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Selection of drought-tolerant rice genotypes under cajuput (*Melaleuca cajuputi* subsp. *cajuputi*) agroforestry system

TARYONO[1,♥], SUPRIYANTA[1], PANJISAKTI BASUNANDA[1], RANI AGUSTINA WULANDARI[1], NURMANSYAH[1], ERLINA AMBARWATI[1], I GUSTI KOMANG DANA ARSANA[2], VINA EKA ARISTYA[2], ARDIAN ELONARD PURBA[2], ALWA WIDI AISYA[1], TAUFAN ALAM[1]
[1]Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora No. 1, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia.
Tel.: +62-274-588688, ♥email: tariono60@ugm.ac.id
[2]National Research and Innovation Agency. Jl. M.H. Thamrin No. 8, Jakarta Pusat 10340, Jakarta, Indonesia

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Abstract. *Taryono, Supriyanta, Basunanda P, Wulandari RA, Nurmansyah, Ambarwati E, Arsana IGKD, Aristya VE, Purba AE, Aisya AW, Alam T. 2023. Selection of drought-tolerant rice genotypes under cajuput (*Melaleuca cajuputi *subsp.* cajuputi*) agroforestry system. Biodiversitas 24: 4791-4802.* Rice production can be increased by expanding rice cultivation area on rainfed agroforestry system. However, planting rice in agroforestry system, which is generally rainfed, might cause drought stress and eventually can reduce rice yields. The study aimed to select rice genotypes under drought stress conditions in a rainfed agroforestry system with cajuput or *kayu putih* (*Melaleuca cajuputi* subsp. *cajuputi* Powell). Ten promising rice lines and four control cultivars were grown during the dry season from March-July 2022 at the Menggoran Forest Management Resort, Playen Forest Section, Yogyakarta Forest Management, Indonesia. The observation was conducted on drought stress tolerance (sensitivity and recovery), molecular identification, and yield of rice cultivars. The results showed that G5 was the genotype with drought tolerance and had the best recovery compared to the other promising rice lines and control cultivars. The molecular identification of the 14 rice genotypes using 20 SSR markers detected 108 alleles with a PIC value of 0.707. The G5, G4, and G9 had higher yields than the control cultivars. The BLUE showed that the yield of G5, G4, and G9 were 6.59, 5.93 and 5.50 tons ha^{-1}, respectively, while for BLUP by 6.45, 5.87 and 5.43 tons ha^{-1}, respectively. Furthermore, there were four clusters consisting of cluster 1 (G9), cluster 2 (G7), cluster 3 (G5 and Inpago 12), and cluster 4 (Inpari 42, G6, Inpari 30 Ciherang Sub 1, Situ Bagendit, G3, G4, G10, G2, G1, and G8). The findings of this study recommend G4, G5, and G9 as promising rice lines which tolerant to drought stress under *M. cajuputi* agroforestry system and can be utilized in future plant breeding programs.

Keywords: Agroforestry, drought stress, *kayu putih*, rice genotypes, SSR marker

Abbreviations: BLUE: Best Linear Unbiased Estimator; BLUP: Best Linear Unbiased Prediction; PIC: Polymorphic Information Content; SSR: Simple Sequence Repeat; SES: Standard Evaluation System

INTRODUCTION

Rice is the main staple food for more than half of the world's population, most of whom live in less developed countries, such as Asia, Africa, and Latin America (USDA 2022). The increasing world's population, which is predicted to reach more than 9 billions in 2050 (European Commission 2022), will pose the challenge in meeting the demand of rice and its production. In Indonesia, for example, the rice production in 2019 diminished by 4.60 million tons (7.76%) compared to 2018 due to global climate change and the conversion of paddy fields to other land uses (Statistics Indonesia 2020). The conversion rate of paddy fields to non-paddy fields was recorded at 96.5 thousand hectares per year, while the average annual establishment of new paddy fields was only 20-30 thousand hectares (Mulyani et al. 2017). Therefore, alternative strategy to increase rice production is by expanding and intensifying rice cultivation on rainfed areas because the total rainfed areas in Indonesia is around 144.47 million hectares (Ministry of Agriculture 2022).

Rice cultivation on rainfed areas can be applied by planting paddy under trees using agroforestry system. One tree species that can be combined with paddy is *kayu putih* or cajuput (*Melaleuca cajuputi* subsp. *cajuputi* Powell). The *M. cajuputi* agroforestry has great potential for rice development since there are around 284,756 hectares of *M. cajuputi* plantation in Indonesia (Kartikawati et al. 2014). There are several advantages of rice cultivation under *M. cajuputi* stands, namely (i) there is no sunlight competition between *M. cajuputi* and rice because the leaves and branches of the *M. cajuputi* are pruned twice annually; (ii) there is minimal competition for nutrients and water due to differences in the rhizosphere zone between rice and *M. cajuputi*; (iii) *M. cajuputi* stand can be maintained in a relatively long period because the productive age of *M. cajuputi* is up to 30 years (Suryanto et al. 2017; Alam et al. 2019; Alam et al. 2020a,b; Suryanto et al. 2020a,b; Alam et al. 2021a,b; Alam et al. 2022a,b). However, rice cultivation in *M. cajuputi* agroforestry system produces low yield because of the low water availability, which is only obtained from rainfall. The average productivity of rice in a rainfed agroforestry system with *M. cajuputi* is 3.28 tons per hectare, which is significantly lower than the average productivity of paddy fields with 6.59 tons per hectare (Suryanto et al. 2017; Suryanto et al. 2020a,b; Alam et al. 2021a,b; Faridah et al. 2021; Ministry of Agriculture 2022).

Drought stress can occur in all phases of rice growth, from the vegetative to the generative phases. The critical phases of drought stress in rice growth include tillering, flowering, and seed filling (Yang et al. 2019; Bhandari et al. 2023). In general, drought stress affects morphological appearances (e.g., reduced root length and area, plant height, number of tillers, dry weight), biochemical processes (e.g., increased reactive oxygen species and accumulation of osmoprotectants), physiological mechanisms (e.g., decreased photosynthetic activity, stomatal conductance, transpiration, relative water content, membrane stability, and water use efficiency), molecular processes (changes in gene expression especially in the process of transcription and protein breakdown), and decreased rice yield potential (Pandey and Shukla 2015; Upadhyaya and Panda 2019; Verma et al. 2019; Gupta et al. 2020; Melandri et al. 2020).

