MOLECULAR AND BIOCHEMICAL CHARACTERISATION OF AROMA IN *BIRIYANICHEERA* RICE GENOTYPE

By

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(2017-11-003)

THESIS

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DECLARATION

I, hereby declare that the thesis entitled **"Molecular and biochemical characterisation of aroma in *Biriyanicheera* rice genotype"** is a bonafide record of research done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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ABBREVIATIONS

%	Percentage
>	Greater than
µg	Microgram
µl	Microlitre
µM	Micromole
2-AP	2- Acetyl-1-pyrroline
BADH2	Betaine Aldehyde Dehydrogenase 2
bp	Base pair
cDNA	Complementary Deoxyribonucleic acid
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethylammonium Bromide
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphates
DUS	Distinctness, Uniqueness, Stability
EDTA	Ethylene Diamine Tetra Acetic acid
EST	Expressed Sequence Tag
EtBr	Ethidium Bromide
fgr	Fragrance
GC-MS	Gas Chromatography-Mass Spectrometry
IIRR	Indian Institute of Rice Research
KAU	Kerala Agricultural University
kb	Kilobase

L	Litre
M	Molar
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minutes
ml	Millilitre
mm	Millimetre
mM	Millimole
mRNA	messenger RNA
MSA	Multiple Sequence Alignment
MT	Metric tonnes
ng	Nanogram
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion Concentration
PoP	Package of Practices
Prob.	Probability
PVP	Poly Vinyl Pyrrolidone
QTL	Quantitative Trait Loci
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per minute
sec.	Seconds
SNP	Single Nucleotide Polymorphism
t.	Tonnes

TAE	Tris Acetate EDTA
Taq	*Thermus aquaticus*
Viz.	Namely
^{o}C	Degree Celsius

Introduction

1. INTRODUCTION

Rice is the main staple food for as high as 62.8 per cent of world population and it contributes to 20 per cent of the total calorific intake of the people. The quality of milled rice produced worldwide touched 495.9 million metric tons, of which India accounted for 112.9 million metric tons of production (www.statista.com). Rice possesses many unique qualities which determine its value and preference in the market. The fragrance of rice has been one such a special character which provides extra preference to scented rice and fetches premium price in rice market. Even though having higher marker price, the demand for aromatic rice has never seen a downhill.

Ever since ancient times, India has been well renowned as a major rice growing country, being next to China. It also holds the pride for being the homeland for the world famous Basmati rice cultivar, along with erstwhile part of Indian union, the Pakistan. Basmati stands acclaimed as the major aromatic rice genotype around the globe, together with Jasmine genotypes from Thailand. During 2018-19, the production of Basmati in India was recorded to be 5.3 million tonnes and fetched an income of 32,804.14 crores from an export of 4.4 million MT (Metric tonnes) (*www.dgciskol.gov.in*).

Along with Basmati, India is the home for many aromatic non-basmati cultivars, which are comparable with basmati genotypes in their aroma. The short grains, low yield, and lack of prominent improvement programmes have kept the non-basmati cultivars, behind the line. There is an immense potential for many non-basmati genotypes to hold a major share in aromatic rice market, if the genotypes are improved with high yield and other preferred qualities.

The fragrance in rice is reported to be produced from the blend of several volatile compounds present in the grain. However, Buttery *et al.* (1983) was the first person to analyse the aroma of cooked rice, in which 2-acetyl-1-pyrroline (2-AP) was identified sas a major contributor of aroma. But recent studies revealed the presence of many other volatile compounds, other than 2-AP to be responsible for the aroma in rice grains (Widjaja *et al.,* 1996b; Tava *et al.,* 1999; Jezussek *et al.,* 2002; Sansenya *et al.,* 2018). The aroma present in different aromatic rice genotypes originated from diverse places

and its level of expression tend to vary with genetic as well as environmental parameters.

There has been an extensive effort to understand the molecular basis of aroma in rice. Several markers have been developed to utilise the aroma trait in breeding programmes (Ahn *et al.,* 1992; Garland *et al.,* 2000; Jin *et al.,* 2003). The fragrance nature in rice is controlled by a recessive trait in homozygous condition on chromosome 8 (Bradburry *et al.*, 2005). The mutation in *BADH2* gene leads to non-functional enzyme synthesis, which is responsible for aroma production.

The aromatic rice cultivars are found to possess higher fragrance, when grown in relatively cooler conditions in hilly regions. Hence the production of such plant types is confined to only a few locations of north western and central parts of India. The aromatic rice cultivars such as Jeerakasala, Gandhakasala, Velumbala, Chomala, Kayama, Kothampalarikkayama, Pookkilathari *etc.* are native to Kerala. There has been decrease in total aroma when the same cultivars grown in regions other than their normal traditional growing regions. This poses difficulty in popularising them in other major rice growing tracts of the country. If a genotype with good aroma that can be grown in normal tropical conditions is available, it can be introduced in other parts also.

The early maturing selection from a Kerala local landrace with short grains, named '*Biriyanicheera*', when grown in normal tropical conditions was observed to have sufficient aroma. In this context, the present study focuses on the analysis of aroma in '*Biriyanicheera*' rice genotype through molecular and biochemical methods.

Review of literature

2. REVIEW OF LITERATURE

The investigation entitled 'Molecular and biochemical characterisation of aroma in *Biriyanicheera* rice genotype' was carried out between 2017-2019 at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellannikkara. The review of literature for the study is presented in the chapter under the aspects.

- Biochemical characterisation of aroma in rice varieties
- *fgr* gene characterisation of aromatic rice varieties
- Marker Assisted Selection (MAS) programs for aromatic trait.

2.1 Biochemical characterisation of aroma in rice varieties

The attraction of rats towards Philippine rice fields was confirmed to be due to volatile flavor contents of unprocessed rice. The volatile profile was analysed by feeding the powdered rice tripped at 50°C by a modified rotary evaporator and drifted by helium gas to a liquid nitrogen collection port covered by dibutyl phthalate solvent. The volatiles trapped in dibutyl phthalate solution was analyzed by combined Capillary-Column Gas Chromatography and Mass Spectroscopy method, which yielded 73 compounds including alcohols, aldehydes, furans, ketones and alkyl aromatics *etc.* (Bullard and Holguin, 1977).

Yajima *et al.* (1978) studied volatile components of cooked rice through steam distillation, which was dissolved in methylene chloride and fractions were separated from the extract. The neutral fraction was further resolved into hydrocarbons and oxygenated compounds through column Chromatography combined with Mass Spectroscopy. Numerous compounds belonging to hydrocarbons, alcohols, aldehydes, ketones, phenols *etc.* were identified.

Buttery *et al.* (1983) analysed uncooked rice grains through 2-h atmospheric pressure steam distillation continuous extraction process for isolation of volatile compounds. The obtained volatile oil was analysed by capillary GLC-MS and concentration of 2-AP was derived from its peak areas. A wide range of 2-AP from 0.006

ppm to 0.09 ppm for calrose and malagkit sungsong rice varieties respectively was observed. The description panel for odour evaluation referred 2-AP as 'pop-corn' like odour.

