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### Introgression of Allelopathic Traits into Drought-Tolerant MR1A1 Rice Variety through Backcross Breeding and Validation via the Relay Seeding Technique

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## Abstract

Backcross breeding is a widely used approach for introgressing desirable genes into elite cultivars. In rice production, weeds and drought are two major constraints that substantially reduce rice yield. Although herbicides are commonly used for weed control, their excessive application poses risks to human health, disrupts ecological balance, and promotes the evolution of herbicide-resistant weed populations. Therefore, developing rice varieties that combine allelopathic potential with drought tolerance represents a sustainable breeding strategy for reducing weed pressure while improving climate resilience. The novelty of this study lies in the marker-assisted introgression of allelopathic quantitative trait loci (QTLs) from the Bangladeshi rice cultivar BR17 into the drought-tolerant Malaysian variety MR1A1, followed by the validation of allelopathic performance using the relay-seeding technique and the development of improved allelopathic breeding lines. The experiment was conducted from March 2020 to July 2022 at Ladang 15 and the Seed Technology Laboratory, Faculty of Agriculture, Universiti Putra Malaysia. BR17 was used as the donor parent for allelopathic traits, whereas MR1A1 served as the recurrent parent because of its high yield potential and drought tolerance. Marker-assisted backcross breeding was employed to develop BC<sub>1</sub>F<sub>1</sub>, BC<sub>2</sub>F<sub>1</sub>, BC<sub>2</sub>F<sub>2</sub>, and BC<sub>2</sub>F<sub>3</sub> populations through foreground and background selection. The selected BC<sub>2</sub>F<sub>2</sub> introgression lines were subsequently evaluated for allelopathic activity to identify superior lines with enhanced weed-suppressive potential while retaining the desirable agronomic characteristics of the recurrent parent. This study successfully developed improved drought-tolerant rice lines with enhanced weed-suppressive potential, providing valuable genetic resources for breeding climate-resilient rice. These findings demonstrate the effectiveness of integrating marker-assisted backcross breeding with phenotypic validation to combine allelopathy and drought tolerance in elite rice backgrounds. The developed lines provide valuable breeding materials for developing climate-resilient, weed-competitive rice cultivars, with the potential to reduce herbicide dependence, support sustainable weed management, and improve rice productivity under changing climatic conditions.

**Keywords:** Allelopathic; Drought; Backcrossing; Validation; Relay Seeding Technique; Rice

## 1. Introduction

Rice (*Oryza sativa* L.) is the primary staple food for more than half of the world's population and provides the majority of dietary energy for nearly two billion people in Asia (Chaudhari *et al.*, 2018). However, sustaining rice production under increasing biotic and abiotic stresses has become a major challenge for global food security, particularly in the face of climate change and rising food demand (Rezvi *et al.*, 2023). Consequently, developing climate-resilient and weed-competitive rice cultivars has become a key priority for sustainable agriculture (Mohanavel *et al.*, 2024). Allelopathy, a naturally occurring biological phenomenon in which plants release secondary metabolites (allelochemicals) that influence the growth and development of neighbouring plants, has emerged as a promising eco-friendly alternative for weed management (Macías *et al.*, 2019). Globally, weeds, pests, and pathogens account for approximately 25% of agricultural production losses (Abouzienna & Haggag, 2016; Kalra *et al.*, 2021). Although chemical herbicides remain the primary method of weed control, their intensive use has accelerated the evolution of herbicide-resistant weeds, increased production costs, and caused environmental contamination, emphasizing the urgent need for sustainable weed management strategies. Unlike synthetic herbicides, these naturally occurring phytochemicals are biodegradable, leave minimal environmental residues, and therefore offer a safer approach to weed suppression (Das *et al.*, 2021). Moreover, increasing knowledge of the molecular basis of allelopathy, quantitative trait loci (QTLs), and the genetic regulation of allelochemical production has strengthened the potential for incorporating allelopathic traits into modern rice breeding programmes (Palanivel *et al.*, 2021; Chung *et al.*, 2020; Nocito *et al.*, 2025).

Backcross breeding is an effective strategy for introducing desirable traits from a donor parent into an elite recurrent parent while preserving the superior agronomic performance of the recurrent genotype

(Vogel, 2009). During successive backcross generations, the proportion of the donor genome is progressively reduced while the recurrent parent genome is recovered, enabling the development of improved lines carrying the target trait with minimal linkage drag (Lewis & Kernodle, 2009). Recent advances in molecular breeding and genomics have substantially accelerated the development of climate-resilient rice cultivars through marker-assisted introgression and precise QTL deployment. Marker-assisted backcross breeding (MABB) has been successfully applied to introgress multiple abiotic- and biotic-stress tolerance genes into elite rice cultivars while minimizing linkage drag and preserving desirable agronomic characteristics (Mohanavel *et al.*, 2024). Furthermore, genome-wide analyses of early backcross selective introgression populations have identified several novel QTLs associated with weed competitiveness, seedling vigour, and early growth traits, providing valuable genomic resources for breeding rice with enhanced weed-suppressive ability (Nocito *et al.*, 2025). These recent advances demonstrated that integrating molecular markers, QTL mapping, and genomics-assisted breeding can significantly improve the efficiency of developing allelopathic, weed-competitive, and climate-resilient rice cultivars for sustainable productivity.

