PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.7595

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RESEARCH ARTICLE

Development of herbicide-tolerant lines through marker assisted backcross breeding

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OPEN ACCESS

ARTICLE HISTORY
Received: 04 February 2025
Accepted: 19 March 2025
Available online
Version 1.0 : 30 March 2025

Check for updates

Additional information
Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

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Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc
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CITE THIS ARTICLE
Anushya Ravi, Bharathi A, Sudha M, Rajeswari S, Manonmani S, Pushpam R, Senthil KG, Senthil A, Raveendran M. Development of herbicide-tolerant lines through marker assisted backcross breeding. Plant Science Today (Early Access).
https:/doi.org/10.14719/pst.7595

Abstract

The widespread adoption of direct-seeded rice cultivation over traditional puddled transplanted rice has become necessary due to labor shortages and water scarcity. Amidst several biotic and abiotic challenges faced by direct seeded rice cultivation, weeds remain as a major bottleneck which negatively influences the performance of rice crop. Chemical method of weed control is one of the most economical ways compared to other weed management techniques. Development of rice lines which are resistant to new classes of broad-spectrum herbicides have paved way to introduce them into direct seeded rice cultivation. In this study, the herbicide-tolerant mutant named Robin-HTM, developed at Tamil Nadu Agricultural University and tolerance to imazethapyr, was used as a donor parent to introgress herbicide tolerant into the popular rice variety ADT 43. Initial evaluation with herbicide was done with the parents alone and subsequent evaluation was done in the advance backcross progenies of ADT 43 x HTM cross. The promising lines which perform better than the recurrent parent is selected and forwarded through backcrossing. Weed related parameters were also calculated in this study, to understand the weed dynamics present and the efficiency of the herbicide used.

Keywords

backcross breeding; direct seeded rice; herbicide tolerant lines; imazethapyr

Introduction

Rice is the staple food for more than 50 % of the world's population, belonging to the family Poaceae, having terminal panicle-type inflorescence and the fruit produced is a caryopsis (1, 2). As of the 2024/2025 agricultural year, global rice production is estimated at approximately 532.66 million metric tons, with China and India (145 million metric tons each) contributing about 27 % to this total (3). To meet the rising demand for food, it is estimated that rice production must grow by 2.4 % annually (4). However, the current rate of productivity increase is only half of this expected rate (5). As the primary food source after wheat, it provides 43 % of the calorie intake for more than two thirds of the Indian population (6). To meet the demand for increasing global population, more efficient means of rice cultivation need to be considered to significantly boost rice productivity exponentially. In early days, transplanted rice was the major form of cultivation used for rice cultivation. Due to rising labor costs and water shortage, direct seeded rice has already been adopted in Malaysia, Vietnam, Sri Lanka, Thailand, Cambodia and the Philippines (7).

Rice crops face numerous challenges from both biotic (e.g., blight, blast, brown plant hopper, stem borer) and abiotic factors (e.g., drought, salinity, submergence, flooding). Among them, weeds are the main constraint limiting the productivity of rice. Management of weeds through chemical herbicides is often the most cost-effective method compared to the costs associated with manual and mechanical weeding. Imidazolinones are a group of herbicides that control a broad spectrum of weeds mainly used in pulses, especially soybeans. With its exceptional flexibility, imazethapyr can be used as post-emergence, at planting, or early preplant herbicide (8). However, for rice cultivation, it is commonly used as post-emergence herbicide i.e., after 21 days of sowing. Imidazolinone herbicides inhibit the production of Acetolactate synthase (ALS enzyme) which is responsible for the production of branched chain amino acids *i.e.,* valine, leucine and isoleucine (9). These amino acids in turn produce proteins, which play a crucial role in cell division (10). ALS is the target of many herbicides, such as sulfonylureas and imidazolinones, which work by blocking the enzyme's function. This inhibition stops amino acid production, leading to plant death (11). However, some rice varieties have developed mutations in the ALS gene, making them resistant to these herbicides and allowing them to survive chemical treatments.