Buttery *et al.* (1988) attempted analysis of odour thresholds of 64 known rice varieties in water solution. Aldehydes such as (E)-2-nonenal (T=0.08ppb), (E,E)-2-4-decadienal (T=0.07ppb), (E)-2-decanal (T=0.4ppb), octanal (T=0.7ppb), nonanal (T=1ppb), decanal (T=2ppb) produced very low thresholds, justifying their contribution to aroma along with the 'popcorn' flavored compound 2-acetyl-1-pyrroline (T= 0.1ppb). Ratio of concentration of compounds to its odor threshold indicated the major constituents as 2-acetyl-1-pyrroline, (E,E)-2-4-decadienal, nonanal, hexanal, (E)-2-nonenal, octanal, decanal, 4-vinyl-guaiacal and 4-vinylphenol.

Paule *et al.* (1989) evaluated the volatiles in rice through GC-MS separation using a packed silica column and sniffing port for collection of compounds and identified two peaks as hexanal and 2-AP. Findings from evaluation panel confirmed hexanal to be negatively and 2-AP to be positively correlated with rice aroma.

Cooked rice grains of variety Della had 300 ng of 2-acetyl-1-pyrroline per gram (dry weight), Lemont white rice had only 4 ng per gram (Lin *et al.*, 1990).

Tanchotikul *et al.* (1991) developed an advanced method for quantification of 2-AP in ground rice powder. The improved method entailed less amount of sample of about 1 gram and higher chromatographic separation efficiency through use of selected ion monitoring (SIM) and greater detection sensitivity. The concentration of 2-AP in rice cultivars Della, Basmati 370 and Jasmine rice was found to be 76.2±1.8 ppb, 87.4±3.4 ppb, 156.1±8.4 ppb respectively.

Widjaja *et al.* (1996a) used modified Likens - Nickerson simultaneous distillation-extraction unit (SDE) to analyse volatile compounds from dehulled paddy grains and grains which were stored for three months storage at proper conditions. Lipid oxidation process enhanced the levels of aldehydes and ketones which resulted in increase of total volatile compounds after storage. However, 2-AP was found to be decreased upto the

levels of 40-50per cent during storage process. The study identified (E)-2, (E)-4-decadienal as an important contributor in aroma at relatively lower concentrations. During the storage, hull or bran helped to avoid off-flavor by inhibiting oxygen contact and showed that loss of 2-acetyl-pyrroline was inevitable during preservative storage.

Widjaja *et al.* (1996b) identified hexanol as a main candidate compound to distinguish fragrant rice from non-fragrant rice. Non-fragrant rice grains produced more of heptanol, hexanol, 1-octen-3-ol, nonanal *etc.* than that of fragrant rice. Hexanol and 2-pentylfuran produced grass-like aroma and nutty odour respectively at dilute concentrations where as higher levels led to unpleasant odour.

The aroma volatiles from aromatic rice Italian Line B5-3 along with commercial Basmati rice were studied. The class of compounds identified shown Hexanal as the most abundant followed by pentanal, 2-AP, hexanal, benzaldehyde, 4-vinylguiacol *etc.* (Tava *et al.*, 1999).

Bergman *et al.* (2000) was successful in developing a rapid method for quantification of 2-AP and hexanol in rice to help large scale analysis and quality checking procedures. The method consumed 0.3 gram of powdered rice held in methylene chloride at 85° C for 2.5 hour duration. The extraction process showed ~80 per cent and 56 per cent of solubility for 2-AP and hexanol respectively.

An indirect steam distillation at lower pressure and precise temperature was performed to extract the volatile components of Khao Dawk Mali 105 brown rice by preventing the cooking of rice grains (Mahatheeranont *et al.*, 2001). More than 140 volatile compounds along with 2-AP were unveiled from the analysis of fresh extract through GC-MS. Further the quantification was improved by adopting solvent extraction procedure, followed by capillary gas chromatographic detection combined with flame ionization detector. The use of high selective CP - Wax 51 column led to improvement in detection sensitivity for 2-AP from as low as 0.5 gram of uncooked brown rice.

Aroma Extract Dilution Analyses (AEDA) identified 41 odor-active compounds in three aromatic varieties of which eleven were reported newly. The compound 2-

aminoacetophenone (medicinal and phenolic odor), which was unperceived earlier in rice aroma shown greater flavor dilution (FD) factor. Another unique compound 3-hydroxy- 4, 5-dimethyl-2(5H)-furanone shown differential FD factors among varieties, where as it was maximum in Basmati 370 (Jezussek *et al.,* 2002).

Aroma potency of aromatic rice deviates with genetic and environmental conditions. A study conducted by Itani *et al.* (2004) reported variation of 2-AP concentrations in aromatic rice grains of single variety in a place diverged due to cultivation practices. The rice plant grown in different temperatures shown that the low temperatures across ripening and early harvesting stages might elevate 2-AP levels in brown rice. The fertilizer application dosage also found to influence 2-AP concentrations. Rice plants harvested earlier than other cultivars shown good aroma development.

The influence of drying conditions and storage duration on the aroma level of rice variety Khao Dawk Mali 105 was studied by Wongpornchai *et al.* (2004). The rice grains were subjected to modified air blown at temperature 30, 40, 50 and 70° C and also sun-drying. The low temperature drying ensured higher 2-AP concentrations and lesser off-odor compounds on contrary to that of sun-drying method. Increase in storage time tends to decrease 2-AP content while n-hexanal and 2-pentylfuran to be increased.

Sunthonvit *et al.* (2005) investigated the influence of high temperature drying on Thai Jasmine rice variety through rewetting and drying. A two-stage drying process consisting of a fluidized bed heated to a high temperature was employed, subsequently shade drying was followed. Volatile compounds from rice grains dried at temperatures 100°C, 125°C and 150°C was analyzed by Likens and Nickerson apparatus combined with Gas Chromatography-Mass Spectroscopy. As high as 94 volatile compounds, including 2-AP displayed an increased concentration with corresponding higher drying temperature. A set of new compounds were to be produced due to increase in drying temperature along with loss of some favorable volatiles.

Thirty six aromatic and four non-aromatic varieties were classified and confirmed for their aroma by sensory test using 1.7per cent KOH solution (Hien *et al.,* 2006). A wide range of presence of 2-AP (0 - 430.7ppb) was observed by GC-MS-SIM analysis. The

absence of 2-AP in rice varieties was attributed to genetic difference caused by segregation or mutation during evolution. It confirmed that the aroma strength might be influenced by environmental conditions such as photoperiod and humidity. Integrated influence of several volatile compounds/ substrates may also have inhibited 2-AP production.