Despite the growing interest in allelopathic rice, limited information is available on rice accessions possessing strong allelopathic activity against barnyard grass (*Echinochloa crus-galli*), one of the most destructive weeds in rice ecosystems. Although considerable progress has been made in identifying allelopathic rice germplasm, mapping quantitative trait loci (QTLs), and characterizing allelochemicals associated with weed suppression, most previous studies have focused on either the identification of allelopathic donor genotypes or the genetic analysis of allelopathic traits. Limited attention has been given to introgressing allelopathic QTLs into elite drought-tolerant rice cultivars. The novelty of this study lies in the integration of backcross breeding with biological validation to develop rice lines combining drought tolerance and allelopathic potential in the elite MR1A1 background. Allelopathic QTLs derived from the donor parent BR17 were introgressed into MR1A1 through successive backcrossing followed by validation of weed-suppressive ability using the relay seeding bioassay against *Echinochloa crus-galli*. Therefore, the objectives of this study were (i) to introgress allelopathic QTLs from BR17 into the drought-tolerant MR1A1 genetic background using backcross breeding and (ii) to validate the allelopathic potential of the selected lines using the relay seeding technique against barnyard grass. The resulting breeding lines are expected to contribute to the development of climate-resilient, weed-competitive rice cultivars that support sustainable rice production while reducing reliance on chemical herbicides.

## 2. Materials and Methods

### 2.1 Experimental location and source of plant materials

The study was conducted at the Seed Technology Laboratory, Universiti Putra Malaysia (UPM), Malaysia, from March 2019 to September 2022. Parental lines were initially obtained from accessions stored in UPM's Seed GenBank. Crossing programs were performed at the Glasshouse, Ladang 15, and molecular screening was carried out at the Molecular Laboratory, UPM, from March 2020 to July 2022.

### 2.2 Soil sampling and crop management practices

The experimental soil was collected from a paddy field in Malaysia and thoroughly mixed to ensure uniform distribution of weed seeds in each pot. The soil was then carefully examined, with clay being the predominant soil type. Fertilizers were applied according to the recommended fertilizer and pesticide rates provided by the Malaysian Agricultural Research and Development Institute (MARDI). Fertilizers applied were 80-57-42-50-100 kg/ha of N-P-K-NPK Blue and NPK Green. However, the rice plants were infested by Brown Plant Hopper (BPH) and rice bugs. To address this, Diazinon and Malathion were applied at a rate of 2 mL/L to the affected plants.

### 2.3 Pre-propagation steps

Initially, rice seeds were dried in an oven at 50°C for three consecutive days to break seed dormancy. Following this, the seeds were disinfected. The seeds were then placed in a Petri dish filled with water,

ensuring that all necessary precautions were taken to prevent contamination. After soaking, the water was drained, and the seeds were covered with a piece of soaked cotton cloth and kept in the Petri dish for an additional three days. During this period, the seeds were allowed to germinate. Once pre-germinated, the seeds were transferred to a prepared tray containing a 1:1 mixture of sterile paddy soil and compost, as described by (Wayayok *et al.*, 2015). The tray was maintained under 100% moisture conditions. To prevent rodent and insect damage, the seeds were enclosed in nets. After three weeks, the seedlings were transplanted into well-prepared plastic pots, with three seedlings per pot, spaced appropriately. The seedlings were then allowed to grow in a controlled glasshouse environment until harvest.

## 2.4 Plant materials

Two rice varieties were used in this study: BR17, an allelopathic cultivar developed by the Bangladesh Rice Research Institute (BRRI), Bangladesh, and MR1A1, a drought-tolerant cultivar developed by the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. BR17 served as the donor parent for allelopathic traits, whereas MR1A1 was used as the recurrent parent because of its superior drought tolerance and agronomic performance. The characteristics of both parental lines are presented in **Table 1**.

**Table 1: Characteristics of the parental rice varieties used in the breeding programme**

| Parameter/Variety        | BR17                                                           | MR1A1                                                                       |
|--------------------------|----------------------------------------------------------------|-----------------------------------------------------------------------------|
| Developed by             | Bangladesh Rice Research Institute (BRRI), Gazipur, Bangladesh | Malaysian Agricultural Research and Development Institute (MARDI), Malaysia |
| Origin                   | KN-1B-361-1-8-6-10 (Indonesia)                                 | Mutation of IR76569-259-1-2-1                                               |
| Year of release          | 1985                                                           | 2014                                                                        |
| Main characteristics     | Plant height 115cm, clean rice, medium bold, white in colour   | Plant height 92-99 cm, fine rice, white in colour                           |
| Planting season and time | Rabi (winter rice), late October to Mid-November               | Year-round                                                                  |
| Harvesting time          | Early to Mid-April                                             | Year-round                                                                  |
| 1000-grain weight        | 25.00 g                                                        | 27.80 g                                                                     |
| Yield (t/ha)             | 6                                                              | 4-5                                                                         |
| Maturity (Days)          | 155 (130-135 days in Malaysia)                                 | 79-88                                                                       |

## 2.5 Crossing method and development of breeding lines

### 2.5.1 Backcross Breeding Scheme

The breeding programme followed a marker-assisted backcross breeding (MABC) strategy to introgress allelopathic traits from BR17 into the genetic background of MR1A1 (Figure 1). To synchronize flowering, parental lines were sown at 7-day intervals beginning on 21 May 2021. F<sub>1</sub> hybrids were produced by crossing MR1A1 (♀) with BR17 (♂) through manual emasculation and hand pollination. Emasculation was performed before anthesis by removing all six anthers without damaging the stigmas. Fresh pollen from BR17 panicles was transferred onto the emasculated stigmas around 11:00 a.m., after which pollinated panicles were immediately covered with butter paper bags to prevent contamination. Pollinated panicles were labelled with the cross combination and pollination date. Seed set was confirmed seven days after pollination, and mature F<sub>1</sub> seeds were harvested approximately 28–30 days later.

F<sub>1</sub> plants were screened and confirmed the presence of targeted genes using the tightly linked SSR markers. Phenotypic selection was also performed based on morphological characters including plant height, first flowering date, leaf angle, etc. Twenty-seven confirmed F<sub>1</sub> plants were backcrossed with the recurrent parent (MR1A1) to generate the BC<sub>1</sub>F<sub>1</sub> population.