Identifying or developing rice varieties tolerant or resistant to this group of herbicides can be very useful for controlling weeds. Herbicide resistant rice enables efficient management of broad-spectrum weeds and reduced crop injury. Besides, herbicide resistant rice can be utilized for direct sowing which reduces the usage of water resources. This tolerance is achieved through several mechanisms (12):

Target-Site Resistance (TSR)

Herbicides function by targeting specific enzymes essential for plant growth. Mutations in the genes encoding these enzymes can alter their structure, reducing the herbicide's ability to bind effectively. Consequently, the herbicide becomes less effective, allowing the plant to survive. For instance, glyphosate targets the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Mutations in the EPSPS gene can confer resistance to glyphosate.

Non-Target-Site Resistance (NTSR)

This mechanism doesn't involve changes at the herbicide's specific action site. Instead, plants enhance their ability to detoxify or sequester the herbicide, preventing it from reaching its target. For example, some plants can increase the activity of enzymes that degrade the herbicide before it can cause harm.

Enhanced herbicide metabolism

Some plants can metabolize herbicides more rapidly, rendering them non-toxic. This accelerated breakdown prevents the herbicide from accumulating to harmful levels within the plant.

Overproduction of the target enzyme

By producing higher amounts of the enzyme targeted by the herbicide, plants can ensure that enough functional enzyme remains active, even if some are inhibited. This surplus allows the plant to maintain essential functions despite the presence of the herbicide.

Imazethapyr resistant rice varieties can be identified by screening germplasm or through conventional mutagenesis which can then be utilized to develop potential high yielding rice varieties through Marker Assisted Selection or Marker Assisted Backcross Breeding. Emerging gene editing technologies like CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats - CRISPR-associated protein 9) can also be used to create mutations in binding sites of herbicides which may result in herbicide resistance. Other alternate approaches include tissue culture and transgenic approaches (13). An Imazethapyr tolerant rice cultivar developed in USA (Clearfield®) through Ethyl methane sulfonate (EMS) induced mutagenesis is being widely commercialized and cultivated in American regions and some parts of Asia and Europe. The mutant ALS gene in Clearfield® rice makes it insensitive to imidazolinone herbicides (14).

Through mutagenesis, Tamil Nadu Agricultural University developed herbicide-tolerant mutant, Robin-HTM, which is tolerant to imazethapyr. Robin HTM was developed through EMS induced mutagenesis from a drought tolerant upland cultivar, Nagina 22. A single tolerant plant withstanding imazethapyr spray was identified among 100000 plants in M_2 population (15). It was then found to possess a mutated version of ALS enzyme which alters the binding site of the imazethapyr herbicide, thus responsible for the herbicide tolerance (15). This mutant is free from IPR infringements and can be used as a potential donor for transferring herbicide tolerance trait into leading commercial varieties, through marker assisted selection or marker assisted backcross breeding. Robin-HTM was used as a donor parent and crossed with ADT 43 parent. The true F1's was then backcrossed with the recurrent parent and the backcrossed progenies were forwarded through Marker Assisted Backcross Breeding (MABB) scheme. An SSR marker on chromosome 2, located 0.16 Mbp upstream of the AHAS (LOC_Os02g30630) locus and reported to co-segregate with herbicide tolerance (15), was used as foreground marker in this study.

Materials and Methods

Evaluation of the parental genotypes for herbicide (Imazethapyr) tolerance

In this study, the imidazolinone tolerant mutant, Robin-HTM have been employed as a donor parent and backcrossed with an elite cultivar ADT 43, suitable for direct seeding (16). ADT 43 rice variety, developed by Tamil Nadu Agricultural University, is a high-yielding, short-duration rice that matures in 110-115 days. It offers a yield potential of 5.5-6.5 tons per hectare and features medium-slender grains with good cooking quality.

Three different rice genotypes *viz.,* ADT 43, Nagina 22 and HTM were used in this preliminary study. ADT 43 is a fine grain short duration rice variety used as a recipient for introducing herbicide tolerance gene from HTM. Nagina 22 is the wild type cultivar from which HTM was developed through mutagenesis. All the three genotypes were raised under direct seeded condition during summer 2024 at Paddy Breeding Station (PBS), Department of Rice, Tamil Nadu Agricultural University, Coimbatore. Each genotype was raised in three plots of 2m^2 area and maintained normally up to 21 days after sowing. Imazethapyr (commercial name: Pursuit 10 % SL) herbicide was sprayed at the rate of 2.5 mL/L along with the appropriate adjuvant (commercial name: outright) 2.5 mL/L with the help of a backpack sprayer. The performance of genotypes after herbicide spray was recorded and normal fertilizer applications were followed.