Sriseadka *et al.* (2006) developed a rapid method using static Headspace Gas Chromatography (HS-GC) for quantitative analysis of 2-AP in rice grains. The method avoids the wet extraction of sample and directs the volatiles automatically into GC analysis. High recovery and sensitivity was achieved by modifying conditions of auto sampler. Megabore fused silica capillary column combined with nitrogen-phosphorous detector (NPD) was utilized to enhance the sensitivity. The method ensured effective evaluation of aroma quality in comparison with 2-AP concentrations.

The volatile profiles of black rice, an important aromatic rice predominant in asia was analyzed by GC-MS through a dynamic headspace system fitted with tenax trapping system (Yang *et al.,* 2008a). Quantity of aldehydes and aromatics posed highest abundance of 80.1 per cent to the total concentration of volatiles. Principal component analysis of odor-active compounds displayed substantial differences in aroma between black and regular white rice of about 93 per cent of total variation. The aroma in black rice was largely determined by 2-AP, guaiacol, indole and *p*-xylene than to white rice and contributed in unique odor character in black rice.

The aroma profile of six different rice cultivars was analyzed by a dynamic headspace system attached with Tenax trap in combination with GC-MS and GC-olfactometry (GC-O) (Yang *et al.*, 2008b). The analysis revealed twenty-five odorants as important odor-active compounds based on their moderate to high intensity. The odor thresholds determined by GC-O exhibited lowest odor threshold (0.02ng/L) for 2-acetyl-1-pyrroline followed by aldehydes, guaiacol and 1-octen-3-ol. Overall 97 per cent of the OAVs in each rice plants was governed by 2-AP, hexanal, (E)-2-nonenal, octanal, heptanal and nonanal. Multivariate analysis helped in separation and characterization of individual rice types based on aroma profile present in it. The three Japanese rice varieties, Nihonbare, Koshihikari and Akitakomachi were used to understand the impacts of cooking

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stages on aroma volatiles. GC-MS analysis identified an increase in major odor-active compounds and reduction of the low-boiling compounds during cooking process. The compound Indole was more abundant in Nihonbare and varieties Koshihikari and Akitakomachi possessed higher 4-vinylphenol compound (Zeng *et al.*, 2008).

Odour-active volatiles (OAVs) from specialty rice such as premium-quality, waxy and black pigmented was analyzed to observe distinct pattern of their aroma. Hexanal as a main contributor to aroma in both premium-quality and waxy cultivars was found to be 16 times higher in waxy cultivars than premium-quality depicting its lower aroma strength. The total aroma strength of black-pigmented rice grains were contributed by 2-AP followed by guaiacol. A clear distinction of all three types of specialty rice was made possible by the use of multivariate analysis of the compounds 2-AP, guaiacol, hexanal, (E)-2-nonenal, octanal and heptanal (Yang *et al.*, 2010).

Aromatic intensity of rice is believed to vary highly with environmental and cultivation conditions. The effect of soil salinity as a environmental factor on the 2-AP content of rice grains was studied. The fragrant rice plants were grown in fields varying with the salinity level checked by scaling the electrical conductivity (EC) of soil solution. The study supported the probable impact of salinity on 2-AP concentrations and complex nature of mechanisms involved (Gay *et al.,* 2010).

The analysis using headspace SPME in combination with GC-MS of aromatic rice cultivars shown five- fold higher 2-AP levels for Guixiangzhan cultivar compared to that of established aromatic cultivar Peizaruanxiang. The deviation in 2-AP concentration was correlated with combined effects of several components blended in proper proportions (Goufo *et al.,* 2010).

Bryant and McClung (2011) studied the differences in volatile profile of aromatic and non-aromatic rice cultivars. Along with 2-AP, a total of 93 different volatile compounds were identified, 64 of which were discovered newly. Volatiles or groups which were unique to specific aromatic cultivars may play a role in aromatic flavor development other than 2-AP. Majority of compounds were found to be in freshly harvested rice and

subsequent storage and no specific compounds produced in storage process. Study showed a great diversity of volatiles in both aromatic and non-aromatic varieties.

Grimm *et al.* (2011) employed highly sensitive sorptive stir bar placed inside of headspace for headspace sorptive extraction of 2-acetyl-1-pyrroline in rice varieties. The method provided immensely sensitive detection limit for 2-AP of 0.1ppb and produced a semi quantitative data. This helped to detect 2-AP in esteemed aromatic varieties quickly and even at lower concentrations.

Rice varieties from Iran, Afghanistan, and Uzbekistan were classified into aromatic and non-aromatic by analysing their aroma using 1.7per cent KOH sensory test and molecular marker analysis (Vazirzanjani *et al.*, 2011).

The cooked rice grains of Jasmine, Basmati and Jasmati was analysed for aroma by Headspace SPME (Mahattanatawee *et al.*, 2014). These volatile compounds when subjected to Principal Component Analysis (PCA) exhibited a distinguishing pattern of sulphur volatiles. The presence of a characteristic sulphur compound 2-acetyl-2-thiazole was identified in cooked rice grains of variety Jasmine. The compounds producing nutty, sweet floral odor such as 3 methyl-2-butene-1-thiol, genyl acetate, β-damascone, β-damascenone and α-ionone were detected in cooked aromatic rice.

Quantitative analysis of selected 23 volatiles along with 2-AP in non-basmati scented rice, basmati and non-scented rice varieties from different locations was performed and observed for the nature of production. Higher 2-AP content was exhibited by the non-basmati scented cultivars compared to basmati varieties. A positive correlation of 2-AP with the compounds 1-tetradecane and indole and negative correlation with benzyl alcohol was observed (Mathure *et al.*, 2014).

The influence of single nutrient element on aroma strength was studied by growing rice variety Khao Dawk Mali 105 under soilless conditions and varying concentrations of N, P, Ca, Zn, Mn and Mg nutrient solution was provided. The 2-AP measured through Headspace extraction with Gas Chromatography-Nitrogen Phosphorous Detector (HS-GC-NPD) showed increase of upto 12.69, 16.68, 13.82, 13.06ppm for N, P, Ca and Mn

respectively, whereas control yielded 1.42ppm of 2-AP. Results shown positive effects of addition of single nutrient element in rice plants (Monggoot *et al.*, 2014).

Poonlaphdecha *et al.* (2016) studied the role of 1- pyrroline through feeding experiments using rice calli cultures to analyze the vital steps in 2-acetyl -1-pyrroline (2AP) biosynthesis. Fresh calli from fragrant rice variety Aychade and a non-fragrant variety (Gladio×Fidji K2) were incubated with 1-pyrroline. It was found that 2AP in both varieties was increased, especially 2AP in the non-fragrant variety was greatly elevated by supplementation. It was proved that 1-pyrroline as a limiting factor for 2AP synthesis in rice. Stable isotope labeled substrates of glucose, sodium acetate and sodium octanoate were used to find the origin of acetyl donor. The presence of labeled 2AP in calli hypothesized the possibility of those compounds as acetyl group-donors for 2AP.