The BC<sub>1</sub>F<sub>1</sub> population was screened using the tightly linked SSR markers linked to allelopathy and drought tolerance. Five heterozygous plants carrying the desired alleles and exhibiting high phenotypic similarity to MR1A1 were selected and backcrossed with the recurrent parent to produce BC<sub>2</sub>F<sub>1</sub>. Subsequently, selected BC<sub>2</sub>F<sub>1</sub> plants were self-pollinated to develop the BC<sub>2</sub>F<sub>2</sub> generation, and superior BC<sub>2</sub>F<sub>2</sub> plants were further selfed to obtain BC<sub>2</sub>F<sub>3</sub> breeding lines.

The schematic diagram of crossing population development is illustrated in **Figure 1**.

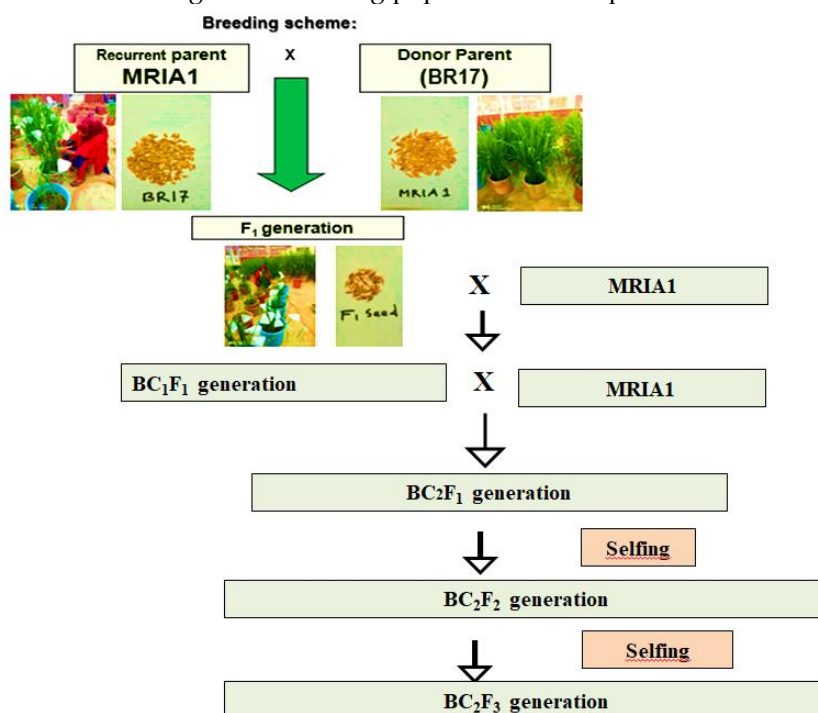


**Figure 1: Crossing and Selection Scheme of BC<sub>2</sub>F<sub>3</sub> generation**

## 2.5.2 Development of Backcross Populations

Seeds from each backcross generation were soaked in distilled water for 24 h and incubated for 48 h to promote germination. Pre-germinated seeds were sown in seedling trays, and 24–26-day-old seedlings were transplanted into prepared field plots. Standard agronomic, irrigation, pest, and disease management practices were followed throughout the experiment.

At each backcross generation, plants were selected based on a combination of foreground marker analysis, recurrent parent phenotypic similarity, and agronomic performance before advancing to the next generation.

## 2.6 Molecular screening:

A total of 476 simple sequence repeat (SSR) markers were screened to assess polymorphism between the donor parent BR17 and the recurrent parent MR1A1 for foreground and background selection. Of these, 71 SSR markers were identified as polymorphic and subsequently used for marker-assisted selection. Functional SSR markers linked to allelopathy (Jensen *et al.*, 2008; El-Naby *et al.*, 2019) and drought tolerance were included in the screening. The selected polymorphic markers were used to confirm the target alleles across the F<sub>1</sub>, BC<sub>1</sub>F<sub>1</sub>, BC<sub>2</sub>F<sub>2</sub>, and BC<sub>2</sub>F<sub>3</sub> generations.

## 2.7 Validation of Allelopathic Potential of Selected Rice Lines

### 2.7.1 Allelopathy Screening

The allelopathic potential of selected BC<sub>2</sub>F<sub>2</sub> lines was evaluated using *Echinochloa crus-galli* (barnyard grass) as the indicator species following the relay seeding bioassay described by Navarez and Olofsdotter (1996), with slight modifications. Twenty surface-sterilized rice seeds from each selected line, together with the donor (BR17) and recurrent (MR1A1) parents were placed individually in 9-cm Petri dishes lined with Whatman No. 1 filter paper. Each dish received 17 mL distilled water and was incubated under laboratory conditions (30 ± 2°C, 12-h photoperiod). Distilled water was replenished every 2–3 days to maintain adequate moisture.

### 2.7.2 Relay Seeding Technique

Rice seedlings were allowed to grow for 12 days before being thinned to ten seedlings per Petri dish. Subsequently, twenty sterilized barnyard grass seeds were relay-seeded into each dish containing rice seedlings. Control treatments consisted of barnyard grass grown without rice seedlings. The co-culture continued until rice seedlings reached the four-leaf stage (22 days after rice sowing). During the experiment, 3 mL distilled water was added every two days to maintain moisture (**Figure 2**).

At harvest, germination percentage, shoot length, root length, and dry weight of barnyard grass seedlings were recorded. Mean germination time (MGT) and average percentage inhibition (API) were subsequently calculated to quantify allelopathic activity. Weed seedlings were oven-dried at 72°C for 72 h before dry weight determination.