Rice production in rainfed system or drought prone areas can be increased by using drought-tolerant rice cultivars. The development of rice hybrids that are tolerant to drought stress is a breakthrough for the sustainability of rice production (Pandey and Shukla 2015). In the last decade, the International Rice Research Institute (IRRI) has used a breeding approach for rice selection based on phenotypic traits and markers such as Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Amplified Fragment Length Polymorphism (AFLP), and Single Nucleotide Polymorphism (SNP) for developing drought-tolerant rice cultivars (Kumar et al. 2016; Sandhu and Kumar 2017; Verma et al. 2019).

The most commonly used molecular marker for detecting genetic diversity is SSR marker (Vikram et al. 2011; Dixit et al. 2014). SSR marker is codominant, cheap, easy to analyze, monolocus, reproducible, and can detect high allele variations. SSR marker is relatively easy because they use the Polymerase Chain Reaction (PCR) technique to reach all chromosomes (Nadeem et al. 2018). Therefore, the chance to get markers related to preferable characteristics is higher. This study aimed to select promising rice genotypes that are tolerant to drought stress conditions in a rainfed *M. cajuputi* agroforestry system using the Standard Evaluation System (SES) approach for rice and SSR markers. The results of this study will provide information related to rice lines tolerant to drought stress under the *M. cajuputi* agroforestry system and can be utilized in future plant breeding programs.

MATERIALS AND METHODS

Plant materials

The study used 14 genotypes of rice, consisted of 10 promising rice lines sourced from Universitas Gadjah Mada, Indonesia and 4 rice cultivars as control sourced from the Indonesian Center for Rice Research (ICRR), West Java, Indonesia. The four rice cultivars as control consisted of two lowland cultivars (Inpari 30 Ciherang Sub 1 and Inpari 42) and two upland rice cultivars (Inpago 12 and Situ Bagendit). The pedigree information of rice genotype was presented in Table 1.

Experimental design and agronomic practices

The rice was planted between *M. cajuputi* stands during the dry season from March-July 2022 at Menggoran Forest Management Resort, Playen Forest Section, Yogyakarta Forest Management, Indonesia. The geographical location of the study site was 7°52′59.5992″ S to 7°59′41.1288″ S latitude and 110°26′21.462″ E to 110° 35′7.4868″ E longitude. The experimental design used a Randomized Complete Block Design (RCBD) with three blocks as replications. The experimental plots were placed between *M. cajuputi* stands. The plot size, harvested area, and distance between plots were 20 m^2, 12 m^2, and 0.5 m, respectively. Soil tillage was done minimally. Rice seeds were soaked for ±12 hours and planted using direct seeding with two seeds per planting hole. The plant spacing was 25 cm × 25 cm. The fertilizers were Urea of 300 kg ha^{-1}, *Zwavelzure Ammoniac* (ZA) of 100 kg ha^{-1}, SP-36 of 100 kg ha^{-1}, and KCl of 150 kg ha^{-1}. Urea and ZA were applied twice (5 and 9 weeks after planting/WAP), while SP-36 and KCl were applied entirely at planting. The weed control was done twice at 5 and 9 WAP before applying Urea and ZA. The irrigation was carried out to field capacity and adjusted for drought stress treatments. The drought stress assessment consisted of drought sensitivity (leaf rolling and drying) and drought recovery (plant recovered and spikelet fertility). Drought sensitivity (leaf rolling and drying) was assessed when no watering/rainfall for two weeks during the vegetative phase. Drought recovery (plant recovered) was assessed ten days after watering/rainfall in the vegetative phase. Drought recovery (spikelet fertility) was assessed at harvesting time.

Table 1. Fourteen rice genotypes used in this study with pedigree information

Promising lines and cultivar	Pedigree
G1	Aek Sibundong / M11 (Mutant Anak Daro)
G2	Fatmawati / IR64 // Memberamo / Ciherang
G3	Fatmawati / IR64 // Cimalaya Muncul / Ciherang
G4	Sintanur / IR64 // Memberamo/Ciherang
G5	Mutant Lampung Kuning
G6	Mutant Rajalele Klaten
G7	Mutant Rajalele Klaten
G8	M12 (Mutant Dusel) / Bahbutong
G9	Mutant Mayangsari
G10	Mutant Lakatesan
Inpari 30 Ciherang Sub 1 (Control)	Ciherang / IR64Sub1 // Ciherang
Inpari 42 (Control)	Huangxinzhan / Fenghuazhan
Inpago 12 (Control)	Selegreng / Ciherang // Kencana Bali
Situ Bagendit (Control)	Baturn / S2823-7d-8-1-A // S2823-7d-8-1-A

Data collection

Soil characteristics

The observation of soil quality consisted of physical, chemical, and biological properties. Soil sampling was carried out randomly before the research. Soil variables consisted of soil texture (% clay, % sand, % silt), bulk density, soil moisture (pF 0, 2.54, and 4.2), permeability, pH H_2O, cation exchange capacity, total nitrogen, soil nutrient availability (phosphorus, potassium, calcium, sodium, magnesium, and manganese), soil nutrient exchange (iron and aluminium), total bacteria, and total fungi (Peters 2013; David and Davidson 2014). The observations were conducted at the experimental site, the General Soil Laboratory, and Microbiology Laboratory, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Screening of drought sensitivity and recovery

The screening of drought sensitivity and recovery in rice followed the procedure of the Standard Evaluation System (SES) for Rice (International Rice Research Institute 2014). Drought sensitivity and recovery were randomly assessed on ten clumps samples (Tables 2 and 3).

DNA isolation and amplification

Genomic DNA extraction was isolated from leaves of rice aged 3 WAP. DNA was isolated from the leaves weighing ±0.1 gram. DNA was isolated using Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer (Kouakou et al. 2022). DNA quantification using Nanodrop®. PCR amplification was carried out by the reaction shown in Table 4. The PCR program used was as follows: initial denaturation at 94°C for 4 minutes, followed by 35 cycles consisting of denaturation at 94°C for 1 minutes, annealing temperature at 40-65°C for 1 minute and 72°C for 2 minutes, 2-4 repetitions 13 times with a touchdown program (regular temperature reduction) with a difference of 0.5°C each cycle, then followed by primer elongation finally at 72°C for 10 minutes. The SSR markers used in the study were presented in Table 5.