Mo *et al.* (2015) studied the effect of shading treatment on yield and quality attributes of aromatic rice varieties Yuxiangyouzhan and Nongxiang 18. Shading during the grain filling stage shown notable increase in 2-AP and GABA concentrations in both the varieties. Additional ten more compounds exhibited the similar variations in their concentrations. The study indicates a desirable selective effect of shading on increased aroma and negative impact on yield and grain quality.

Biosynthesis and availability of primary and secondary metabolites including volatile organic compounds (VOC's) is highly uneven in growth phases like vegetative, reproductive, grain filling and maturity (Hinge *et al.*, 2016). Aroma volatiles contributing to aroma were screened at seven developmental stages in scented rice varieties Basmati-370 and Ambemohar-157 along with IR-64 as check variety through HS-SPME-GC-MS analysis. N-heterocyclic compounds were found to be the main discriminating class between scented and non-scented rice cultivars. Most of the compounds were produced during seedling stage and reduced gradually at reproductive and maturity stage.

Hinge *et al.* (2016) performed volatile analysis of scented and non-scented cultivars at various growth stages. 14 odor-active volatiles (OAVs) where N-heterocyclic compounds were found to be characteristic for scented rice. Seedling stage possessed greater number of compounds which was reduced at reproductive and maturity stages.

Among 14 OAVs, eight compounds such as 2-AP, 2-acetyl-1H-pyrrole, β-ionone, (E, Z)-2,6-nonadienal, *p*-xylene and methyl 2-aminobenzoate were involved in characteristic odor development. Ambemohar-157 found to possess 4-methyl decane, 4-cyclopentylidane-2-butanone, toluene, indole and allyl cyclohexane as specific compounds, whereas Basmati-370 showed camphene, a terpene compound as distinct.

Ghosh *et al.* (2018) studied various biochemical pathways and level of numerous metabolites that are important in fragrance production. The results showed that higher levels of proline content was combined with enhanced activity of enzymes such as proline dehydrogenase (PDH), Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and ornithine amino transferase (OAT), which were found to be involved in production of Δ^1-pyrroline-5-carboxylate. Higher diamine oxidase (DAO) activity reduced the accumulation of putrescine producing more GABald in aromatic varieties. Lower BADH2 enzyme activity led to conversion of GABald to Δ^1-pyrroline which is the main precursor of aroma.

The effect of milling on the volatile profile of scented rice Cheonjihyang-1-se was evaluated. Four volatile compounds along with 3-hexonone shown depletion of up to 78 per cent, where as another four compounds including (E)-nonen-2-enal got elevated during milling process. The degree of milling had no role in the level of 2-AP compound. An important variability in projection scores of partial least squares for the compounds Benzene and 2-pentyl furan helped to differentiate white rice based on degree of milling (Mahmud *et al.*, 2018).

The effects of gamma irradiation on the accumulation of 2-AP, GABA and other volatile compounds were analyzed in germinated Thai upland rice. The study resulted an increase of 2-AP up to 23-folds in irradiated rice at 20 Gy compared to that of non-irradiated rice. On the contrary GABA concentration reduced up to 2.6 fold in irradiated rice at 300 Gy than that of non-irradiated rice. Overall volatile compounds were found to decrease with an increase in gamma dosage (Sansenya *et al.*, 2017).

Volatile compounds in two parboiled Nigerian aromatic rice variety (Ofada and Caroline) were extracted by solvent extraction method and analysed through Gas Chromatography - Mass Spectroscopy. The major constituents of aroma in Ofada and

Caroline rice varieties were found to be organic acids, aldehydes, ketones, amine, alcohols, hydrocarbons and esters, whereas 2AP was found to be absent. Different combination and matrices of volatile compound produce different flavor and special aroma perception from rice varieties (Adekoyeni *et al.*, 2018).

The aromatic compounds in 43 Thai local rice varieties was measured and correlated with KDML 105 variety (Sansenya *et al.*, 2018). 2-AP content was lower in all Thai local varieties than KDML 105 except Khao Joo 15 variety, which was on par with KDML 105. The proline content in all Thai varieties had no correlation with 2-AP content which might be due to variation in environmental stresses. Out of 140 volatile compounds recorded, 18 volatiles were found to have role in aroma intensity of rice.

2.2 *fgr* gene characterisation of Aromatic rice varieties

The identification of a DNA marker closely linked to the gene for aroma in rice by Ahn *et al.* (1992) led to early insights into the position of *fgr* gene. In the study the DNA marker was identified by testing 126 mapped rice genomes and cDNAs along with oat cDNA, these clones used as hybridization probes against Southern blots, consisting of DNA from a pair of nearly isogenic lines (NILs) with or without the aroma gene. Introgressed chromosomal segments from the donor genome were distinguished by RFLPs between the NILs. Linkage association of the gene in the clone was verified for aroma using an F_3 segregating population. The presence of linkage between the DNA marker and the gene was indicated by the co-segregation of the scented phenotype and donor-derived allele. RFLP analysis showed that the gene was associated with a single-copy DNA clone, *RG28,* on chromosome 8, at a distance of 4.5 cM.

Lorieux *et al.* (1996) was succeeded in tagging the two QTLs with a major gene controlling grain aroma in rice. It involved both quantification of volatiles in cooking water and molecular mapping by using markers such as RFLPs, RAPDs, STSs, isozymes. Mapping performed on doubled haploid line population shown several markers on choromosome 8 which were closely linked to the major gene controlling the production of 2-acetyl-1-pyrroline, the principle compound in the rice aroma.

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The assessment of a genomic DNA clone RG28 which is linked to fragrance gene (*fgr*) in rice helped to identify polymorphism to construct a PCR based marker for fragrance allele (Garland *et al.*, 2000). A co-dominant PCR-based marker SCU-Rice-SSR-1 was developed from a mono-nucleotide repeat which was polymorphic between fragrant and non fragrant cultivars.

Pattern of inheritance of aroma in rice helps in breeding programme for the selection of high yielding aromatic varieties having strong aroma. Dong *et al.* (2001) conducted experiment to find the inheritance, allelic nature and chromosomal location of the aroma genes in rice cultivars 'Shiroikichi and Della'. Crossing of the aromatic cultivars with non aromatic plants to obtain F_1 and F_2 populations resulted in the absence of leaf aroma in all non aromatic and aromatic F_1 plants indicating recessive nature of aroma trait in cultivars. The aromatic nature of the F_1 and F_2 plants produced from the crosses between those cultivars revealed that the aroma genes in both cultivars were allelic.

RFLP and microsatellite markers flanking fragrance gene were identified *via* DNA sequence alignment of ESTs against BAC clones bound to the same region in chromosome 8. Aligned DNA sequence from PCR products of fragrant cultivar *Kyeema* and non-fragrant cultivar *Dongara* produced a single SNP 'RSP04' (C/T transition) within BAC clone AP003885 (Jin *et al.*, 2003).

