset of aside for validation may be wasteful and the tree selected depends partially on the set of data selected to be held out. Following Breiman *et al*. (1984), it is advisable to use a form of cross-validation to imitate this kind of validation process without wasting data.

The data set is randomly partitioned into ten approximately equal parts and each part is held out in turn. A sub tree T' is then re-estimated on the remaining 90% of the data and the re-estimated tree is used to forecast the 10% of data that was held out, assuming that $CV_i(T')$ denote the Sum Squared Error (SSE) for the ith partition of the process.

The process is repeated for all ten subsets (10-fold cross-validation) of the data and a total cross-validation score:

$$C(T') = \sum_{i} CVi(T')$$

is computed for the sub tree. A sub tree that minimizes the CV (T') is a satisfactory final selection for a tree that is appropriate for the data. It was not possible to sample all possible classification combinations because many combinations did not exist on the landscape and thus result.

All statistical analyses were carried out using SAS Version 9.2 (2008). The Classification and Regression Tree (CART) analysis was carried out using SPSS, V. 16. Models were constructed with 470:250 (total 720) training to test data split. Sixty five percent of the observations was used to construct the model training in the model development stage. The performance of the model on the data set was assessed by 10-fold cross-validation. This procedure involved randomly dividing the data set into 10 partitions. At each step, nine of these partitions were used to fit the model and the performance assessed on the remaining partition was held back as test data. A separate test data set containing 35% of total cases was then used to predict the classification of different treatment. The performance of the models was assessed in terms of Root Mean Square Error (RMSE). For a validation data set (test data set) with a size of n, the RMSE is defined as:

$$RMSE = \sqrt{\sum_{j=1}^{n} \left(y_i - \bar{y}_j\right)^2 / n}$$

Where, denotes the observed values and denotes the predicted value.

RESULTS AND DISCUSSION

The results showed that some of the traditional and developed rice varieties in Sri Lanka posses tolerance or resistance to Glyposate.

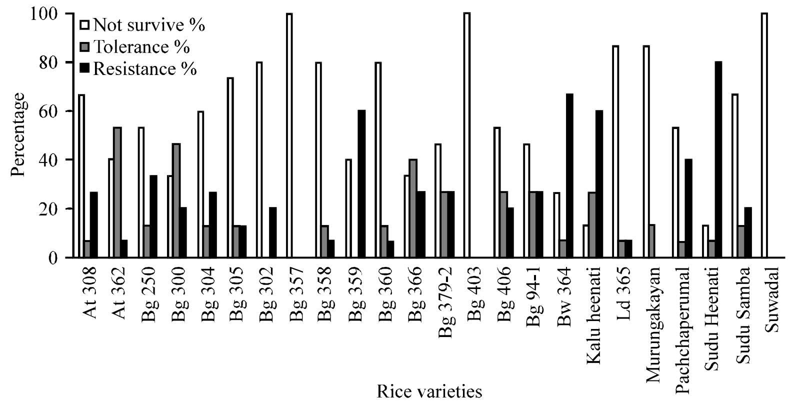

Fig. 4: Presence of resistance/tolerance rice varieties against glyposate at 0.25 g L^{-1}

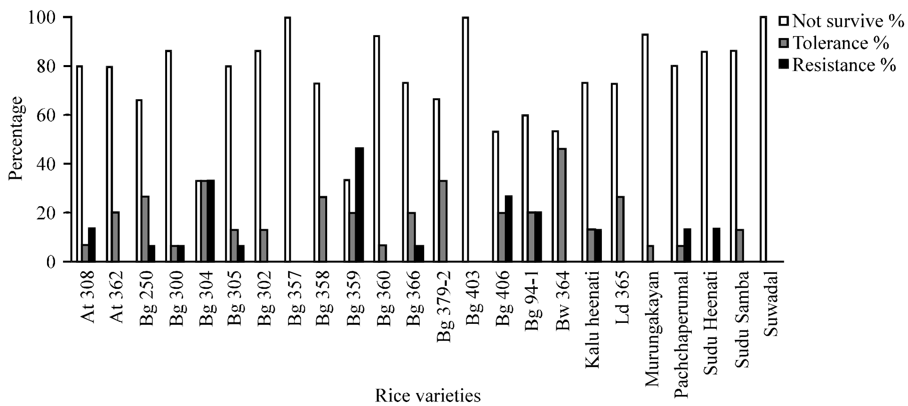

Fig. 5: Presence of resistance/tolerance rice varieties against Glyposate at 0. 5 g L^{-1}

