CHAPTER 3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 Materials

Four globally popular aromatic rice cultivars (Sadri, Gharib, Rato Basmati, and Rambir Basmati) and three advanced homozygous rice lines (Entry 7, Entry 11, Entry 13) supplied by the International Rice Research Institute (IRRI), Philippines were used as parental materials to cross with local rice genotypes MRQ 50 and MR 219 received from the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. Aroma of these parental rice lines was evaluated by Faruq et al. (2010) and they were chosen as they produce good aroma in a tropical environment (Malaysia). A brief description of these rice genotypes is provided in Table 3.1.

3.2 Methods

3.2.1 Crossing Scheme

The experiment was conducted in an experimental field of the Division of Genetic and Molecular Biology, Institute of Biological Science, Faculty of Science, University of Malaya. The planting date started on 15th June 2010. The parental seeds were planted in three batches each with a time interval of two weeks for availability of pollen grain during crossing. Local rice genotypes (MR 219 and MRQ 50) were used as the female parent and the seven other varieties were used as the male parent (Table 3.2).

Table 3.1: Brief description of rice genotypes selected as parental materials for crossing.

Genotypes	Designation	Cross	Origin	Source	Aroma	
					Leaf	Grain
Sadri	Cultivar	unknown	Iran	IRRI	4	4
Gharib	Cultivar	unknown	India/Pakistan	IRRI	3	4
Rato Basmati	Cultivar	unknown	India/Pakistan	IRRI	3	2
Rambir Basmati	Variety	Selection from Basmati 370	India	IRRI	3	3
Entry 7	IR7773493-2-3-2	NSIC RC 148/PSB RC 18//NSIC RC 148	IRRI	IRRI	3	3
Entry 11	IR78554-145-1-3-2	IR 72861-13-2-1-2/IR-68450-36-3-2-2-3	IRRI	IRRI	4	4
Entry 13	IR77512-2-1-2-2	IR 68726-3-3-1-2/IR 71730-51-2	IRRI	IRRI	3	2
MRQ 50	Variety	unknown	Malaysia	MARDI	3	2
MR219	Variety	MR 137/MR 151	Malaysia	MARDI	1	1

Aroma score: 1-absence of aroma, 2-light aroma, 3-moderate aroma, 4-strong aroma. (Faruq et al., 2010)

Table 3.2: Crossing scheme between local rice genotypes with seven selected lines.

Female parent (♀) X	Male parent (♂)		Female parent (♀) X	Male parent (♂)
MR 219	Sadri		MRQ 50	Sadri
	Gharib			Gharib
	Rato Basmati			Rato Basmati
	Rambir Basmati			Rambir Basmati
	Entry 7			Entry 7
	Entry 11			Entry 11
	Entry 13			Entry 13

3.2.1.1 Emasculation

Before normal blooming (before 8.00 am in the morning), a modified hot water treatment technique of Shukla and Chandel (2004) was followed. To begin, the panicles on which the first node had just emerged from the flag leaf were selected. The criterion for the selection is the appearance of yellowish color of anther in the flower when viewed under sunlight. The selected panicles (maximum two each time) were soaked in a thermos flask containing water at 43.5^{o}C for a minimal duration of 5 minutes. The panicles were then gently taken out from the thermos flask. Only the opened flower buds were used for emasculation. The buds were cut off 1/3 from the top to remove the anther and expose the stigma. This was also to allow pollen grains to easily drop into the buds and stick to the stigma.

3.2.1.2 Bagging and Tagging

Immediately after emasculation, the panicles were bagged and labeled as described by Shukla and Chandel (2004). The bags are made up of chocolate sugar

paper (25 x 6 cm) which can fit over the whole panicle. These bags were tied to the base of the panicle with the help of pins designed for this purpose without damaging or banding the panicles. Then, the emasculated panicles were tagged just after bagging. These tags are rectangular in shape of about 15 x 1.5 cm. The tags were attached to the panicles with the help of thread. The following information was recorded on the tags with a carbon pencil: (1) date of emasculation and pollination, and (2) names of the female and the male parents. The name of the female parent was written first, followed by the male parent. Example: MR 219 (♀) x Sadri (♂), where MR219 is the female parent and Sadri is the male parent. Finally, the plants were transferred to a separate protected area to prevent cross-contamination.