Bradburry *et al.* (2005a) showed the presence of a gene having homology with a gene coding for betaine aldehyde dehydrogenase (BAD), consisting significant polymorphisms in the coding region of fragrant plants. Later the accumulation of 2-acetyl-1-pyrroline in fragrant rice was proposed to be due to the presence of mutations resulting in loss of function of the '*fgr*' gene by introducing a stop codon in conserved amino acid sequence of BADs.

An aroma mutant (SA0420) was obtained from a mutation pool of variety 'Tainung 67' developed by sodium azide mutagenesis (Kuo *et al.*, 2005). Aroma detection in F_2 progenies proved that aroma trait of SA0420 was governed by a single dominant locus (χ^2 = 0.0164). SSR mapping unfurled that aroma locus of SA0420 was located on chromosome 8. Cloning and sequencing of three dominant aroma mutants and six RILs

with aroma trait contained reported a deletion in *BAD2* gene, while other few aromatic lines were with normal *BAD2* gene. Hence it was denoted that deletion in *BAD2* gene is not universal.

Mapping analysis using SSR markers confirmed the presence of a single recessive *fgr* gene on chromosome 8 and was found to be located between markers RM8264 and RM3459 was having a physical distance of around 800kb (Chen *et al.,* 2006). A fine mapping of *fgr* region using high density markers such as RM1309 and RM7580 and subsequent analysis of segregant populations shown that the *fgr* locus to be flanked by 69kb region between left marker L02 and the right marker L06.

Molecular analysis of fragrance locus in 229 wild rice accessions from *O. rufipogon* was performed (Prathepha, 2009). Low frequencies of 0.23 for the mutated allele found in the wild rice plants suggested the occurrence of mutation well prior to the domestication. Results supported the hypothesis that domestication of rice through artificial selection by farmers in ancient times led to the production of present aromatic rice varieties.

The analysis of physiological roles OsBADH2 through sequencing approach and RNA interference (RNAi) technique was carried out by Niu *et al.* (2008). The expression levels of OsBADH2 and the fragrant compound in wild type as well as transgenic OsBADH2-RNAi repression lines was examined by semi-quantitative, Real-Time reverse transcription-Polymerase Chain Reaction (RT-PCR) and Gas Chromatography-Mass Spectrometry (GC-MS) respectively. Presence of multiple mutations in *fgr* allele in 13 fragrant rice cultivars of china along with low expression levels in mature roots was observed. Disruption of OsBADH2 by RNAi led to prominent increase in 2-acetyl-1-pyrroline synthesis and variation in expression levels of OsBADH2 gene was found to influence aroma accumulation.

Transformation of three major *Fgr* constructs such as pCAM-Badh2, pCAM-Cah, and pCAM-Mccc2 into the fragrant rice cultivar *Wuxiangjing* through *Agrobacterium tumefaciens*-mediated rice transformation was done by Chen *et al.* (2008). Determination of 2AP concentration in transgenic plants showed no difference in plants having transgene

either Cah or Mccc2 from that of non transgenic plants, where as plants carrying *Badh2* were found to be non fragrant. Further production of plants over-expressing both complete and partial *Badh2* coding sequences driven by CaMV35S promoter produced non fragrant transgenic plants with low 2AP levels from complete *Badh2* expressed plants where as latter had no influence over fragrance production. Aldehyde dehydrogenase activity of intact BADH2 protein producing BADH2 enzyme showed the strong AB-ald dehydrogenase activity. The BADH2 enzyme converts AB-ald into GABA, resulting in 2AP inhibition and absence of the enzyme increased AB-ald accumulation leading to 2AP synthesis.

Shi *et al.* (2008) discovered a new *badh2* allele and was successful in developing a functional marker for the *badh2* locus. The study consists a total of 24 fragrant and ten non-fragrant varieties which were sequenced for the *Badh2/badh2* loci. Out of 24 fragrant varieties, 12 were observed to have previously reported 8bp deletion in *badh2* (*badh2-E7*) allele, while remaining revealed a new null *badh2* allele (*badh2-E2*) having 7 bp deletion in exon 2 but consisting no deletions in exon 7.

The origin of fragrance was originally assumed to be from *Indica* varietal group. Kovach *et al.* (2009) identified eight putative non functional alleles from *BADH2* gene which had distinct geographic and genetic origins. Haplotype analysis of a single predominant *badh2.1* allele, established its origin in *Japonica* varietal group and indicating the introgression of the allele from *Japonica* to *Indica* group. Basmati-like accessions showed an identical 5.3Mb region flanking *Badh2* to that of ancestral *Japonica* haplotype revealing close evolutionary relationship between Basmati varieties and the *Japonica* gene pool.

Fragrance in rice is believed to be associated with two deletion mutations in *Badh2*. Shao *et al.* (2011) showed that fragrance gene in variety Zaimiaoxiangnuo had a new mutation instead of previously identified mutations at exons 2 and 7. Sequencing followed by BLAST analysis of twenty two fragrant and four non fragrant varieties presented a deletion of 803bp between exons 4 and 5 in eight fragrant cultivars. A functional marker

FMbadh2-E4-5 was developed to detect the new mutation in fragrant gene which produced PCR products of 321bp, where as wild type *Badh2* produced 1123bp PCR products.

Myint *et al.* (2012) reported a aromatic allele having a 3-bp insertion in exon 13 of *Os2AP* gene in aromatic cultivars from Myanmar. The insertion resulted in additional tyrosine in the amino acid chain, but however the changes had no effect on the expression of *Os2AP* gene compared to that of non-aromatic varieties. Varieties with 3-bp insertion produced less 2-AP to that of 8-bp deletion (exon 7) varieties. It was validated that 3-bp insertion dents the catalytic efficiency of enzyme encoded by *Os2AP* towards the 4-aminobutanal metabolism.

Production of fragrant rice grain from non fragrant variety through application of transgenic breeding was attempted by Chen *et al.* (2012). Maize ubiquitin driven amiRNA transgenic lines were used for down-regulation of *BADH2* expression to produce fragrance in non fragrant wild type plants which resulted in 2AP accumulation. L- proline content in leaves shown accrual through concurrent down-regulation of *PRODH* (proline dehydrogenase) and up-regulation of *P5CS* (Δ^1-pyrolline-5-carboxylic acid synthetase).

Shao *et al.* (2013) analysed 516 fragrant rice accessions for all three known *Badh2* FNPs i.e., deletions in exon 2, 4-5, and 7, where 62 fragrant accessions were lacking any of the *Badh2* FNPs. 56 accessions had the exon regions similar to that of non-fragrant allele showing an probable chance of presence of polymorphism in non-exonic regions such as promoter region or introns. The re-sequencing of the 62 accessions revealed the presence of 75 bp deletion in exon 2 and a SNP at exons 10 and 13. Diversity analysis of 144 accessions using 48 SSR markers formed three clusters, namely *Indica, Japonica, O. rufipogon.* The allele *badh2.7* was found to be present in both *Indica* and *Japonica,* where as *badh2.2* and *badh2.4-5* was specific to *Japonica* group.