Table 2. Drought sensitivity scoring and criteria

Score	Criteria	Description	
		Leaf rolling	**Leaf drying**
0	Highly tolerant	Leaves healthy	No symptoms
1	Tolerant	Leaves start to fold (shallow)	Slight tip drying
3	Rather tolerant	Leaves folding (deep V-shape)	Tip drying extended up to ¼ length in most leaves
5	Moderate tolerant	Leaves fully cupped (U-shape)	One-fourth to 1/2 of all leaves dried
7	Moderate susceptible	Leaf margins touching (0-shape)	More than 2/3 of all leaves fully dried
9	Susceptible	Leaves tightly rolled	All plants apparently dead

Rice yield variable

The observed variable of rice was rice yield. Rice yield harvested from seed weight in an area of 12 m^2 was converted to tons per hectare with a moisture content of 12% (Ministry of Agriculture 2019).

$$\text{Yield} = \frac{10000}{\text{HA}} \times \frac{100 - \text{MC}}{100 - 12} \times \text{S}$$

Where:
MC : Moisture content at harvested
HA : Harvested area (m^2)
S : Seed dry weight at harvested (kg)

Statistical analysis

Polymorphic Information Content (PIC) was calculated for each SSR marker using the formula developed by Serrote et al. (2020). PIC values were divided into three classes, namely highly informative (PIC>0.5, moderate (0.25>PIC>0.5), and low (PIC<0.25) (Serrote et al. 2020). Molecular data were used to calculate genetic similarity based on the Jaccard coefficient. The variable of rice yield was analyzed using two models, namely, the Best Linear Unbiased Estimator (BLUE) with cultivars given a fixed effect and the Best Linear Unbiased Prediction (BLUP) with genotypes given a random effect (Buntaran et al. 2018; Alam et al. 2021a,b; Taryono et al. 2022). Relationships between rice genotypes were analyzed using heatmap cluster, phylogenetics cluster, Multiple Factor Analysis (MFA), and PCA-Biplot using combined data of (i) sensitivity and drought recovery data; (ii) molecular data, and (iii) yield per hectare data. The third data was collected with the Mahalanobis distance. Mahalanobis distance of an observation of $\vec{x} = (x_1, x_2, x_3, \ldots, x_n)^T$ from a set of observations that had a mean of $\vec{\mu} = (\mu_1, \mu_2, \mu_3, \ldots, \mu_N)^T$ and the covariance matrix S was defined as follows (Ghorbani 2019):

$$D_M(\vec{x}) = \sqrt{(\vec{x} - \vec{\mu})^T}\, S^{-1} (\vec{x} - \vec{\mu})$$

Table 3. Drought recovery scoring and criteria

Score	Criteria	Description	
		Plants recovered	**Spikelet fertility**
1	Tolerant	90-100%	More than 80%
3	Rather tolerant	70-89%	61-80%
5	Moderate tolerant	40-69%	41-60%
7	Moderate susceptible	20-39%	11-40%
9	Susceptible	0-19%	Less than 11%

Table 4. PCR components used for DNA amplification with SSR primers

PCR components	Stock concentration	Volume (µL)
Go tag Green Master Mix 2x		6.3
Forward primer	10 µL	1
Reverse primer	10 µM	1
DNA Template	30 µL	1
Nuclear Free Water		3.7
Total Reaction		13 µL

Table 5. Polymorphic SSR marker used in the study

Primer ID	Annealing temp (°C)	Sequence	Linkages	References
RM 72	55	F: CCGGCGATAAAACAATGAG R: GCATCGGTCCTAACTAAGGG	Leaf rolling	Lin et al. (2007)
RM 186	61	F: TCCTCCATCTCCTCCGCTCCCG R: GGGCGTGGTGGCCTTCTTCGTC	Yield potential in drought stress	Dixit et al. (2014)
RM 228	54.2	F: CTGGCCATTAGTCCTTGG R: GCTTGCGGCTCTGCTTAC	Leaf rolling	Lin et al. (2007)
RM 248	55	F: TCCTTGTGAAATCTGGTCCC R: GTAGCCTAGCATGGTGCATG	Yield potential in the flowering phase in drought stress	Swamy et al. (2017)
RM 263	59.5	F: CCCAGGCTAGCTCATGAACC R: GCTACGTTTGAGCTACCACG	Drought resistant	Vikram et al. (2012)
RM 293	55	F: TCGTTGGGAGGTATGGTACC R: CTTTATCTGATCCTTGGGAAGG	Yield potential when drought stress	Dixit et al. (2014)
RM 431	55	F: TCCTGCGAACTGAAGAGTTG R: AGAGCAAAACCCTGGTTCAC	Yield potential in the flowering phase in drought stress	Vikram et al. (2011)
RM 508	55	F: GGATAGATCATGTGTGGGGG R: ACCCGTGAACCACAAAGAAC	Yield potential when drought stress	Dixit et al. (2014)
RM 510	55	F: AACCGGATTAGTTTCTCGCC R: TGAGGACGACGAGCAGATTC	Yield potential of aerobic soil conditions	Venuprasad et al. (2009)
RM 518	55	F: CTCTTCACTCACTCACCATGG R: ATCCATCTGGAGCAAGCAAC	Leaf rolling	Lin et al. (2007)
RM 541	55	F: TATAACCGACCTCAGTGCCC R: CCTTACTCCCATGCCATGAG	Yield potential when drought stress	Dixit et al. (2014)
RM 552	55	F: CGCAGTTGTGGATTTCAGTG R: TGCTCAACGTTTGACTGTCC	Yield potential in the flowering phase in drought stress	Swamy et al. (2017)
RM 587	55	F: ACGCGAACAAATTAACAGCC R: CTTTGCTACCAGTAGATCCAGC	Yield potential in drought stress	Dixit et al. (2014)
RM 3825	40.9	F: AAAGCCCCCAAAAGCAGTAC R: GTGAAACTCTGGGGTGTTCG	Drought resistant	Salunkhe et al. (2011)
RM 6909	58.8	F: AAGTACTCTCCCGTTTCAAA R: CCTCCCATAAAAATCTTGTC	Yield potential and root characteristic in rainfed area	Vasant (2012)
RM 7424	42.6	F: AGAAGCCCATCTAGCAGCAG R: TCAAGCTAGCCACACAGCTG	Deeper rooting	Pandit et al. (2020)
RM 19367	57.5	F: CGTCATGTCGCGGAGGTAAGC R: AGGCGTACGTGGAGCAGAGTGC	Yield potential of aerobic soil conditions	Venuprasad et al. (2009)
RM 24393	56.9	F: TAGCTGCTTAGCTTTGACTTGG R: ATGTAATCCTACGAGGAGATCG	Deeper rooting	Nogoy et al. (2016)
RM 27933	54.2	F: TCCTCTGTCATATGGCTGTAAACG R: GGACAAGGAGGAACTATTGATTGG	Yield potential in drought stress	Boopathi et al. (2012)
RM 28199	61.5	F: CGGCTTAGGGAGCGTCTGTAGG R: GCATGCTAGTATGGCCACCATATTCC	Yield potential in the flowering phase when drought stress	Mishra et al. (2013)

The coefficient of genetic similarity based on the Jaccard coefficient, BLUE, and BLUP was analyzed with SAS® Studio software using proc corr, proc glm, and proc mixed (SAS Institute 2023). Corrplot, heatmap cluster, phylogenetics cluster, multiple factor analysis, and PCA-Biplot were visualized with Rstudio software using corrplot, FactoMineR, factoextra, GGally, ggplot2, magrittr, mahalanobis, pheatmap, and scatterplot3d packages (R Core Team 2017).