The experiment was conducted in a completely randomized design (CRD) with four replications. Barnyard grass (*Echinochloa crus-galli*) was used as the target weed species, whereas only barnyard grass without rice plants served as the control. The relay seeding bioassay evaluates allelopathic potential by allowing rice seedlings to release allelochemicals into a shared growth environment, thereby affecting the germination and early growth of *E. crus-galli*.

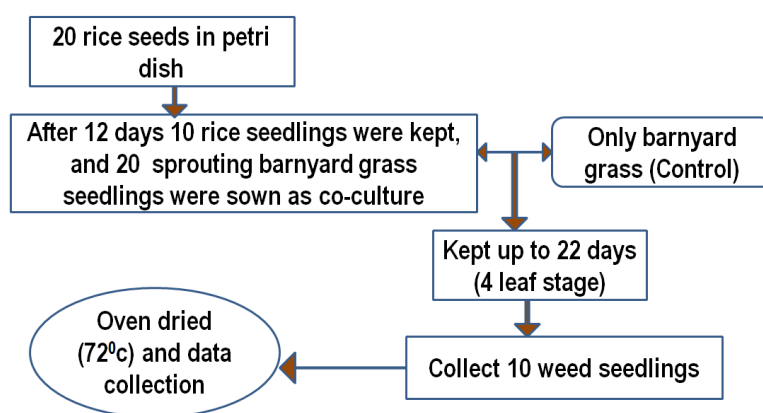


**Figure 2:** Flow chart of Relay seeding method (Navarez and Olofsdotter, 1996)

### 2.7.3 Data Collection

Root and shoot lengths were measured using ImageJ software (Mirmostafae *et al.*, 2020). The germination percentage was recorded starting four days after the seeds were planted. The measured variables were germination percentage, root length, shoot length, dry weight, mean germination time, and average percentage inhibition. The mean germination time (MGT) was calculated using the formula provided by (Nicols & Heydecker, 1968):

$$\text{MGT} = \frac{\sum nT}{\sum nt} \quad (1)$$

where n is the number of newly germinated seeds at time "T" and t is the number of days from sowing. The root and shoot lengths (mm) of five randomly selected *Echinochloa crus-galli* seedlings were measured 17 days after seeding using both techniques. The seedlings were placed in paper bags and oven-dried at 80°C for 72 hours. The percentage reduction in germination, mean germination time, root length, shoot length, and dry matter accumulation was determined using the mathematical equation by (Kabir *et al.*, 2010).

$$\text{Percent reduction} = \frac{C - T}{C} \times 100 \quad (2)$$

Where C represents the value obtained under the control treatment (i.e., without rice) and T denotes the parameters recorded during the rice treatment. The overall impact of rice varieties on the growth of *Echinochloa crus-galli* was analyzed using the average percentage inhibition, as described by (Karim *et al.*, 2012):

$$\text{API} = (\% \text{ root reduction} + \% \text{ shoot reduction} + \% \text{ dry weight reduction} + \% \text{ germination reduction} + \% \text{ mean germination time reduction})/5. \quad (3)$$

## 2.8. Statistical Analysis

Data were analysed using SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was performed according to a completely randomized design (CRD) to determine the effects of rice accessions on the measured traits. When significant differences among treatments were detected, treatment means were separated using the least significant difference (LSD) test at the 5% probability level ( $P \leq 0.05$ ). The correlation between average percentage inhibition (API) and all seedling traits of barnyard grass was calculated using PROC CORR. Agglomerative hierarchical cluster analysis was performed using NTSYSpc version 2.02e (Numerical Taxonomy and Multivariate Analysis System) based on similarity matrices to classify rice accessions according to their allelopathic performance (Sandhu *et al.*, 2013).

## 3. Results

The allelopathic effects of the improved rice lines significantly ( $P < 0.01$ ) influenced the germination percentage, mean germination time (MGT), root length, shoot length, and dry matter accumulation of barnyard grass. The relay seeding technique effectively differentiated the allelopathic potential of the improved lines and identified superior genotypes (**Figures 3–6**).

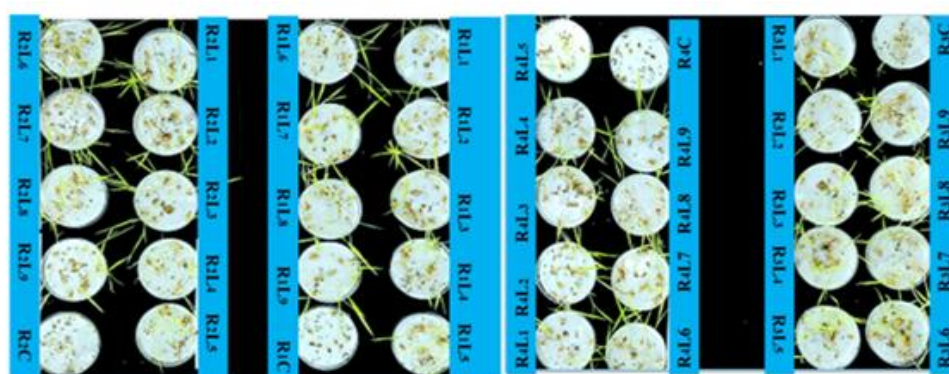


**Figure 3:** Screening of selected improved rice lines using the relay seeding method. Note: R denoted Replication, R1=Replication1, R2=Replication 2, R3=Replication 3, R4=Replication 4, L= lines and C= control, L1= 52-3-3-8, L2= 30-3-3-19, L3=30-3-3-103, L4=20-3-3-9, L5= 52-3-3-8, L6= MRIA1, L7= 52-3-3-7, L8=30-3-3-109, L9= 20-3-3-5,