3.2.1.3 Pollination

After emasculation, pollination was done by following a force flowering method (Singh, 2008). Panicles with at least ¾ heights from the flag leaf, yellow color anther and with buds remaining close were chosen. The stem of the chosen panicles were cut with a length of 6-7 cm below the first panicle node. Immediately, the stems were submerged and were split with some water on the panicles. Then, submerged stems were cut 2 cm from the bottom. Once the buds were open (generally, around 10.00 am), the panicles were gently moved to the emasculated plant. This was carried out after 10.00 am or during flowering time. The covering bag of the emasculated plant was removed. One panicle each was gently approached to the emasculated panicle and was touched to release the pollen grains. This step was repeated with at least 3 panicles. Once pollination was completed, the pollinated panicle was bagged and the plants were moved to a shelter to protect pollinated panicles from rain drops.

3.2.1.4 F_1 seeds collection

After fertilization, the grain filling period was around 21 days. The F_1 hybrid seeds were observed from time to time to avoid moisture inside the cross bag. After harvesting, the seeds bagged in envelope were dried under hot sun for 4-5 hours per day for 3-4 days. A moisture meter was used to measure the moisture percentage in the rice seeds to make sure the seed moisture was 13%. Then, seeds were kept in a glass container in a 4 ℃ refrigerator until used, to avoid moisture and protect the seed from spoilage by fungus and pests.

3.2.2 Field experiment

Before planting, the F_1 seeds were taken out from refrigerator and dried under hot sun for 4-5 hours per day for 2-3 days to break the dormancy. The collected seeds were separated into curve and non-curve shaped for each of the successful crosses. Curve shaped seeds were expected to be the F_1 hybrids. The experiment was conducted at experimental field (net house) of the Institute of Biological Science, Faculty of Science, University of Malaya. The experiment was laid out in a Complete Randomized Design (CRD) with three replications. The planting date was on 24th December 2010. The parental rice lines were planted together as check. Curve and non-curve shaped seeds from each successful cross were planted separately in small pots each containing 500 g black soil and with labels. Water level was maintained at 1-2 cm before transplanting to the experimental field. After 3 weeks, all seedlings were transplanted to a 75 x 100 cm tank (planting plot design, as shown in Figure 3.1) 2/3 filled with loam soil which was prepared one week before transplanting by the addition of 100 g fertilizer per tank (75 x 100 x 30 cm) comprising N: P: K: S (15: 15: 15: 2) as recommended by Hossain et al. (2005). After that, the fertilizer was given in small amount from time to time when needed. Water level for each pot was from base of plant

to 5-7 cm height where the plant was slightly emerged. Water was added twice per day until the plant reached the hard dough stage. At hard dough stage, water level was decreased to 3-4 cm height. A total of 15 g of Furadan 3G was applied to the plants in each tank from time to time when needed (especially before flowering period) to prevent stem borer infection.