RESULTS AND DISCUSSION

Sites characteristics

The study site is located at an altitude of ±150 meters above sea level. It has a shallow solum (<50 cm) and ustic moisture regime. The ustic moisture regime is characterized by limited moisture content but will be sufficient when environmental conditions are suitable for plant growth (Soil Survey Staff 2014; Boettinger et al. 2015). The soil type in the study site is Lithic Haplusters (Suryanto et al. 2017; Alam et al. 2019; Alam et al. 2020a; Alam et al. 2020b; Suryanto et al. 2020a,b; Alam et al. 2021a,b; Alam et al. 2022a,b; Kastono et al. 2022).

This soil type is dominated by a clay fraction >60%, has rock contact <30 cm, and will shrink (thickness >25 cm and cracks: width >5 mm), and expands under dry and wet conditions (Boettinger et al. 2015). The water availability (pF 2.54-pF 4.2) for rice was 9.15%. Total rainfall, average air temperature, and average relative humidity during the study (4 months) were 727 mm, 28.14°C, and 80.32%, respectively. The site selection was based on the relatively extreme conditions of rice cultivation to reflect the tolerance of rice cultivars to drought stress.

The pH of H_2O was classified as alkaline with a very high CEC (59.93 cmol$^{(+)}$ kg^{-1}). Soil organic carbon and total nitrogen in the soil content were included in the low category with values of 1.4 and 0.18%. Phosphorus and potassium availability contents were 11 mg L^{-1} and 0.14 cmol$^{(+)}$ kg^{-1} and classified as moderate and low categories. The availability of sodium, calcium, and magnesium were moderate to very high with values of 0.62, 25.74, and 1.17 cmol$^{(+)}$ kg^{-1}, respectively. The manganese availability and nutrient exchange (iron and aluminium) were 1.18, 1.22, and 1.41 11 mg L^{-1} and classified as low and very low (Table 6). Based on climate and soil quality data, the study site belongs to the marginally suitable class for rice cultivation.

Scoring of drought sensitivity and recovery

The results of drought sensitivity analysis showed that the genotypes of G5 and Inpago 12 were classified as drought tolerant category, while G3, G4, G6, G7, G8, G10 were included in the rather tolerant category. Genotypes classified as moderate tolerant were G1, G2, G9, Inpari 30 Ciherang Sub 1, and Situ Bagendit, while Inpari 42 had the moderate susceptible category (Table 7). Therefore, it can be stated that G3, G4, G6, G5, G7, G8, and G10 are more drought tolerant than the control (Inpari 30 Ciherang Sub 1, Inpari 42, and Situ Bagendit), while G1, G2, and G9 are more drought tolerant than Inpari 42. Leaf performance during drought stress indicated that G5 and Inpago 12 only experienced shallow leaf rolling and dry leaf tips.

Analysis on drought recovery showed that G5, G10, and Inpago 12 were included in the tolerant category. G1, G2, G3, G4, G8, and Situ Bagendit were included in the rather tolerant category, while G6, G7, G9, Inpari 30 Ciherang Sub 1, and Inpari 42 had the moderate tolerant category (Table 7). G5 and G10 had higher drought recovery than control cultivars (Inpari 30 Ciherang Sub 1, Inpari 42, and Situ Bagendit), while G1, G2, G3, G4, and G8 are more tolerant than Inpari 30 Ciherang Sub 1 and Inpari 42. Regarding drought recovery, G5 and Inpago 12 had a relatively low fertility spikelet (>80%), and 90-100% of the plants recovered. G5 and Inpago 12 were drought tolerant and had the best recovery compared to the other promising lines and control cultivars.

Table 6. Soil quality characteristics in rainfed agroforestry land in this study

Soil quality characteristics	Unit	Value	Notes
Soil physical properties			
Soil texture			
Clay	%	60.34	Clay texture class
Sand	%	7.08	
Silt	%	32.58	
Bulk density	g cm^{-3}	1.11	-
Soil Moisture (pF)			
pF 0	%	55.83	-
pF 2.54	%	48.54	-
pF 4.2	%	39.39	-
Permeability	cm h^{-1}	0.01	Very poor drainage
Soil chemical properties			
pH H_2O	-	8.3	Alkaline
Soil organic carbon	%	1.4	Low
Cation exchange capacity	cmol$^{(+)}$ kg^{-1}	59.93	Very high
Electrical conductivity	dS m^{-1}	1.649	Low
Total nitrogen	%	0.18	Low
Soil nutrient availability:			
Phosphorus (Olsen)	ppm	11	Moderate
Potassium	cmol$^{(+)}$ kg^{-1}	0.14	Low
Sodium	cmol$^{(+)}$ kg^{-1}	0.62	High
Calcium	cmol$^{(+)}$ kg^{-1}	25.74	Very high
Magnesium	cmol$^{(+)}$ kg^{-1}	1.17	Moderate
Manganese	mg L^{-1}	1.18	Low
Soil nutrient exchange:			
Fe	mg L^{-1}	1.22	Very low
Al	mg L^{-1}	1.41	Very low
Soil biological properties			
Total bacteria	cfu g^{-1} dry soil	1.91×10^5	-
Total fungi	cfu g^{-1} dry soil	1.78×10^3	-

Table 7. Scoring of drought sensitivity and recovery of fourteen rice genotypes used in this study