Generation of backcross progenies harboring herbicide tolerant loci

The parents ADT 43 and HTM were crossed with each other to produce F_1 seeds. The true F_1s were identified using the foreground marker RM 6844 (Chromosome 2: 18063725-18063917 bp), which has been reported to be linked with the herbicide tolerant loci (15). True F_1s were then backcrossed to the recurrent parent ADT 43 to produce the first backcross generation. Foreground genotyping with the SSR marker RM 6844 was done in the BC_1F_1 generation and the positive plants harboring herbicide tolerant loci were selfed to produce BC_1F_2 seeds. In BC_1F_2 generation, the homozygous positive plants for HTM allele were forwarded to the next generation i.e., BC_1F_3. The selfed seeds of the BC_1F_3 generation were used in the present study giving rise to BC_1F_4 generation (Fig. 1).

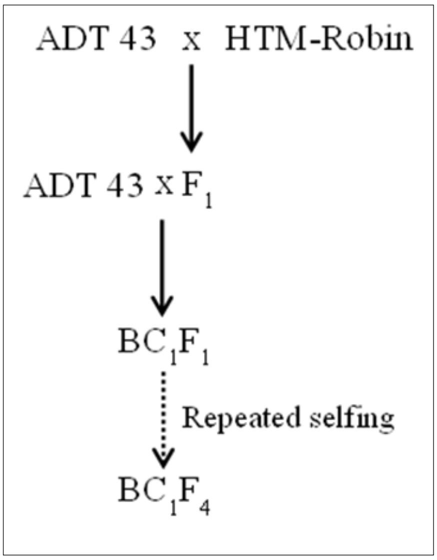

Fig. 1. Schematic representation of markers assisted backcrossing breeding scheme followed for ADT 43 x HTM in the study.

Evaluation of herbicide tolerant lines developed through MABB

During Kharif 2024, BC_1F_4 progenies harboring HTM allele along with parents ADT 43 and HTM were sown in two replications under direct seeded conditions, one being the control (Unsprayed) and the other one being the treatment (Sprayed). The treatment plots were maintained normally up to 21 days and imazethapyr was sprayed at the rate of 2.5 mL/L along with adjuvant at the same after 21 days.

Weed related parameters recorded included weed flora composition, fresh weight of weeds/m^2, dry weight of weeds/m^2 and species density per m^2. Agronomic traits like plant height, days to first flowering, days to 50 % flowering, number of productive tillers, panicle length, flag leaf length, flag leaf width, number of filled grains, number of chaffy grains, single plant yield were recorded from three random plants both under treatment and control plots. Student's t test was used to determine the statistical significance of differences in single plant yield between sprayed and unsprayed plots ($p \leq 0.05$).

Weed control efficiency and relative weed density were calculated by the formula given below (17).

$$\text{Weed control efficiency} = \frac{\text{Dry weight of weeds in control plot - Dry weight of weeds in treated plot}}{\text{Dry weight of weeds in control plot}} \times 100 \quad \text{Eqn. 1}$$

$$\text{Relative weed density} = \frac{\text{Density of a particular species in a community}}{\text{Density of all species in a community}} \times 100 \quad \text{Eqn. 2}$$

Results

Evaluation of the parental genotypes for herbicide (Imazethapyr) tolerance

Direct sowing of parental varieties used in the present study (ADT 43 and HTM) and the wild type cultivar of the Robin mutant (Nagina 22) was taken up in a plot size of 2 m^2 area. Imazethapyr spray was given at 21 days after sowing at the concentration of 2.5 mL/L by using battery operated hand sprayer and performance of the parental lines were evaluated. ADT 43 and Nagina 22 cultivars exhibited high level of susceptibility to imazethapyr spray (Fig. 2) and showed 100 % mortality, whereas the herbicide tolerant mutant "Robin" exhibited complete survival against imazethapyr spray (Fig. 2).

Generation of backcross progenies harboring herbicide tolerant loci

Initial crosses were made between ADT 43 and Robin HTM to obtain F_1 seeds. ADT 43 was then backcrossed with the positive plants genotyped with RM 6844 marker in the F_1 generation. Among 18 BC_1F_1 plants, three were confirmed to carry the target allele based on foreground selection (Fig. 3). These three positive plants were forwarded to BC_1F_2 generation and the 10 homozygous positive plants in the BC_1F_2 generation were selfed and used for further backcrossing with ADT 43. The selfed seeds gave rise to BC_1F_3 generation which was again selfed to produce BC_1F_4 seeds.