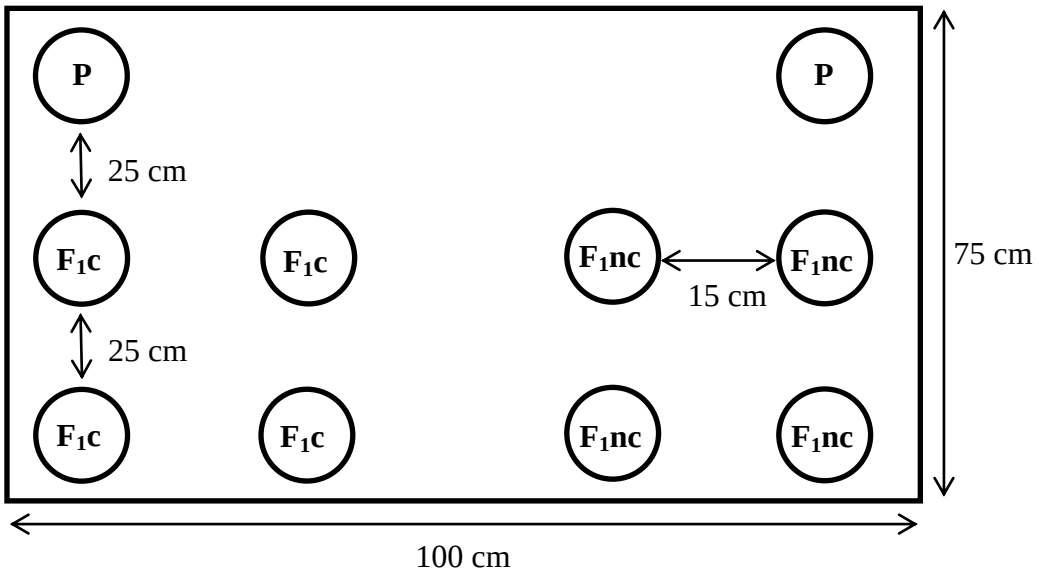

Figure 3.1: Planting plot design for F_1 hybrid separated into curve (F_1c) and non-curve (F_1nc) seeds. P: parental rice line.

3.2.3 Data collection

Three selected plants from both curve and non-curve shaped seeds from each cross were used for agronomic data analysis. The data was taken throughout the whole growing season and after harvesting. Data on days to heading (DH), days to maturity (DM), grain filling period (GFP), plant height (PH), lodging, phenotypic acceptance (PAcp), number of tiller (NT), number of fertile tiller (NFT), panicle length (PL), grain/panicle (GP), fertile grain/panicle (FGP), 1000 grain weight (TGW), grain yield/plant (GYP) and disease were recorded. DH was recorded when 90% of the panicles came out from the flag leave (flowering) of the individual plant. DM was recorded when 90% of the panicles filled with brown color rice grains. GFP was

measured from DH to DM. PH was measured during hard dough state. Plant was measured from the ground level to the top at the middle where bunch of panicles was hold by hand with a scale in centimeters. Lodging and PAcp were recorded visually according to the Rice Standard Evaluation System (SES) described by IRRI. NT and NFT were recorded from each selected plant. Three panicles from each plant were selected to measure their PL, GP and FGP, then the mean for each was calculated.

3.2.4 Aroma Evaluation (Sensory Tests)

3.2.4.1 Preparation of 1.7 % KOH Solution

A total of 17 g KOH tablets were weighed. Then, tablets of KOH were diluted in 1000 ml of distilled water. Finally, the solution was kept at room temperature until used (around one week).

3.2.4.2 Leaf Aromatic Test

Leaf samples (about 5 cm long) from three F_1 individuals of each cross (before flowering stage) were cut and wrapped in a piece of aluminium foil with labelled in the experimental field. On the same day, the leaf samples were taken out from the aluminium foil and wiped with distilled water to remove the dirt on the surface of the leaves. Immediately, the leaf samples of 0.2 g were weighed and cut into tiny pieces (around 1-2 mm width). The assessment for aroma in leaf (Appendix 1.2) used a modification of a method described by Sood and Siddiq (1978). The cut pieces of leaf were soaked in 10 ml of 1.7% KOH solution in a petri dish with cover. After 10 minutes, the cover was opened and the aroma smelled and scored by panelists. They were scored on 1-4 scale with 1, 2, 3 and 4 corresponding to absence of aroma, slight aroma, moderate aroma, and strong aroma respectively. Four to six previously trained panel members, postgraduates and undergraduates from Division of Genetics and Molecular

Biology, Institute of Biological Science, Faculty of Science University of Malaya were invited to score the aroma in each individual, respectively for leaf and grain.

3.2.4.3 Grain Aromatic Test

After harvesting, five grains each were taken from three F_1 individuals of each cross. The grains were dehulled manually. For grain aromatic test (Appendix 1.3), the method used was according to the Rice Gene Discovery Unit, Kasetsart University, Thailand. Five F_2 seeds from F_1 hybrid were placed into 1.5 ml centrifuge tubes. Next, 200 ml of distilled water was added to the tubes and closed. They were then incubated at 65 ℃ for 30 min. After that, the samples were allowed to cool, the lids were individually opened, and the samples were smelled and scored for fragrance by panelists as described in 3.2.4.2 for the leaf aromatic test.