A total of 12 different alleles which are associated with mutations in *badh2* coding regions have already been reported. A work conducted by Shi *et al.* (2013) was successful in discovering a new *badh2* allele having a 3-bp deletion in 5'UTR and an 8-bp insertion in upstream promoter region of *badh2.* The 8-bp insertion in the promoter region of fragrant rice variety might alter the spacing of the *cis*-acting elements which are believed to affect

gene transcription and its level. A new sequence tagged site specific functional marker i.e., Cleaved Amplified Polymorphic Sequence (CAPS) targeting *badh2-p-5'UTR* & *badh2-E7* alleles was developed to differentiate diverse genotypes for fragrance.

Re-sequencing of whole genome of 295 rice accessions by He *et al.* (2015) gave rise to unearthing of another new novel allele (*badh2-E12*) for fragrance from the Korean landrace *Mongdonjaera*. The novel allele carried a 3-bp deletion in exon 12 leading to loss of an amino acid, which contributed fragrance in rice.

Absence of common fragrance mutation of exon 7 in Japanese fragrant landraces revealed the presence of genetic difference from non-japanese fragrant landraces (Ootsuka *et al.,* 2014). Fragrant landraces were found to carry two major mutations groups, one was known SNP in exon 13 and the other was a SNP at exon1-intron1 junction which acted as splicing donor site. The latter was more potential in producing aroma than other mutation in Japanese landraces.

Sun *et al.* (2008) carried out gene fine mapping of aroma in aromatic hybrid rice variety *chuanxiang*-29B and followed by linkage analysis between SSR markers and the aroma locus. The mapping of F2 plants revealed that aroma gene in Ch-29B to a chromosome 8 region flanked by SSR markers RM23120 at 0.52cM and RM3459 at 1.23cM. Molecular mapping data uncovered that the aroma locus was present in a 142.85kb interval on BAC clones AP005301 or AP005537.

Whole genome sequencing of *Seeragasamba*, a short-grain aromatic rice variety from *indica* group revealed a new *badh2* allele with an 8bp insertion in promoter region of BADH2 gene. It was found that the *badh2-p* allele was specific to 13 varieties of *indica* and *japonica* and present in 17 varieties which already had the loss of function allele, *badh2-E7* (Bindhusree *et al.,* 2017).

2.3 Marker assisted selection (MAS) programs for aromatic trait

Jain *et al.* (2004) studied the genetic relationship between germplasms of Indian aromatic rice (*Oryza sativa* L.) with the thirty microsatellite markers which were fluorescently labelled. The study was conducted using sixty nine rice genotypes, of which

fifty two were basmati and other scented and the remaining seventeen were *indica* and *japonica* varieties which were used as controls. Of the 235 alleles which were detected using these thirty SSRs, sixty two of them were unique to basmati and other scented varieties. Around twenty SSRs could differentiate traditional basmati and 8 could distinguish basmati derived by cross-breeding and non-basmati rice varieties. When the varieties were clustered using similarity coefficients, high quality Indian aromatic and quality rice were distinguished from both the *indica* and *japonica* cultivars and the traditional basmati varieties which were cross bred could be differentiated from traditional basmati cross. All of which suggest that Indian aromatic germplasm is genetically different from the groups within *Oryza sativa*. This is the result of a evolution that occurred independently over a long period of time.

Kibria *et al.* (2008) used the SSR markers RM 223, RM 342A and RM 515 for identifying *fgr* locus. The genotypic analysis were carried out using these markers to screen thirty two F_7 rice lines from the segregating population of a cross between Y-1281 and Khalizira. They identified 9, 12 and 17 pedigree lines having *fgr* locus using the markers RM 223, RM 342A and RM 515 respectively. They identified a total of fourteen lines with the *fgr* locus with significant yield, better agronomic performance and grain quality traits.

Baishya *et al.* (2000) studied the genetic variation using 26 RAPD markers at the genomic level in 9 aromatic and 4 non-aromatic rice varieties. Ninety eight polymorphic PCR products were obtained out of a total of 177 PCR products. The primers OPA-03, OPB-01 and OPD-13, *jas* 1.5 were developed for differentiation of non-basmati, basmati and non aromatic rice varieties respectively.

Rai *et al.* (2015) evaluated the usefulness of three SSRs for fragrance in 24 rice genotypes that consisted of aromatic varieties such as Kalanamak 3119, Kasturi Basmati, Basmati LC74-3, Thurumbhog and Jeeraga Samba. The presence of SNPs was also detected. The marker patterns were observed as expected based on the intensity of fragrance in all the genotypes except Kalanamak 3119, Kasturi Basmati, Basmati LC74-3, Thurunbhog and Jeeraga Samba. These rice genotypes were clustered into two based on

the fragrance locus as fragrant and non-fragrant genotypes. Kala Kalanamak 3119, Kasturi Basmati, Basmati LC74-3 formed the fragrant cluster whereas Thurunbhog and Jeeraga Samba formed the non-fragrant cluster. None of these markers could identify all the five genotypes as fragrant. Hence it was concluded that there exists another gene that imparts fragrance in rice.

$(AT)_{40}$, a SSR marker was developed to identify fragrant and non-fragrant alleles of the *fgr* gene. A total of 50 F_2 individuals were genotyped from the mapping population for this polymorphic marker, with a PIC of 0.9. They could also identify a marker, SCU015RM found to be closely linked to the *fgr* gene. This study was found to be an effective approach for the marker identification so as to enable them for rice improvement (Cordeiro *et al.,* 2002).

Previous studies revealed that the aroma in rice is caused as a result of mutations in *badh2* gene. The exons 1, 2, 7, 10, 13 and 14 of *badh2* were found to be the hotspots for numerous mutations. Based on this, Dissanayaka *et al.* (2014) attempted to sequence the 14^{th} exon of *badh2* gene of Srilankan aromatic rice varieties. These variation were found to lack the *badh2.1* allele. It was found that 4 accessions possessed 'G' insertion in the 14^{th} exon. This was the novel mutation and classified as *badh2.7* allele. The 3D protein structure of the mutant showed partly loss of oligomerization and coenzyme binding domains which is considered to impart fragrance to rice. Also a new CAPS based marker *badh2.7* CAPS was also developed in order to identify the varieties supposed to possess *badh2.7* allele.

A mono-nucleotide repeat was identified to be polymorphic between a pair of frequent and non-fragrant cultivars, was developed into a co-dominant marker for PCR. It is good to be highly useful to distinguish the fragrant alleles and non-fragrant alleles lying in the *fgr* locus.