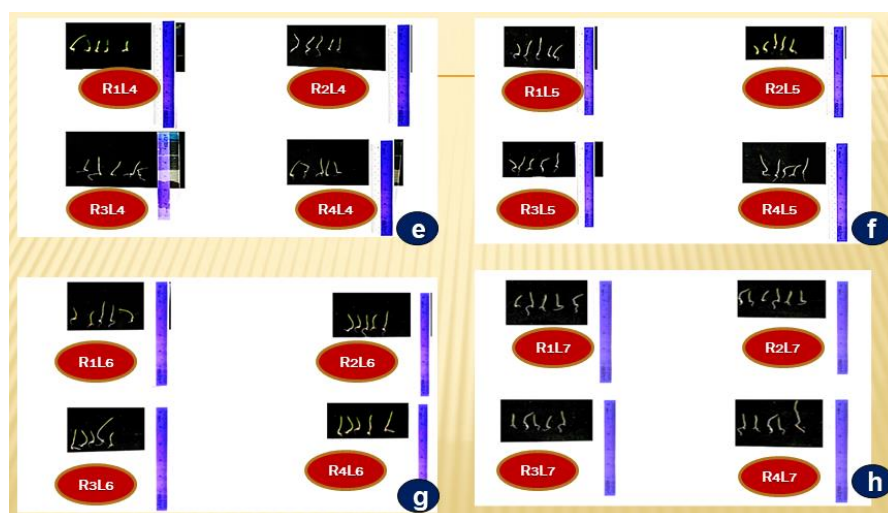


**Figure 4:** Allelopathic effect of best improved rice lines on barnyard grass using relay seeding technique.

Note: a= control, b=L1= 52-3-3-8, c= L2= 30-3-3-19, d= L3=30-3-3-103

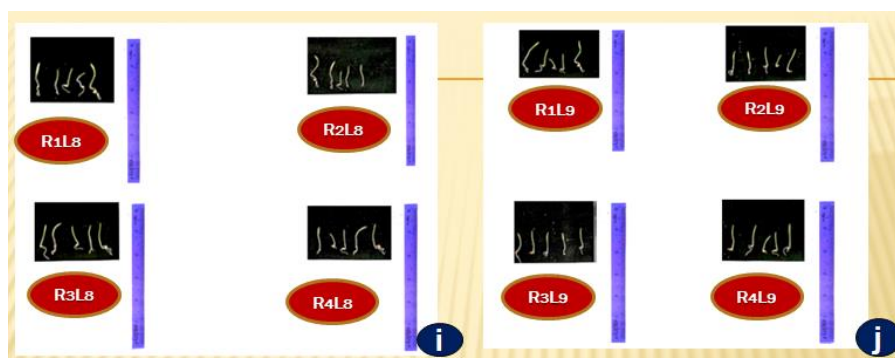


**Figure 5:** Allelopathic effect of best improved rice lines on barnyard grass using relay seeding technique. Note: e=L4=20-3-3-9, f= L5= 52-3-3-8, g= L6= MR1A1, h= L7= 52-3-3-7

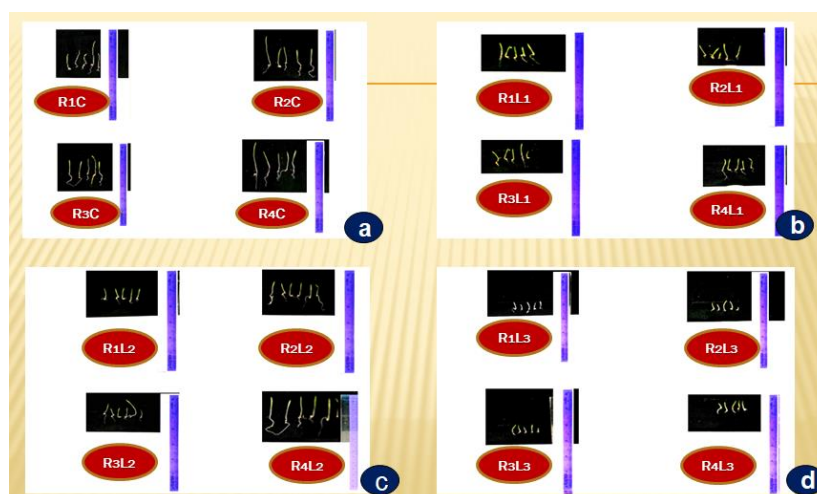


**Figure 6:** Allelopathic effect of best improved rice lines on barnyard grass using relay seeding technique.

Note: i= L8=30-3-3-109, j=L9= 20-3-3-5

Barnyard grass germination was highest in the control treatment (100%) and lowest in **L3** (**Table 2**). Root and shoot growth were also markedly reduced by the improved rice lines, with **L3** producing the shortest root (0.53 cm) and shoot (1.54 cm) lengths compared with the control, which recorded the greatest values (2.05 cm and 4.38 cm, respectively). Similarly, dry matter production was highest in the control (10.0 mg) and lowest in **L3** (4.2 mg), confirming the strong phytotoxic effects of this line (**Table 2**). **L3** differed significantly from the remaining lines for root length, shoot length, dry matter accumulation, and API (**Table 3**).

The improved lines also differed significantly in MGT. The control recorded the longest MGT, whereas **L4** exhibited the shortest MGT, indicating stronger inhibition of barnyard grass germination. Conversely, **L6** showed the weakest effect on germination time.