Promising lines and cultivars	Drought sensitivity				Drought recovery			
	Leaf rolling	Leaf drying	Mean	Criteria	Plant recovered	Spikelet fertility	Mean	Criteria
G1	5	5	5	Moderate tolerant	3	3	3	Rather tolerant
G2	5	5	5	Moderate tolerant	3	3	3	Rather tolerant
G3	3	3	3	Rather tolerant	3	3	3	Rather tolerant
G4	3	3	3	Rather tolerant	3	3	3	Rather tolerant
G5	1	1	1	Tolerant	1	1	1	Tolerant
G6	3	3	3	Rather tolerant	5	5	5	Moderate tolerant
G7	3	3	3	Rather tolerant	5	5	5	Moderate tolerant
G8	3	3	3	Rather tolerant	3	3	3	Rather tolerant
G9	5	5	5	Moderate tolerant	5	5	5	Moderate tolerant
G10	3	3	3	Rather tolerant	1	1	1	Tolerant
Inpari 30 Ciherang Sub 1	5	5	5	Moderate tolerant	5	5	5	Moderate tolerant
Inpari 42	7	7	7	Moderate susceptible	5	5	5	Moderate tolerant
Inpago 12	1	1	1	Tolerant	1	1	1	Tolerant
Situ Bagendit	5	5	5	Moderate tolerant	3	3	3	Rather tolerant

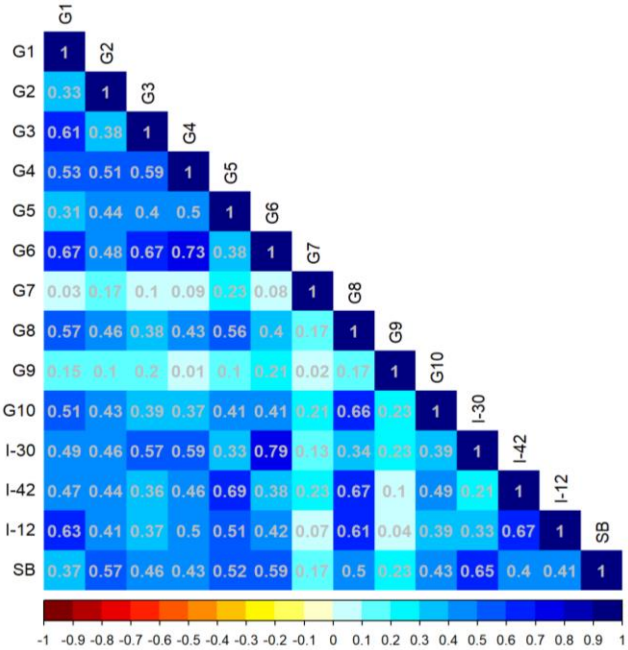
Molecular identification of drought sensitivity and recovery

The total alleles produced were 108 with average of 5.4. The resulting allele sizes ranged from 110 bp to 1250 bp. PIC values ranged from 0.231 for RM 248 to 0.891 for RM 552, with a mean of 0.707 for all rice genotypes. PIC was needed to select markers that can provide good polymorphic information. Based on the average PIC value, the 20 markers were classified as very informative for identifying resistance to drought stress. There was one marker that was classified as low (RM 248) and two markers classified as moderate (RM 72 and RM 228), while the other 17 markers were classified as highly informative (Table 8).

Genetic relationship between rice genotypes were measured based on the Jaccard coefficient. The coefficient of genetic similarity ranged from 0 to 1 with value of 0 indicates no similarity between genotypes, while 1 indicates similar genotypes. The result of analysis showed that the genetic similarity coefficient between rice genotypes ranged from 0.02-0.79 (Figure 1). Genetic similarity between promising rice lines with control cultivars with value above >50% were G1 and Inpago 12 (*r* = 0.63**), G2 and Situ Bagendit (*r* = 0.57**), G3 and Inpari 30 Ciherang Sub 1 (*r* = 0.57 **), G4 and Inpari 30 Ciherang Sub 1 (*r* = 0.59**), G5 and Inpari 42 (*r* = 0.68**), G5 and Inpago 12 (*r* = 0.51*), G5 and Situ Bagendit (0.52*) , G6 and Inpari 30 Ciherang Sub 1 (*r* = 0.79**), G6 and Situ Bagendit (*r* = 0.59**), G8 and Inpari 42 (*r* = 0.67**), G8 and Inpago 12 (*r* = 0.61**). Several SSR markers that showed polymorphisms related to the drought stress tolerant in each rice genotype are shown in Figure 2.

Table 8. Polymorphic of 20 SSR markers

Primer	Number of Chromosome	Size Range (bp)	Number of Alleles	PIC
RM 72	8	120-125	2	0.489
RM 186	3	210-880	7	0.808
RM 228	10	110-120	2	0.493
RM 248	7	290-350	2	0.231
RM 263	2	180-600	11	0.872
RM 293	3	200-250	3	0.667
RM 431	1	250-800	7	0.794
RM 508	6	200-220	3	0.640
RM 510	6	120-1150	9	0.843
RM 518	4	190-550	8	0.862
RM 541	6	150-750	6	0.804
RM 552	11	250-1250	11	0.891
RM 587	6	190-210	3	0.555
RM 3825	1	310-1200	7	0.818
RM 6909	4	125-400	4	0.612
RM 7424	9	150-1250	10	0.885
RM 19367	6	110-420	3	0.640
RM 24393	9	390-550	3	0.645
RM 27933	12	225-400	4	0.710
RM 28199	12	170-190	3	0.602
Total		110-1250	108	
Mean			5.4	0.707



	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	I-30	I-42	I-12	SB
G1	1													
G2	0.33	1												
G3	0.61	0.38	1											
G4	0.53	0.51	0.59	1										
G5	0.31	0.44	0.4	0.5	1									
G6	0.67	0.48	0.67	0.73	0.38	1								
G7	0.03	0.17	0.1	0.09	0.23	0.08	1							
G8	0.57	0.46	0.38	0.43	0.56	0.4	0.17	1						
G9	0.15	0.1	0.2	0.01	0.1	0.21	0.02	0.17	1					
G10	0.51	0.43	0.39	0.37	0.41	0.41	0.21	0.66	0.23	1				
I-30	0.49	0.46	0.57	0.59	0.33	0.79	0.13	0.34	0.23	0.39	1			
I-42	0.47	0.44	0.36	0.46	0.69	0.38	0.23	0.67	0.1	0.49	0.21	1		
I-12	0.63	0.41	0.37	0.5	0.51	0.42	0.07	0.61	0.04	0.39	0.33	0.67	1	
SB	0.37	0.57	0.46	0.43	0.52	0.59	0.17	0.5	0.23	0.43	0.65	0.4	0.41	1