The concentration of 0.25 g L^{-1} of Glyposate affected the growth of most rice varieties and rest of them showed resistance or tolerance to Glyposate (Fig. 4). There were five varieties (Bg359-60%, Bw364-65%, "Kalu Heenti" -60%, "Pachchaperumal"-40%, "Sudu Heenati"-80%) showing ≥40% resistance to 0.25 g L^{-1} treatment of Glyposate and another three varieties (At362-50%, Bg300-50%, Bg366-40%) expressed considerable tolerance (≥40%) to Glyposate. There are reports available on developed rice cultivars resistant to broad-spectrum herbicides, imidazolinone and glufosinate in the USA and Latin America (Olofsdotter *et al*., 2000). IMI rice ("Clearfield") which is resistant to imidazolinone has been adopted for its use in USA, Costa Rica, Colombia and Uruguay (Annou *et al*., 2001). However, there are limited or no studies reported on the existence of herbicide tolerant and/or resistant rice varieties used worldwide. Similarly, there are no comparative studies carried out on the existence of natural herbicide resistance among wild rice, traditionally cultivated and inbred-cultivated rice varieties grown in Sri Lanka.

The treatment of 0.5g L^{-1} Glyposate affected highly to the growth of rice variety (Fig. 5) and there was only one variety (Bg359-45%) which showed the ≥40% resistance and one variety which showed tolerance (Bw364-45%).

The result of the regression tree analysis was use to explore the pattern reflected from the data and to verify the conclusions derived from the conventional statistical analyses and plotting of the data.

The result obtained from the CART analysis is shown in Fig. 6 and the tree indicated that the entire dataset was split into six terminal nodes based on different splitting criteria based on the different variables. The first split was based on the absence or presence of Glyphosate treatment (node 1, 2). The node 1 includes a considerable number of rice varieties those served as control in the experiment. The second split which was further split based on the rice varieties and treatment groups and produced node 3 and 4.

The node three consists of glyphosate-intolerant rice varieties and the node 4 included the higher percentage of glyphosate intolerant rice varieties. The last split of the right branch of the tree indicated the effectiveness of two different Glyphosate concentrations (0.25 and 0.5 g L^{-1}) on the development of resistance and tolerance in different rice varieties (node 5, 6). In the fourth split rice varieties were separated into node 7 and 8 based on the percentage improvement of tolerance and resistance to Glyphosate. Fifth (node 9, 10) split indicated the efficiency of two different Glyphosate concentrations (0.25 and 0.5 g L^{-1}) to develop the resistance and tolerance of different rice varieties.

In summary, the findings of the analysis make it easier to explain the variation of natural Glyphosate resistance across rice varieties. The rice varieties grown under control set up of the experiment were well-separated from the treatment group which further grouped into two separate nodes (3 and 4). The Node 8, of the tree included the rice varieties such as Bg357, Bg403 and "Suwadel" which were not survived under the either concentrations of Glyphosate. The node 7 (which subsequently split into two nodes (node 9, 10) of the tree included the rice varieties such as Bg352, Bg300, Bg3.5, Bg358, At305,