Development of quantitative trait loci (QTLs) through anchor markers for genes of interest can enhance QTL detection. Hashemi *et al.* (2015) developed an F2 mapping population from cross between MRQ 74 and MR 84, which are scented and non-scented cultivar respectively. Two QTLs having 3.2 per cent to 39.3 per cent total fragrance

phenotypic variance were identified on chromosomes 4 and 8. An important putative allele was located on chromosome 8 between markers RM223 and SCU015RM.

Hossain *et al.* (2007) used 30 microsatellite markers in 21 rice cultivars for characterization of rice varieties. Based on Polymorphic Information Content (PIC) values RM 223 was identified as the best markers for characterization of all 21 genotypes and possessed a frequency of 24 per cent.

The allelic diversity of 12 aromatic rice varieties was analysed using microsatellite markers (Hossain *et al.,* 2012). Twenty four SSR markers, of which 10 was polymorphic for various chromosomes. Higher PIC values were identified for RM180 (0.765), RM207 (0.746), RM224 (0.680) *etc.* The highest genetic dissimilarity was observed between Gondhocosturi and Lalrodha dhan (81.03 per cent) from the pairwise genetic dissimilarity studies, which helps in selection of higher diverse cultivars for creating novel background population in backcross breeding studies.

Evaluation of genetic diversity of chromosome 8 present in 33 rice genotypes consisting traditional basmati, basmati crosses and non-basmati crosses was performed using 26 SSR markers. Out of 106 alleles detected, 21 were found to be unique for a single genotype. Traditional basmati was distinct from other rice cultivars (*indica* and *japonica*) and numerous markers were developed for distinguishing them from other poor varieties (Jain *et al.,* 2006). SSR markers are evolved into a major tool for diversity analysis of aromatic varieties (Joshi and Behera, 2007; Sajib *et al.,* 2012; Patel *et al.,* 2015).

Twenty one rice genotypes were subjected for aroma detection using SSR markers RM223, RM515 and RM342. Highest number of *fgr* locus was detected using RM223, which could also identify genes with higher yield and grain quality traits (Jewel *et al.,* 2011). Madhav *et al.* (2010) also identified novel SSR markers ARSSR-3, which is highly linked (0.3cM) to the aroma trait.

Single Nucleotide Polymorphism (SNP) markers for *fgr* gene was identified from whole rice genome sequence. Sequence alignment of EST sequence with BAC clones covering *fgr* gene was used to identify Restriction Fragment Length Polymorphism (RFLP)

and microsatellite markers flanking the same region in chromosome 8. Re-sequencing and comparison identified a single SNP (C/T transition) in 14 genes. This could create a way for efficient gene discovery of elite cultivated rice varieties (Jin *et al.,* 2003).

Apart from identifying various genes, Marker assisted selection (MAS) has numerous application in conventional breeding method. MAS using allele specific amplification marker was employed for genotypic selection of F_2 population derived from crosses of high yielding varieties Neda and Nemat with local aromatic cultivars Sang tarom and Tarom deylamani (Kiani *et al.*,2011). Plants exhibiting homozygous condition for aroma gene combined with good agronomic performance was identified and used for developing new aromatic rice lines.

Bradburry *et al.* (2005b) developed allele specific amplification (ASA) markers, a low cost and robust technique to distinguish alleles differing by SNPs in a single PCR tube. The ASA assay was successful in discriminating fragrant and non-fragrant rice varieties in both homozygous and heterozygous condition of fragrant trait.

The markers FMbadh2-E2, FMbadh2-E7 and a novel marker FMbadh2-E4-5 for deletions in region lying between exon 4 and 5 was attempted for genotyping of fragrant genes in rice (Shao *et al.,* 2011). A single co-dominant functional marker for *fgr* trait was developed for validation of Basmati and non-Basmati rice cultivars (Siwach *et al.,* 2004; Steele *et al.,* 2008; Sakthiwel *et al.,* 2009). The marker amplifying InDel polymorphic regions of badh2 region produced 95 and 103 bp fragments in aromatic and non-aromatic cultivars respectively. This was believed to be high efficient and co-segregating with the fragrance trait.

Linkage analysis through SSR markers Aro7, RM23120 and RM3459 corresponding to aroma locus in the F2 aromatic plants revealed the presence of aroma gene on chromosome 8 of variety Chuanxiang-29B. The aroma locus found to be present in a 142.85Kb interval regions in BAC clones BP005301 or AP005537 (Sun *et al.,* 2008).

The whole genome BAC-End Sequence (BES) and Finger Print Contig (FPC) was used to develop 40 SSR markers for aroma gene in crossbred rice plants. A tightly linked

marker was mapped to aroma trait within a region of 1.1 cM *i.e.,* 170 Kb in the genome flanked by 10L03_FW and CP04133. The method was successful in explaining the use of *in silico* approaches for physical mapping and marker enrichment (Wanchana *et al.,* 2005).

Singh *et al.* (2004), used Sequence Tagged Microsatellite Site (STMS) marker for describing the DUS application in aromatic rice. A total of 55 STMS markers developed from 12 linkage groups of rice genome was employed to observe uniqueness of 23 aromatic rice cultivars. Fourty one markers were showed polymorphism in all the varieties along with a very low probability of occurrence of identical match by chance.

Materials and Methods

3. MATERIALS AND METHODS

The study on 'Molecular and biochemical characterisation of aroma in *Biriyanicheera* rice genotype' was carried out at Centre of Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Thrissur during the period 2017-2019. The details of research work, along with materials used and the methodologies followed, are discussed in this chapter.

3.1 Materials

3.1.1 Plant materials

The aromatic rice genotypes *Biriyanicheera* and Gandhakasala (check genotype) grown in three different locations, such as Mulangunnathukavu (10.5989^0 N, 76.2146^0 E and MSL- 26 metre) from Thrissur district, Pattambi (10.8114^0 N, 76.1904^0 E, MSL- 63 metre) Palakkad district and Kolenchery (9.982968^0 N, 76.476388^0 E and MSL- 12 metre) from Ernakulam district of Kerala along with a Non-aromatic genotype Triveni grown in Mulangunnathukavu from Thrissur during the period 2017-2018 were used for the study. The duration of rice genotypes *Biriyanicheera*, Gandhakasala and Triveni variety is 105, 120 and 105 days respectively.

3.1.2 Laboratory chemicals, glassware and plastic wares

The chemicals used in the study were obtained from companies like Merck India Ltd., HIMEDIA, and Sigma Aldrich Pvt. Ltd. The primers for PCR amplification were supplied by Sigma Aldrich Pvt. Ltd. The components for PCR such as, *Taq* polymerase, dNTPs, *Taq* buffer, $MgCl_2$, DNA ladder were obtained from GeNei Pvt. Ltd. The plastic wares were procured from Axygen, Tarsons India Ltd. and glass wares used were obtained from Borosil and Vensil.