**Table 2:** The allelopathic effect of improved rice lines on some parameters of barnyard grass using the Relay seeding technique

| Treatment | WSG (%) | RL (cm) | SL (cm) | DW (mg) | MGT (No.day <sup>-1</sup> ) |
|-----------|---------|---------|---------|---------|-----------------------------|
| C         | 100a    | 2.05a   | 4.38a   | 10a     | 3.59ab                      |
| L1        | 83.75b  | 1.26b   | 2.85bc  | 6.5b    | 3.50ab                      |
| L2        | 88.75b  | 1.08b   | 1.82de  | 6.0b    | 3.62ab                      |
| L3        | 71.25c  | 0.53c   | 1.54e   | 4.2c    | 3.53ab                      |
| L4        | 88.75b  | 1.04b   | 2.11cde | 6.3b    | 3.36b                       |
| L5        | 85.0b   | 1.02b   | 2.47bcd | 6.6b    | 3.45ab                      |
| L6        | 87.50b  | 1.12b   | 3.13 b  | 7.3b    | 3.76a                       |
| L7        | 90.00b  | 1.14b   | 2.32cd  | 7.3b    | 3.57ab                      |
| L8        | 85.0b   | 1.04b   | 2.34bcd | 6.8b    | 3.56ab                      |
| L9        | 87.50b  | 0.94b   | 2.39bcd | 6.5b    | 3.59ab                      |
| SEM       | 2.25    | 0.112   | 0.239   | 0.263   | 0.125                       |
| PV        | <.0001  | <.0001  | <.0001  | <.0001  | 0.745                       |

Note: Means with different letters are significantly different, \*\* indicates significance at the  $p < .001$  level, and \* indicates significance at the  $p < 0.05$  level of significance. WSG = Weed seed germination, RL = Root Length, SL = Shoot Length, DW = Dry Weight, MGT = Mean Germination Time

Among the evaluated lines, **L3** consistently exhibited the strongest allelopathic activity against barnyard grass. It presented the greatest inhibition of root length (71.28%), shoot length (64.27%), and dry matter accumulation (59.91%), and recorded the highest Average Percentage Inhibition (API) value (45.16%) (**Table 3**). In contrast, **L3** showed the greatest suppression of seed germination and the lowest dry weight, although the reduction in germination percentage was not significantly different from that of the other improved lines.

**Table 3:** Percent reduction in some parameters of barnyard grass due to the allelopathic effect of different rice varieties under the relay seeding method.

| Treatment | WSG (%) | RL (cm)  | SL (cm)   | DW (mg)  | MGT (day <sup>-1</sup> ) | API      |
|-----------|---------|----------|-----------|----------|--------------------------|----------|
| L1        | 16.250b | 34.24b   | 34.15cd   | 38.20b   | 2.66ab                   | 25.20b   |
| L2        | 11.250b | 46.96ab  | 58.09ab   | 44.06b   | -1.03ab                  | 31.87b   |
| L3        | 28.75a  | 71.28a   | 64.27a    | 59.91a   | 1.6ab                    | 45.16a   |
| L4        | 11.25b  | 47.54ab  | 51.62abc  | 41.98b   | 6.28a                    | 31.74b   |
| L5        | 15.00b  | 48.97ab  | 43.43bdc  | 39.21b   | 3.94a                    | 30.11b   |
| L6        | 12.50b  | 42.24b   | 28.79d    | 32.57b   | -4.81b                   | 22.26b   |
| L7        | 10.00b  | 40.27b   | 46.43abcd | 31.88b   | 0.73ab                   | 25.86b   |
| L8        | 15.00b  | 45.39 b  | 46.24abcd | 36.61b   | 0.97ab                   | 28.84b   |
| L9        | 12.50b  | 49.46a   | 45.55bcd  | 39.203b  | 0.097ab                  | 29.36b   |
| SEM       | 2.50041 | 8.008547 | 5.728087  | 4.255772 | 2.480929                 | 3.355657 |
| PV        | >0.001  | >0.2274  | >0.0155   | >0.0106  | >0.2518                  | >0.0076  |

Note: Means with different letters are significantly different, \*\* indicates significance at  $p < .001$  level, and \* indicates significance at  $p < 0.05$  level of significance. WSG = Weed Seed Germination, RL = Root Length, SL = Shoot Length, DW = Dry Weight, MGT = Mean Germination Time, API = Average Percent Inhibition

Eventually, based on the average percent inhibition, L3 achieved the highest value (45.16%), followed by L4 (31.74%) and L5 (30.11%). The plant with the lowest API value was L6 (22.26%), followed by L1 (25.20%) and L7 (25.86%) (**Table 3**).

### 3.2 Correlation analysis among the quantitative traits of rice lines

Weed seed germination showed a highly significant and positive correlation with root length and dry weight. However, only a positive correlation with shoot length was observed (**Table 4**). On the other hand, root length presented a highly significant and positive correlation with dry weight at a 1% level of probability, but merely a positive correlation was seen with shoot length and mean germination time at a 5% level as well. Additionally, shoot length had a highly positive correlation with dry weight. In contrast, dry weight showed an exclusively positive and significant correlation with average percent inhibition (API) at a 1% probability level. Moreover, API had a highly significant along with a positive correlation with all the parameters at a 1% probability level except mean germination time.