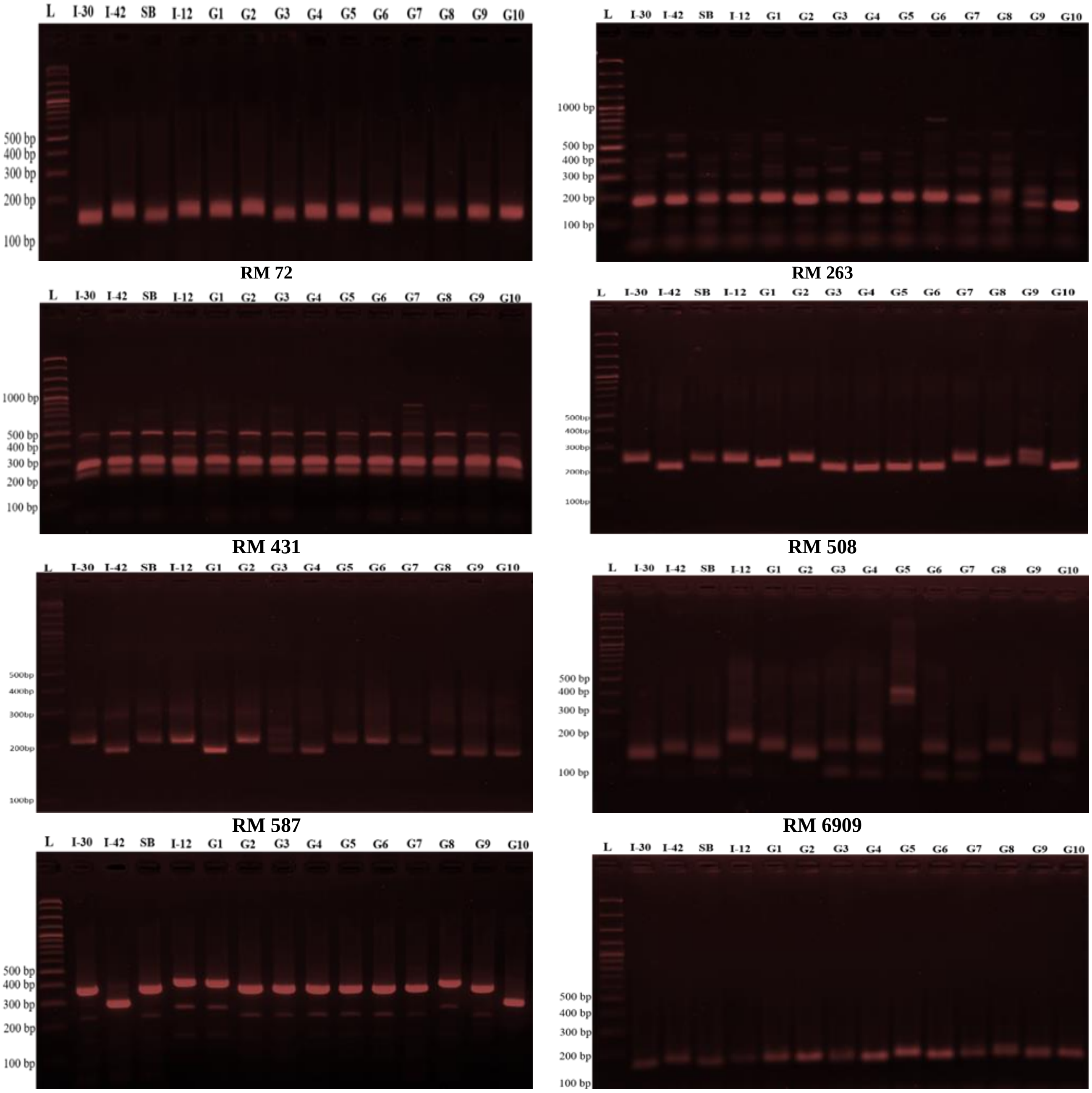
Figure 1. The coefficient of genetic similarity based on Jaccard coefficient. I-30: Inpari 30 Ciherang Sub 1; I-42: Inpari 42; SB: Situ Bagendit; I-12: Inpago 12

BLUE, BLUP, and ranking of 14 cultivars

Estimation, prediction, and ranking of rice yield across genotypes were carried out using the Best Linear Unbiased Estimator (BLUE) and Best Linear Unbiased Prediction (BLUP). There was no difference in the result of ranking between BLUE and BLUP. The promising rice lines with higher yields than the control cultivars (Inpari 30 Ciherang Sub 1, Inpari 42, Inpago 12, and Situ Bagendit) were G5, G4, and G9, respectively. BLUE showed that the yields of G5, G4, and G9 were 6.59, 5.93 and 5.50 tons ha^{-1}, respectively, while for BLUP by 6.45, 5.87 and 5.43 tons ha^{-1}, respectively (Table 9).

Table 9. Adjusted yield based BLUE and BLUP of fourteen rice genotypes used in this study

Rice genotypes	**Adjusted Yield (tons ha^{-1})**			
	BLUE	**SE**	**BLUP**	**Ranking**
G1	2.26	0.18	2.32	13
G2	2.32	0.05	2.41	12
G3	3.77	0.20	3.78	7
G4	5.93	0.09	5.87	2
G5	6.59	0.22	6.45	1
G6	3.52	0.23	3.53	8
G7	3.34	0.40	3.35	9
G8	1.58	0.14	1.68	14
G9	5.50	0.53	5.43	3
G10	3.22	0.13	3.28	10
Inpari 30 Ciherang Sub 1	4.13	0.28	4.12	6
Inpari 42	2.91	0.36	2.94	11
Inapago 12	4.39	0.17	4.37	5
Situ Bagendit	5.36	0.35	5.29	4