Fig. 2. ADT 43, Robin-HTM and Nagina 22 after Imazethapyr spray.

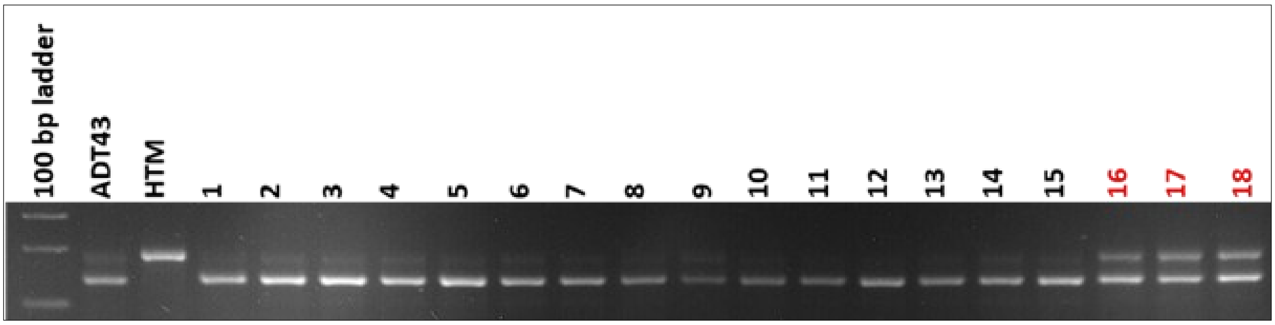

Fig. 3. Foreground marker genotyping of BC_1F_1 generation of ADT 43 x HTM cross.

Agronomic evaluation of HT-BILs

Advance backcross progenies belonging to BC_1F_4 generation of the ADT 43 x HTM cross, were used for agronomic evaluation (Fig. 5). Imazethapyr spray was given in the treatment plot at 21 days after sowing and agronomic performance of the progenies belonging to BC_1F_4 generation of ADT 43 x HTM cross was evaluated. All the eight progenies survived the imazethapyr spray, whereas the recurrent parent ADT 43 showed susceptibility and died. The progenies in the treatment plot showed increased plant height when compared to the control plot where the growth would have been suppressed due to competition from weeds.

Data on agronomic characters i.e., days to first flowering, days to 50 % flowering, plant height, number of productive tillers, panicle length, flag leaf length, flag leaf width, number of filled grains in primary panicle, number of chaffy grains in primary panicle and single plant yield were recorded from three plants taken at random from each plot under sprayed and unsprayed conditions. Mean data for all the characters from herbicide treated plots are given in Table 1 along with single plant yield data taken from unsprayed plots. Recurrent parent data alone corresponds to the data taken from unsprayed conditions, since it exhibited 100 % mortality after imazethapyr spray.

Student's t test was performed between single plant yields obtained from sprayed and unsprayed plots and there is no significant difference ($p \leq 0.05$) present between them. Significant difference from the recurrent parent ADT 43 was observed for the characters like plant height, number of productive tillers, panicle length, flag leaf length, number of chaffy grains in the primary panicle and single plant yield under sprayed and unsprayed conditions. Lines 13-17-13-1, 13-17-13-2, 13-17-13-3, 13-18-31-1 and 13-17-23-1 exhibited significant increase than recurrent parent ADT 43 in number of productive tillers and single plant yield under sprayed condition. Line 13-17-23-1 exhibited significant increase than ADT 43 in single plant yield under both sprayed and unsprayed conditions (Fig. 4).