**Table 4.** Pearson's correlation coefficient of improved lines using the relay seeding technique

|     | WSG | RL     | SL    | DW     | MGT      | API    |
|-----|-----|--------|-------|--------|----------|--------|
| WSG | 1   | 0.58** | 0.33* | 0.65** | 0.094ns  | 0.69** |
| RL  |     | 1      | 0.37* | 0.7**  | 0.34*    | 0.85** |
| SL  |     |        | 1     | 0.76** | -0.002ns | 0.75** |
| DW  |     |        |       | 1      | 0.13ns   | 0.93** |
| MGT |     |        |       |        | 1        | 0.31ns |
| API |     |        |       |        |          | 1      |

Note: \*\* indicates significance at  $p < .001$  level, and \* indicates significance at  $p < 0.05$  level of significance. Here, WSG = Weed Seed Germination, RL = Root Length, SL = Shoot Length, DW = Dry Weight, MGT = Mean Germination Time, API = Average Percent Inhibition

### 3.3. Clustering

The dendrogram revealed that nine rice accessions clustered into four groups with a similarity coefficient of 0.40, and this implied a high level of agro-morphological variation among the rice lines (**Figure 7**). The UPGMA dendrogram generated by cluster analysis indicated the differences in the relationship among the developed lines. As a consequence, selecting any of these lines based on their relationship will help in future breeding programs for rice improvement. Cluster I had only one accession, viz., L1 (52-3-3-8) and similarly, cluster II, L6 (MR1A1). Cluster III took 3 accessions, viz., L2 (30-3-3-19), L4 (20-3-3-9), L7 (52-3-3-7) (**Table 5**). Moreover, cluster IV included four accessions: L3, L5, L8, and L9. In comparison to other clusters, the dendrogram demonstrates that variations in one cluster are essentially identical and have less variability. The variations in one cluster shared a lot of the same traits and had little variance.

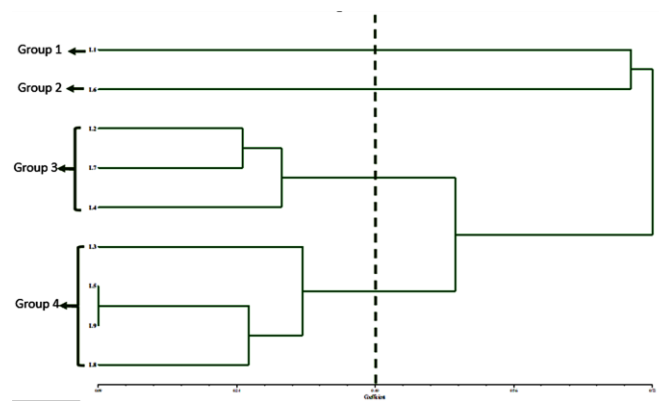


**Figure 7.** Cluster analysis on the average percentage of inhibition (API) values of all indicator rice accessions, showing the similarity among indicator rice accessions.

**Table 5:** Cluster analysis showing the similarity groups among the rice accessions.

| Cluster | No. of Accessions        | Entry no.                                   |
|---------|--------------------------|---------------------------------------------|
| I       | L1(one)                  | 52-3-3-7                                    |
| II      | L6(one)                  | MRIA1                                       |
| III     | L2, L4, L7(three)        | (30-3-3-19), (20-3-3-9), (52-3-3-7).        |
| IV      | L3, L5 L8, and L9 (Four) | 30-3-3-103, 52-3-3-8, 30-3-3-109, 20-3-3-5, |

The principal component analysis (PCA) is a re-validation tool for cluster analysis, as shown in **Figure 8**. According to Jobson, (2012) PCA is an effective tool. The two-dimensional (**Figure 8**) and three-dimensional (**Figure 8**) graphical elucidations revealed that the majority of rice accessions were dispersed at short distances, while two rice accessions were dispersed at long distances, as indicated by the eigenvectors. L1 and L6 were the accessions furthest from the centroid. Other rice accessions were also close to the centroid.

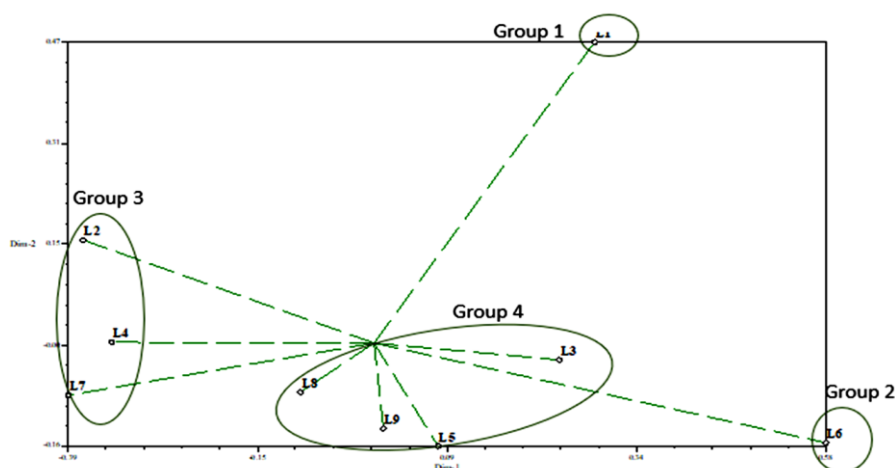


**Figure 8.** Principal component analysis (PCA)-2D graphical relationship among the improved rice lines based on Euclidean distance

Overall allelopathic performance, as measured by API, ranked the improved lines in the order **L3 > L2 > L4 > L5 > L9 > L8 > L7 > L1 > L6** (**Table 3**). These findings demonstrate that L3, developed through backcross breeding, combined superior allelopathic activity with the targeted drought-tolerance

background, while the relay seeding technique proved to be an effective and reliable method for identifying elite allelopathic rice lines.

#### 4. Discussion

The relay seeding bioassay demonstrated that the improved rice lines differed significantly in their allelopathic potential against barnyard grass. Across all measured traits, including seed germination, shoot length, root length, root dry weight, and Average Percent Inhibition (API), the selected backcross-derived lines exhibited stronger inhibitory effects than the recurrent parent. Among the evaluated genotypes, L3 consistently produced the greatest suppression of barnyard grass growth, as reflected by its highest API value (45.16%), followed by L2 and L4. These results indicate that the introgressed allelopathic genomic regions were successfully expressed in the improved genetic background and contributed to enhanced weed-suppressive ability.