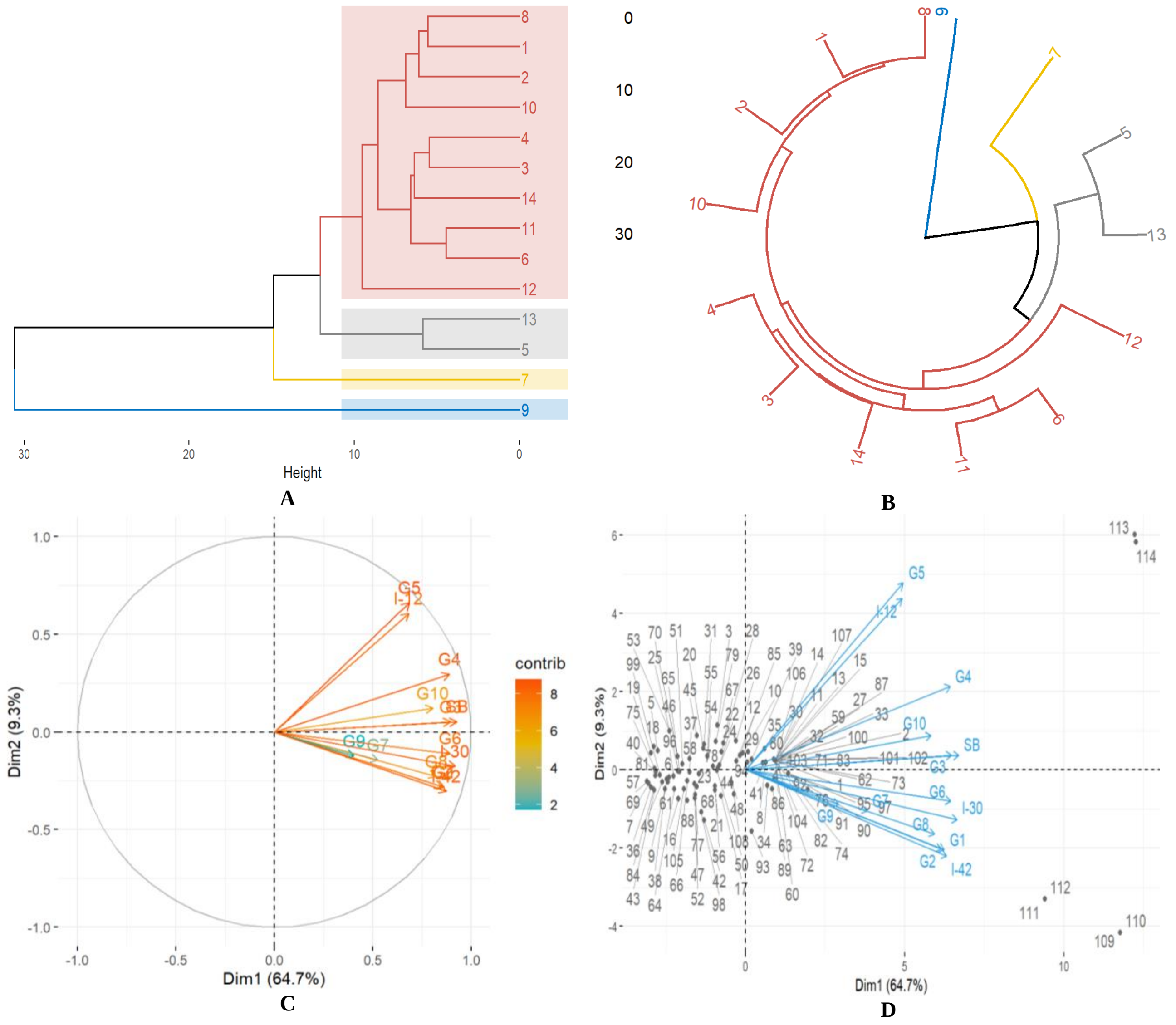
RM 72 **RM 263**

RM 431 **RM 508**

RM 587 **RM 6909**

RM 27933 **RM 28199**

Figure 2. Agarose gel electrophoresis of PCR amplified fragments for the polymorphic SSR markers RM 72, RM 263, RM 431, RM 508, RM 587, RM 6909, RM 27933, and RM 28199. L: 100 bp DNA ladder; I-30: Inpari 30 Ciherang Sub 1; I-42: Inpari 42; SB: Situ Bagendit; I-12: Inpago 12



A
B
C
D

Figure 3. Rice genotypes grouping. A. Heatmap Cluster; B. Phylogenetics Cluster; C. Multiple Factor Analysis (MFA), and D. PCA-Biplot. I-30: Inpari 30 Ciherang Sub 1; I-42: Inpari 42; SB: Situ Bagendit; I-12: Inpago 12

Based on the grouping using the heatmap cluster and the phylogenetic cluster of rice genotypes, it showed that there were four clusters, consisting of cluster 1 (G9), cluster 2 (G7), cluster 3 (G5 and Inpago 12), and cluster 4 (Inpari 42, G6, Inpari 30 Ciherang Sub 1, Situ Bagendit, G3, G4, G10, G2, G1, and G8) (Figures 3.A-3.B). The results of Multiple Factor Analysis (MFA) showed that the contribution of total variables to total genotypes were 75.00% (Figure 3.C). PCA-Biplot showed that the variables contributing to rice genotypes were in the same quadrant. The genotypes G5, Inpari 42, G4, G10, Situ Bagendit, and G3 were grouped in quadrant 2, while the others were grouped in quadrant 4 (Figure 3.D).

Discussion

The main challenge of rice cultivation in rainfed agroforestry systems with *M. cajuputi* is the dependence on water sources from rainfall, thus it has the risk of experiencing drought stress (Suryanto et al. 2017; Suryanto et al. 2020a,b; Alam et al. 2021a,b; Faridah et al. 2021). Soil moisture is a crucial factor in the uptake and translocation of nutrients for plants. Soil moisture conditions affect nutrient availability in the soil and nutrient uptake by plants (Scherer et al. 2017). The short-term mitigation strategy is to select drought-tolerant rice genotypes (DeChant and Moradkhani 2015). This can be done by selecting rice genotypes by planting trials in the actual environment. Several methods developed for selecting preferable rice genotypes include the Standard Evaluation System (SES) for Rice approach developed by International Rice Research Institute (IRRI) and SSR DNA markers (International Rice Research Institute 2014; Kumar et al. 2016; Sandhu and Kumar 2017; Verma et al. 2019).