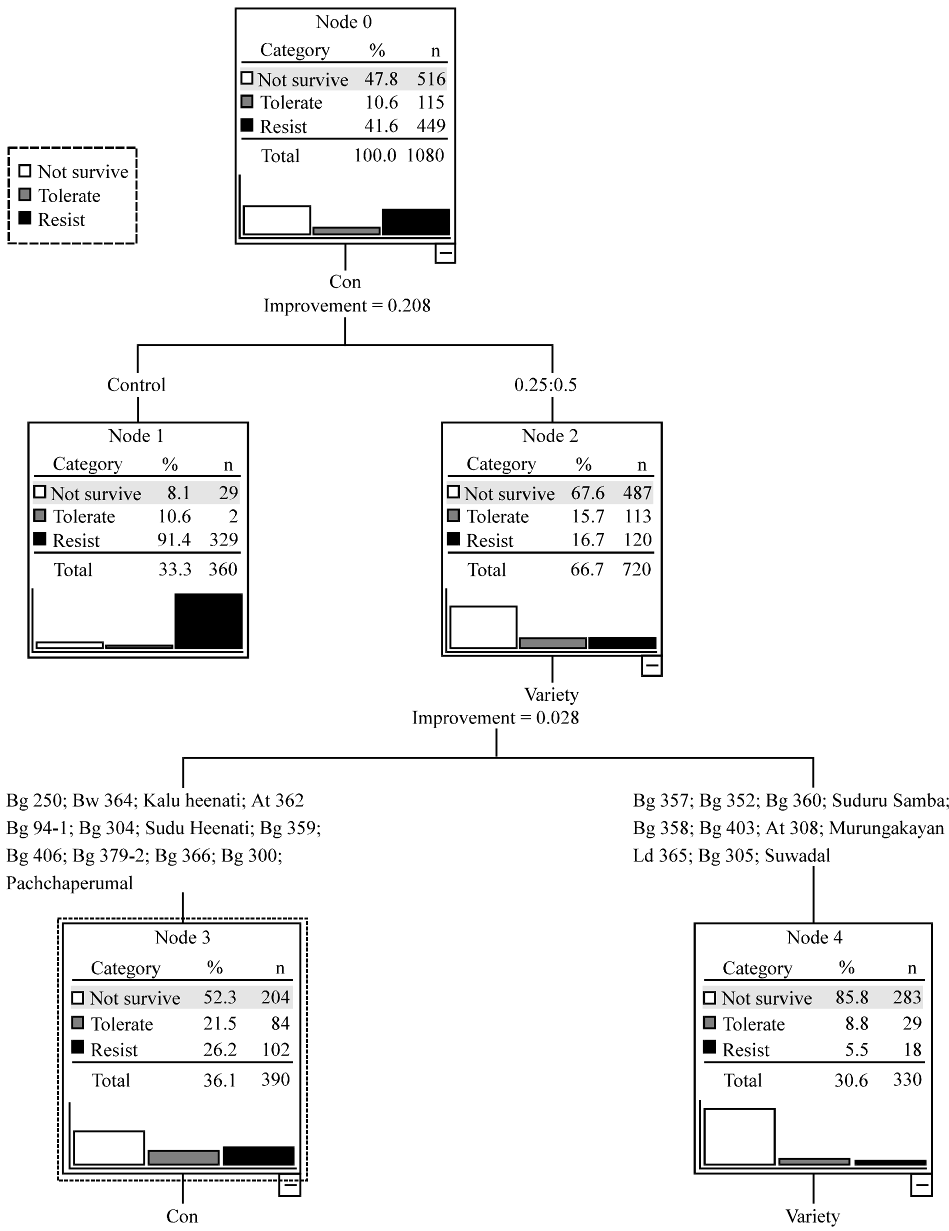

Fig. 6: Continue

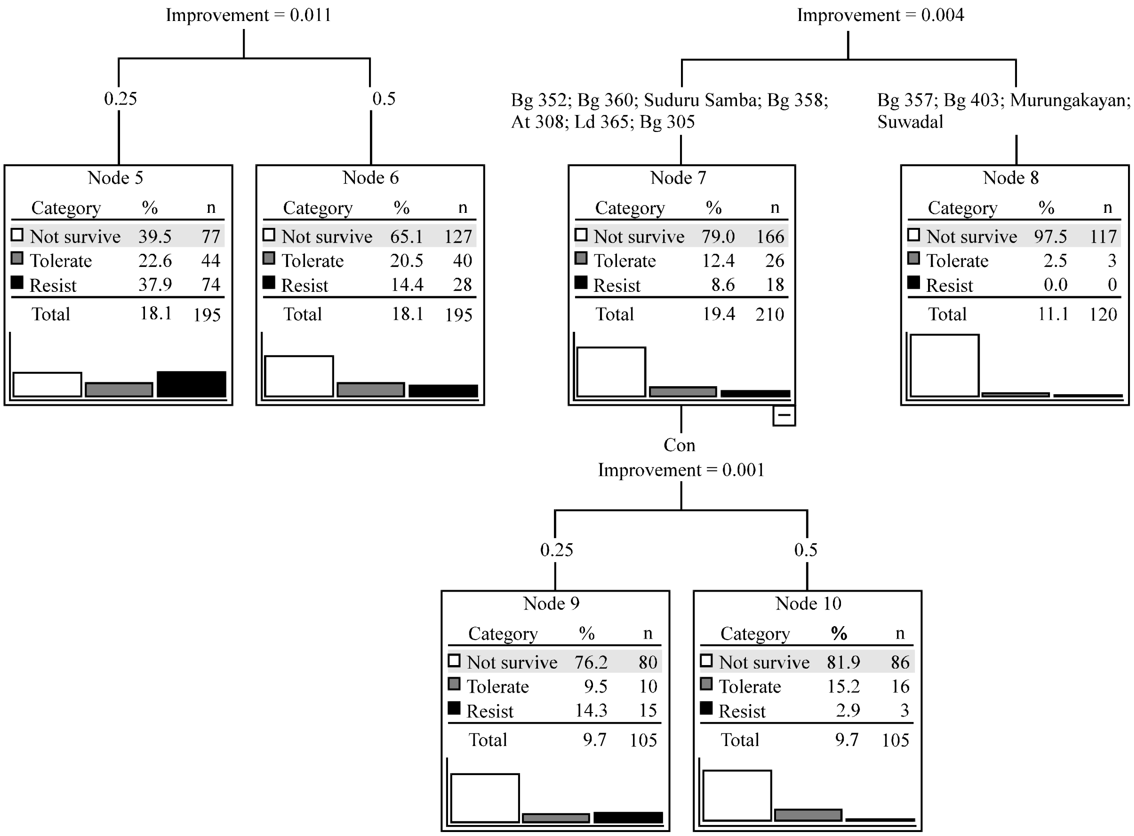

Fig. 6: Regressions tree (CART) constructed for rice varieties for presence of resistance/tolerance to glyphosate

Ld365 and "Sudu Heenati" and based on the Glyphosate concentration used in the experiment this node was split into to terminal nodes which represent higher percentages of Glyphosate intolerance rice varieties This group included the varieties such as Bg357, Bg403, "Murungakayan" and "Suwadel". Under natural conditions the rice varieties, Bg250, Bg94-1, Bg304, Bg359, Bg406, Bg379-2, Bg366, Bg300, Bw364, At362, "Kalu Heenti", "Pachchaperumal", "Sudu Heenati" had a considerable resistance and tolerance to 0.25 g L^{-1} Glyposate. However, all of these varieties showed a less resistance and tolerance to the concentration of 0.5g L^{-1} Glyposate. Comparatively the regression tree analysis made it easier to visualize and explain the variation of natural Glyphosate resistance in the rice varieties than the use of conventional statistical analysis.

CONCLUSION

The present study revealed that thirteen inbred rice varieties (Bg250, Bg94-1, Bg304, Bg359, Bg406, Bg379-2, Bg366, Bg300, Bw364, At362) and three traditional rice varieties ("Kalu Heenti", "Pachchaperumal", "Sudu Heenati") posses considerably high natural HR against Glyposate. The higher percentage of HR was resulted in 0.25 g L^{-1} Glyposate concentration and 0.5 g L^{-1} concentration caused reduction in the percentage of resistance due to inhibited seed germination. In addition, the study showed that use of modern statistical methods such as classification and regression tree analysis (CART) in exploring and visualizing the patterns reflected in the larger experimental database (a large number of rice varieties) on herbicide resistance in future.

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