3.2.5 DNA extraction

Total genomic DNA from young leaves was extracted using QuickExtract™ plant DNA extraction solution (Epicentre® Biotechnologies, Madison, USA). Leaf disc around 3-5 mm diameter was cut by using the cap of a 500 µl centrifuge tube. The leaf disc was then put into 200 µl PCR tube and 100 µl of QuickExtract™ plant DNA extraction solution was added and immersed the leaf tissue. The samples were then heated at 65 ℃ for 6 minutes then at 98 ℃ for 2 minutes in a C1000 Thermal Cycler (BioRad, USA). Then, the samples were used as template for Allele Specific Amplification.

3.2.6 Primer Design, Synthesis and Dilution

Primers were designed (Table 3.3 and Figure 3.2) based on Bradbury et al. (2005b) and synthesised by BioBasic, Canada. The tube with primer was then spun for a

short time to ensure that the primer was at the bottom of the tube before opening. A volume equal to the number of the autoclaved distilled water written at the tube with primer was added to the tube to produce a stock solution of 100 µl. After that, the tube was vortex gently and spun for a short time before storing in -20 ℃.

For PCR running, the primer was further diluted. A new tube was labelled and 10 µl of the primer was transferred from the stock solution to the new tube. Next, 90 µl of autoclaved distilled water was added to the tube. The tube was then vortex gently and followed by spun for a short time. The concentration of the diluted primer was 10 µM, which is suitable concentration for running the PCR. Finally, the diluted primers were stored in a -20 ℃ freezer.

Table 3.3: Primers for analysis of fragrance in rice. (Bradbury et al., 2005b)

Primer name	**5' Primer sequence 3'**
External Sense Primer (ESP)	TTGTTTGGAGCTTGCTGATG
Internal Fragrant Antisense Primer (IFAP)	CATAGGAGCAGCTGAAATATATACC
Internal Non-fragrant Sense Primer (INSP)	CTGGTAAAAAGATTATGGCTTCA
External Antisense Primer (EAP)	AGTGCTTTACAAAGTCCCGC

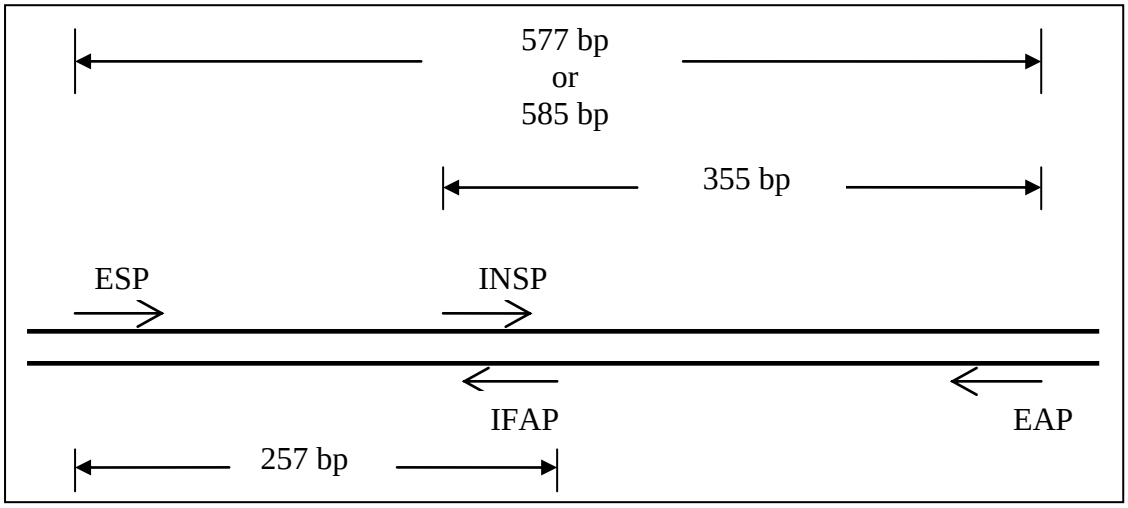

Figure 3.2: Relative position of PCR primers used in fragrance PCR. (Bradbury et al., 2005b)

3.2.7 Allele Specific Amplification (ASA) PCR assays

Allele specific amplification PCR assay was performed using 2.0 µl of 10X reaction buffer (with 20 mM Mg^{+}), 0.2 µl of 10 mM dNTPs mix, 0.25 µl of YEAtaq DNA Polymerase (Yeastern Biotech Co., Ltd, Taiwan), 5.0 µl of DNA template (150-280 ng/µl), 0.4 µl of each primer ESP (10 µM) and EAP (10 µM), 0.5 µl of each primer INSP (10 µM) and IFAP (10 µM), in a total volume of 20 µl. Amplification was carried out using a C1000 Thermal Cycler (BioRad, USA). Cycling conditions were 5 min at 94 ℃, followed by 35 cycles of 30 s at 94 ℃, 30 s at 53 ℃, 1 min at 72 ℃, concluding with a final extension of 7 min at 72 ℃ and a hold at 4 ℃ until recovery.