Table 1. Agronomic performance of ADT 43 HT-BILs in comparison with the recurrent parent ADT 43

Genotype	DF[a]	DFF[b]	PH[c]	NT[d]	PL[e]	FLL[f]	FLW[g]	FG[h]	CG[i]	SPY[j] (SPRAYED)	SPY[j] (UNSPRAYED)
13-17-13-1	74	78	91	25.00*	22.53	25.00	1.23	77.67	65.33*	18.00*	12.33
13-17-13-2	76	79	85.67	31.67*	19.40	19.40*	1.27	75.67	56.33	25.67*	11.33
13-17-13-3	74	78	96.67*	29.00*	20.10	23.90	1.23	69.00	53.67	24.67*	10.67
13-18-31-1	76	79	95.67*	22.00*	20.63	24.47	1.13	67.33	41.33	20.33*	14.67
13-17-23-1	76	79	91.00	23.00*	23.57	24.80	0.87	72.67	57.00	18.33*	24.67*
13-17-15-1	74	77	84.33	19.00	25.17	24.20	1.20	58.00	53.00	15.33	14.33
13-17-15-2	76	79	77.67*	13.67	25.27	21.50*	1.03	61.67	62.33*	14.67	19.67*
13-17-14-1	75	78	90.00	10.33	26.07*	23.93	1.27	61.00	59.33	10.33	12.00
HTM	60	65	96.33	21.00	16.00	21.27	0.97	31.00	32.00	16.34	14.33
ADT 43	72	76	85.67	14.00	21.67	27.00	1.07	78.33	40.33	-	10.33
CD (5%)			**6.95**	**6.51**	**3.65**	**4.41**	**0.24**	**25.3**	**21.4**	**7.39**	**5.91**

[a] - Days to first flowering; [b] - Days to 50% flowering; [c] - Plant height in cm; [d] - Number of tillers; [e] - Panicle length in cm; [f] - Flag leaf length in cm; [g] - Flag leaf width in cm; [h] - Filled grains in primary panicle; [i] - Chaffy grains in primary panicle; [j] - Single plant yield in grams. *- Significant at 5% level

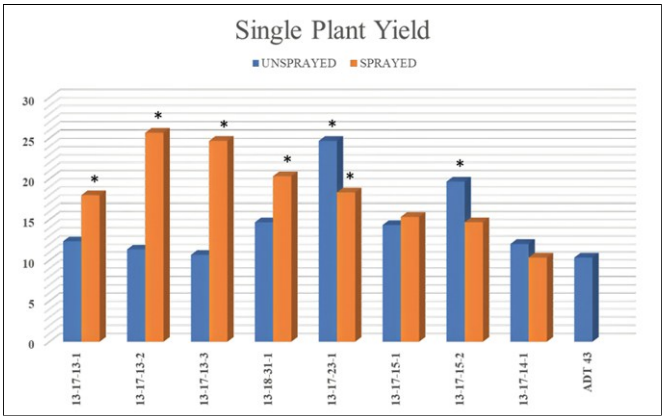

Fig. 4. Comparison of single plant yield (g) between ADT 43 HT-BILs and their parents in unsprayed and herbicide sprayed condition; *Significantly superior to the recurrent parent, ADT 43.

Weed indices

The spectrum of weed flora observed in the experimental field included species like *Cyperus difformis* and *Cyperus rotundus* under sedges, *Dinebera retroflexa*, *Echinochloa colona*, *Echinochloa crusgalli* and *Fimbristylis aestivalis* under grasses and *Alternanthera paronychioides* under dicots. Application of imazethapyr herbicide resulted in 100 % mortality of all these weeds.

Weed control efficiency signifies the ability of herbicide in controlling the weed biomass in the treated plot when compared to untreated control. Dry matter of weeds was found to be higher in the untreated control plot than in the treated plot demonstrating the effective control of weed species by imazethapyr herbicide. The weed control efficiency of the imazethapyr herbicide ranged from 76.2-89.1 % (Table 2).

Relative weed density was calculated 60 DAS and the chronological order of major weed species present were as follows: *Cyperus rotundus, Cyperus difformis, Dinebera retroflexa, Alternanthera paronychioides, Echinochloa colona, Echinochloa crusgalli* and *Fimbristylis aestivalis* (Table 3).

Fig. 5. Field view of imazethapyr sprayed plots and unsprayed control plots.

Discussion

Increase in the adoption of direct seeded rice cultivation rather than traditional puddled transplanted rice poses high risk of weed infestation which hinders the yielding potential of rice crop. The critical weed-free period for DSR extends from 11 to 83 days after sowing, which is longer than that for transplanted rice, indicating a heightened need for effective weed management strategies in DSR systems (18). Potential yield losses due to weed infestations in dry DSR can reach up to 50 %, underscoring the importance of integrating various weed management approaches to ensure sustainable rice production (19). Among several weed management techniques, chemical methods of weed control appear to be an effective way to control the broad spectrum of weeds. Imazethapyr herbicide, well known for its broad spectrum of weed control, is mainly used in pulses. Whereas, rice crops are sensitive to this herbicide, restricting its use in rice cultivation. Developing herbicide resistant rice lines will enable us to use these broad-spectrum herbicides in rice cultivation.