Drought stress causes changes in the biochemical, physiological, molecular, and morphological properties of plants. Plant's resistance to stress depends on the plant species and its intraspecific variation. Each plant species and its variety have a mechanism to survive during drought stress. Accordingly, intraspecies variation in rice represented by genotypes show different responses to drought stress. The response of several rice genotypes to drought stress is due to differences in morpho-physiological and biochemical characteristics (Bhandari et al. 2023). The ability of plants to tolerate and recover from drought stress depends on differences in genotype responses, stress levels, and plant growth phases (Abid et al. 2018). Changes in phenology and yield of rice genotypes indicate differences in plant response to drought stress. The screening results of this study showed that G5 and Inpago 12 were drought-tolerant genotypes and had the best recovery capacity compared to the other promising rice lines and cultivars. G5 and Inpago 12 only experienced shallow leaf rolling and dry leaf tips. Leaves can be an early indicator of drought stress, indicated by faster leaf rolling and maturity (Yang et al. 2021). Drought stress disrupts water transport through the xylem and low turgor pressure (Hussain et al. 2018). This impact changes in leaf anatomy and ultrastructure (Upadhyaya and Panda 2019; Zhu et al. 2020). These changes include decreased area and number of leaf stomata, enlarged cell walls and cutinations on the leaf surface (Rollins et al. 2013). Some properties used to screen genotypes for drought include leaf area index, flag leaf area, leaf moisture content, and high leaf pigment content (Mishra and Panda 2017; Hussain et al. 2018).

Plants with good recovery from drought stress are indicated by osmotic adjustment through increasing enzymatic and non-enzymatic antioxidant activity, proline, amino acids, and accumulation of dissolved sugars to increase inward water diffusion and maintain high turgor potential (Hou et al. 2021). This response has implications on the increasing physiological processes such as the assimilation of stomata opening, assimilation of CO_2, and cell development. Production of proline and other amino acids correlates with a decrease in leaf water potential, indicating the contribution of these solutes in osmotic adjustment. Proline will play its role in adjusting osmotic potential and osmolytes to stabilize cell membranes and reduce oxidative damage (Yi et al. 2016; Serraj and Sinclair 2018). The activities of Superoxide Dismutase (SOD), Ascorbate Peroxidase (APX), Catalase (CAT), and Glutathione Peroxidase (GPX) in plants that are tolerant to drought stress can reduce the activity of Reactive Oxygen Species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical (OH^-) and decreased malondialdehyde (MDA) content. SOD converts O_2^- to H_2O_2, which CAT further catabolizes to prevent oxidative damage (Movludi et al. 2014). APX is also a major enzymatic scavenger for H_2O_2 (Ozyigit et al. 2016). SOD and CAT levels increase during periods of stress (Rossatto et al. 2017).

DNA markers were used for accurate identification to screen drought-tolerance varieties and for crop improvement in general. Markers could identify certain regions on a chromosome. Linkage analysis showed that a marker was linked to a specific phenotype, depicted that the regions close to these markers control the phenotypes tested (Yohana et al. 2018). DNA markers could be used to differentiate genes involved in rice drought tolerance (Kumar et al. 2016; Barik et al. 2019; Prakash et al. 2019; Upadhyaya and Panda 2019; Panda et al. 2021). The PIC value provided information regarding determining marker values to detect polymorphisms (El-Wahsh et al. 2016). There was one marker that was classified as low (RM 248) and two markers classified as moderate (RM 72 and RM 228), while the other 17 markers were classified as highly informative. Serrote et al. (2020) showed that PIC values were divided into three classes, namely highly informative (PIC>0.5, moderate (0.25>PIC>0.5), and low (PIC<0.25).

In this study, all markers produced polymorphic bands and various information related to the markers used in this study related to rice drought stress tolerance. Lin and Hour (2012) stated that the marker RM 72 is located on chromosome 8 and has a QTL lr8.1 (Leaf Rolling 8.1). This QTL contributes to the leaves rolling and relative water content. In addition, QTL is associated with osmotic pressure and one of the main mechanisms in drought tolerance. RM 6909 marker is located on chromosome 4. Febre et al. (2016) stated that the RM 6909 marker could be used to select plants carrying the qTSN4 locus (total spikelet number). RM 24393 marker also has the potential to detect deep roots in rice (Uga et al. 2011). Boopathi et al. (2012) showed that the RM 27933 marker is on chromosome 12. The marker contains a QTL with the name *qtl*12.1. *qtl*12.1 has a significant effect on yield components under drought stress. In addition, *qtl*12.1 is found in most upland rice varieties which show highly tolerant to drought stress. The markers used in this study were SSR markers related to drought stress tolerance properties, thus rice clustered in one cluster could be said to have almost the same drought stress tolerance properties (Widyawan et al. 2020). Anupam et al. (2017) provided that cluster analysis based on 30 markers resulted in five rice accession clusters.

The adjusted yield was analyzed using the Best Linear Unbiased Estimation (BLUE) and Best Linear Unbiased Prediction (BLUP). The difference in these methods lies in the cultivar modelling, namely the fixed effect for BLUE and the random effect for BLUP. BLUP is more recommended than BLUE because BLUE is too over-optimistic and has a high error potential (Smith et al. 2001; Buntaran et al. 2018). In addition, the advantages of BLUP compared to BLUE include: (i) the expected mean square errors are smaller; (ii) BLUP is suitable for evaluating a variety of trials; and (iii) BLUP is suitable for estimating small areas (Robinson 1991). Nonetheless, in this study, the adjusted yield using the BLUE and BLUP methods showed the same results. BLUE showed that the yield of G5, G4, and G9 were 6.59, 5.93 and 5.50 tons ha^{-1}, respectively, while for BLUP by 6.45, 5.87 and 5.43 tons ha^{-1}, respectively. G5 had the highest ranking compared to other promising rice lines and cultivars. This aligns with the drought sensitivity and recovery screening results which classified G5 as tolerant.

The genotype G5 is developed by the mutation of the Lampung Kuning cultivar. Lampung Kuning is a local cultivar originating from Lampung Province, Indonesia which has excellent drought-stress and aromatic properties (Hairmansis et al. 2005). The mutation is an effective breeding technique for increasing productivity and quality, improving plant physicality, and resisting pests and diseases (Oladosu et al. 2014). Rice plant breeders still use the mutation technique to produce superior varieties (Oladosu et al. 2019). Soe et al. (2016) stated that in Myanmar, the rice cultivars MK-D-2 and MK-D-3 result from a mutation of the Manawthukha variety, which physiologically shows tolerance to drought. Two superior cultivars from Malaysia, namely MR219-9 and MR219-4, which have high yields and are drought-tolerant, resulted from mutations from the MR219 variety (Rahim et al. 2012). In Indonesia, mutation results were shown by mutant super green rice, which has superior, efficient properties for low fertilization, drought tolerance, and high yields (Efendi et al. 2017). This study concluded that the genotypes G4, G5, and G9 are proposed as promising rice lines for drought stress and can be cultivated in rainfed areas. Furthermore, this genotype can be utilized in future plant breeding programs, namely the multi-environmental trial (MET).

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