3.2.8 Agarose Gel Preparation

To prepare 1.0 % agarose gel, a specific amount of agarose powder and a volume of 1x TBE buffer were prepared according to table 3.4. Appropriate amount of agarose powder was weighed and added into the appropriate volume of 1x TBE buffer (preparation as presented in table 3.5) in a flask. Then, the mixture was heated in an oven until there was no crystal-like granule. The flask then taken out from oven was hold with a holder and spun gently to let it cool for a minute. Finally, the mixture was poured into the tray which had set up with cassette and comb. The mixture was left at room temperature for 30 minutes to solidify.

Table 3.4: Agarose gel mixture condition.

TBE buffer 1X	Agarose (g)			
	0.8 %	1 %	2 %	4 %
20	0.16	0.20	0.40	0.80
25	0.20	0.25	0.50	1.00
30	0.24	0.30	0.60	1.20
35	0.28	0.35	0.70	1.40
40	0.32	0.40	0.80	1.60

Table 3.5: Components for preparation of 10X TBE buffer.

Component	**Volume**
Tris Base	108 g
Boric Acid	55 g
EDTA	40 ml
dH_2O	Top up to 1 Litre

*To dilute 10X TBE buffer into 1X solution, for every 100 ml of the 10X TBE buffer used, add 900 ml of distilled water to top up to 1 Litre.

3.2.9 Genotyping

PCR products were analysed by electrophoresis in 1.0 % agarose gels. Firstly, 5 µl of PCR products was mixed with 1 µl of 6x loading buffer on a parafilm paper and then loaded into the well of the agarose gel. Then, the electrophoresis was operated at 80 V, 135 mA, for 40 minutes in 1x TBE buffer and followed by staining in ethidium bromide. A 100 bp ladder (Vivantis, USA) was used to estimate PCR product size.

3.2.10 Statistical Analysis

The collected data were analysed with SAS Version 9.2 (Statistical Analysis System) program for analysis of variance (ANOVA) and mean differences adjudged with Duncan's Multiple Range Test (DMRT).

Genetic analysis of the agronomic traits for F_1 hybrids was done as suggested in Singh (2008). Genotypic variance (V_g), phenotypic variance (V_p), error variance (V_e), genetic advance under selection (Gs), phenotypic coefficient of variation (PCV), genotypic coefficient of variation (GCV) and broad sense heritability (H_b) were calculated by using Microsoft Excel with the formulae below:

Genotypic variance (V_g) *= (MS due to genotypes – MS due to error)/R*

Phenotypic variance (V_p) $= V_g + V_e$

(Where R is the replication, MS is mean square and V_e is the MS due to error)

Genotypic coefficient of variation (GCV) *= (Square root of V_g / X) x 100*

Phenotypic coefficient of variation (PCV) *= (Square root of V_p / X) x 100*

(Where X is the grand mean respectively to trait under consideration)

Genetic advance under selection (Gs) *= (k) (Square root of V_p) (H)*

Heritability (H_b) *= V_g / V_p*

(Where k is selection differential based on mean phenotypic values of the selected lines and of the base population. The intensity of selection is 5 per cent, that is, the extreme 5 per cent of the population will be saved to raise the next generation. While, H= H_b , the proportion of phenotypic variance that is due to genotype, expressing as the ratio of genetic variance to the total variance, i.e. phenotypic variance for the trait.)

Germination rates were calculated for curve shaped seeds and non-curve shaped seeds respectively by using formula as below:

Germination Rate *= (No. of seedlings/ No. of seeds) x 100 %*

Simple correlation between yield and yield contributing components was also carried out by using Microsoft Excel.

Finally, the aroma scores from panelists were calculated to obtain the mean and analyzed with Microsoft Excel.