Robin-HTM, an EMS induced mutant, developed from Tamil Nadu Agricultural University, is tolerant to imazethapyr herbicide have been used as the donor parent in this study to introgress herbicide resistance trait into

Table 2. Weed control efficiency observed in 6 plots of 1 m^2 area

Replication	Dry matter content of weeds in the control plot	Dry matter content of weeds in the treatment plot	Weed control efficiency
1	450	136	**69.8**
2	260	62	**76.2**
3	573	88	**84.6**
4	625	85	**86.4**
5	731	80	**89.1**
6	670	86	**87.2**

Table 3. Relative weed density of major weed species observed in 3 plots of 1 m^2 area

Name of the weeds	Weed count^1	Weed count^2	Weed count^3	Relative weed density^1	Relative weed density^2	Relative weed density^3	Average weed density
Cyperus difformis	184	151	153	23.2	19.0	17.8	**20.0**
Cyperus rotundus	308	281	462	38.9	35.3	53.8	**42.7**
Dinebera retroflexa	146	143	96	18.4	18.0	11.2	**15.9**
Echinochloa colona	38	42	50	4.8	5.3	5.8	**5.3**
Echinochloa crusgalli	19	14	22	2.4	1.8	2.6	**2.2**
Fimbristylis aestivalis	18	10	13	2.3	1.3	1.5	**1.7**
Alternanthera paronychioides	79	154	63	10.0	19.4	7.3	**12.2**

Numbers indicate the replication plots

ADT 43 rice variety. Initial screening and evaluation of parental lines with imazethapyr spray validated the presence of herbicide tolerant loci in the donor parent Robin HTM (20,21).

The progenies of ADT 43 x HTM cross belonging to BC_1F_4 generation survived imazethapyr spray and agronomic evaluation indicated significant difference of several traits from the recurrent parent ADT 43. The line 13-17-23-1 exhibited significant increase in single plant yield from ADT 43 under both sprayed and unsprayed conditions which tends to be promising. This line can be used for further backcrossing with ADT 43 to increase the recurrent parent recovery percentage. A total of seven predominant weed species have been observed in the experimental field, including grasses, sedges and broad-leaved weeds. Another study (20) reported a similar spectrum of weed species in their study. A study conducted in the farmer's fields in North-west Cambodia reported the presence of *E. crusgalli, E. colona, C. iria*, *Melochia corchorifolia* L., *L. chinensis, F. miliacea, Oryza sativa* f. *spontanea* and *C. difformis* L (22). Weed dynamics were calculated in a study conducted in Colombia, which reported the predominance of *E. colona* followed by *Digitaria ciliaris, Cyperus iria* and *Ischaemum rugosum* (23). Weed control efficiency was found to be high, indicating the efficient management of weeds by imazethapyr herbicide. Relative weed density indicated the predominance of sedges followed by grass and broad-leaved weeds.

Conclusion

This study indicates that Robin-HTM can be a promising donor for the introgression of herbicide tolerance trait into popular rice varieties. Unlike gene edited or transformed lines, those which are developed through conventional mutagenesis are free from extra regulatory protocols for their release. Hence, superior lines identified in this study could be evaluated under multi-location trials and other yield trials and can be recommended for direct seeded rice cultivation regions. Also, the herbicide imazethapyr proves to be an efficient herbicide in controlling a wide range of weed species from grasses, sedges to broad-leaved weeds.

Acknowledgements

The support provided by Centre for Plant Molecular Biology and Biotechnology, Paddy Breeding Station and Centre for Plant Breeding and Genetics to carry out this research is highly acknowledged. The funding provided by Department of Biotechnology; Government of India (BT/PR40997/ATGC/127/62/2020) is acknowledged.

Authors' contributions

RM, MS and SM aided in planning the outline of this study. RA carried out the research and drafted the manuscript. BA reviewed and edited the manuscript. RM, MS, SM, RS, PR, SKG and SA overviewed the research and finalised the manuscript. All the authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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