The reduction in seed germination observed in the improved lines suggests that rice root exudates released biologically active compounds capable of interfering with the early establishment of barnyard grass. Similar reductions in weed germination have been reported in previous studies, where rice accessions possessing strong allelopathic potential significantly inhibited the germination of several weed species compared with non-allelopathic cultivars (Alam *et al.*, 2018; Ma *et al.*, 2014). Phenolic compounds such as *p*-coumaric acid have been identified among the allelochemicals responsible for this inhibitory activity, although their effectiveness varies depending on concentration and target weed species (Rimando *et al.*, 2001). Therefore, the reduced germination observed in the present study is likely attributable to the cumulative action of multiple allelochemicals released from rice roots rather than to a single phytotoxic compound.

The improved rice lines also markedly reduced shoot elongation of barnyard grass. Since shoot growth depends on successful germination, cell division, nutrient uptake, and photosynthetic development, inhibition at the early growth stage can substantially limit weed competitiveness. Previous studies similarly demonstrated that allelopathic rice cultivars significantly suppress shoot development of lettuce and barnyard grass through the release of phytotoxic metabolites (Chung *et al.*, 2018; Mazid *et al.*, 2018). Momilactone B, one of the best-characterised rice allelochemicals, has been reported to inhibit the growth of neighbouring plants after being released into the rhizosphere (Noguchi, 2004). These findings support the observed reduction in shoot length in the present study.

Root growth was more strongly inhibited than shoot growth in all improved lines, indicating that roots are the primary target of rice allelochemicals. Because roots are directly exposed to allelochemicals in the rhizosphere, inhibition of cell division and elongation can rapidly impair water and nutrient acquisition. Similar observations have been reported previously, where allelopathic rice cultivars significantly reduced barnyard grass root elongation, and the inhibitory effects were reversed by activated charcoal, confirming their chemical origin (Olofsdotter *et al.*, 2002a). Microscopic studies further showed that allelopathic rice not only suppresses root elongation but also inhibits secondary root development (Olofsdotter *et al.*, 2002b). The pronounced reduction in root length observed in the present study therefore provides strong evidence of enhanced allelopathic activity in the selected backcross-derived lines.

Root dry weight was also significantly reduced, indicating that prolonged exposure to rice allelochemicals impaired overall biomass accumulation. Reduced dry matter production is a common consequence of allelopathic stress because inhibition of germination and root development ultimately limits nutrient uptake and plant growth. Comparable reductions in dry biomass have been reported in lettuce, spinach, and barnyard grass exposed to allelopathic rice cultivars (Azmi *et al.*, 2000; Kabir *et al.*, 2010; Chung *et al.*, 2001). Mushtaq *et al.* (2020) further reported that allelopathic stress causes abnormal root morphology, tissue discolouration, and reduced biomass accumulation. The present findings are therefore consistent with previous reports demonstrating that allelopathic rice primarily restricts weed establishment by suppressing early growth and biomass production.

Among the evaluated traits, API effectively integrated the inhibitory effects of rice on germination and seedling growth and clearly distinguished the allelopathic performance of the improved lines. The highest API recorded for L3 confirms its superior weed-suppressive ability relative to the other genotypes. Previous studies have similarly recognised API as a reliable indicator for screening rice germplasm for allelopathic potential (Karim *et al.*, 2014; Mazid *et al.*, 2018). Jung *et al.* (2004) likewise reported that several highly allelopathic rice varieties exhibited API values exceeding 70%, demonstrating the usefulness of this parameter for identifying superior donor genotypes.

The enhanced inhibitory effects observed in the present study are likely associated with increased production of rice allelochemicals. Rice plants are known to perceive chemical signals released from barnyard grass roots, which stimulate the biosynthesis and secretion of allelochemicals such as momilactone B (Noguchi, 2011; Li *et al.*, 2010). Previous studies have shown that the allelopathic activity of rice seedlings increases more than fivefold when grown in association with barnyard grass (Noguchi, 2011). In addition, abiotic stresses have been reported to enhance the accumulation of phenolic compounds and upregulate genes involved in allelochemical biosynthesis, thereby strengthening rice allelopathic potential (Li *et al.*, 2010). These mechanisms may explain the strong inhibitory responses observed in the improved lines carrying the introgressed allelopathic loci.

From a breeding perspective, the superior performance of line L3 demonstrates that backcross breeding can successfully introgress allelopathic traits into an elite drought-tolerant genetic background. Such lines provide valuable parental materials for developing climate-resilient rice cultivars with enhanced natural weed suppression. From a weed management perspective, incorporating allelopathic rice cultivars into integrated weed management programs could reduce early barnyard grass infestation, decrease reliance on synthetic herbicides, lower production costs, and contribute to more environmentally sustainable rice production.

## 5. Conclusions

The screened allelopathic improved lines showed excellent inhibitory effects against the barnyard grasses in this research. In light of the API value, the improved line L3 (30-3-3-103) took a prominent position (45.16 %). The developed allelopathic-tolerant rice line is an eco-friendly approach to dealing with biotic stress like a weed infestation. To battle weed competition and maintain biodiversity, it is a better choice instead of herbicide application. It would ensure better yield while preserving biodiversity in a safe manner. In the era of climate change, the integration of crop allelopathy and drought tolerance offers a novel strategy for developing climate-resilient rice varieties and promoting sustainable weed management strategies. The findings of this study will provide valuable insights for future plant breeding programs for developing allelopathic and drought-tolerant rice varieties to enhance sustainable rice productivity, reduce reliance on pesticides, and promote global food security.

## Availability of Data and Materials

All relevant data are available in all Tables and Figures within the manuscript.

## Declarations

## Ethics Approval and Consent to Participate

Not applicable.

## Consent for Publication

Not applicable.

## Competing Interests

The authors declare that they have no competing interests.

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