3.1.3 Equipment and machinery

The study was carried out using molecular biology facilities at CPBMB, College of Horticulture. The instruments such as electronic balance (Shimadzu), pH meter

(EuTech Instruments PC 510), micropipettes (Eppendorf), Icematic (F100 compact), high speed refrigerated centrifuge (KUBOTA 6500) and Thermocycler (ProFlex PCR System by Life Technologies), were employed for the research work. Nanodrop spectrophotometer (NanoDrop ND-1000) was used for the quantity analysis of DNA. The quality of DNA was analysed using gel electrophoresis unit (BioRad) and the agarose gels were visualised using Gel documentation unit (BioRad Gel DocTM XR+).

3.2 Methods

3.2.1 Raising of rice plants

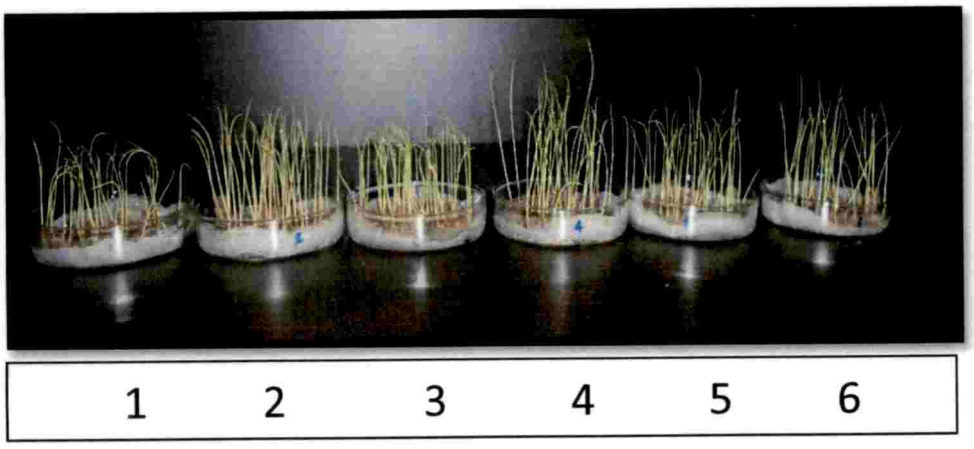
Seeds of aromatic rice genotypes, *Biriyanicheera* and Gandhakasala were collected from three locations *viz*., Thrissur, Palakkad and Ernakulam districts of Kerala. They were grown along with a non-aromatic rice genotype Triveni (seeds collected from Thrissur). The seeds were germinated in petri plates lined with moist tissue paper and allowed to germinate (Plate 1(A)). After a week, the germinated seedlings were transferred to a pot (Plate 1(B)). Later, well grown seedlings (21 days old) were further transplanted into new pots at three seedlings per pot. This procedure was followed for all the three genotypes from different locations.

3.2.2 Biochemical characterisation

Biochemical analysis included GC-MS and a set of tests to determine the cooking quality in rice such as gelatinisation temperature test, determination of amylose content, gel consistency test were performed. Sensory evaluation test was also conducted to determine the aroma.

3.2.2.1 GC-MS analysis

The husk of the seeds was gently removed with the help of a pestle and mortar. These dehusked seeds were powdered finely and 10 gram of the fine powder of each sample was taken for GC-MS analysis at TNAU, Coimbatore after carefully packing in zip lock covers to avoid the loss of aroma. The instrument used was the Perkin Elmer Clarus SQ8C with



1- *Biriyanicheera* (Pattambi)

2- Gandhakasaala(Pattambi)

3- *Biriyanicheera* (Thrissur)

4-Gandhakasaala (Thrissur)

5- *Biriyanicheera* (Ernakulam)

6- Gandhakasaala (Ernakulam)

Plate 1 (A). Rice seedlings grown in the petriplates

Plate 1 (B). Transplanted *Biriyanicheera* seedlings (7 days old) in pot

DB-5 ms capillary standard non-polar column. The injection volume was 1 µl and the carrier gas was helium.

3.2.2.2 Cooking quality analysis

3.2.2.2.1 Gelatinisation temperature analysis by Alkali spreading test

The procedure for alkali spreading test in rice was followed as per Little *et al.* (1958). Six whole milled rice grains with duplicates were spaced evenly in petri plates containing 10 ml of 1.7 per cent potassium hydroxide. The petri plates were kept undisturbed at 30°C for 23 hours in an incubator. After incubation the petri plates were observed for kernel spreading and recorded against seven point scale. The value was expressed as an average of six values corresponding to individual grains. Scoring matrix for alkali spreading value is mentioned below:

Scoring matrix for alkali spreading value

1	Kernel not affected
2	Kernel swollen
3	Kernel swollen, collar incomplete and narrow
4	Kernel swollen, collar complete and wide
5	Kernel split or segmented, collar complete
6	Kernel dispersed, merging with collar
7	All kernel dispersed and intermingled

Classification based on scoring matrix for alkali spreading value

Alkali spreading value	Classification	Gelatinisation temperature	Score
6-7	High	Low	1
4-5	Medium	Medium	3
3	Low, medium	High, medium	5
1-2	Low	High	7

3.2.2.2.2 Determination of amylose content

The amylose content was determined as per the procedure mentioned by Sadasivam and Manickam (1992).

Materials required

- 100 per cent ethanol
- 1N NaOH
- 0.1 N HCl
- 0.1 per cent phenolphthalein
- Iodine reagent (1 g of I and 10 g of KI in water and volume made to 500 ml)
- Standard amylose solution (100 mg of amylose in 10 ml of 1 N NaOH and volume made to 100 ml)

Procedure

1. To 100 mg of finely powdered sample, 1 ml of 100 per cent ethanol, 10 ml of 1 N NaOH was added and left overnight.
2. The volume was made up to 100 ml.
3. 2.5 ml of extract was taken and to this, 20 ml of distilled water and 3 drops of phenolphthalein were added.
4. 0.1 N HCl was added in drops until the pink colour just disappeared.

5. 1 ml of iodine reagent was added and the volume was made to 50 ml. The colour of the solution was read at 590 nm in a spectrophotometer.
6. 0.2, 0.4, 0.6, 0.8 and 1 ml of standard amylose solution were taken and the colour was developed as that of the sample. Based on this, standard graph was prepared.
7. The amount of amylose present in the sample was calculated using standard graph.
8. 1 ml of iodine reagent was added to 50 ml distilled water and used as blank.

Calculation

The amount of amylose present in the sample was calculated using the formula:

Absorbance corresponds to 2.5 ml of the test solution = x mg amylose 100 ml contain

$$= \frac{x}{2.5} \times 100 \text{ mg} \quad \text{amylose} = \% \text{ amylose}$$

3.2.2.2.3 Gel consistency test

Gel consistency was measured as per the method proposed by Cagampang *et al.* (1973). The rice samples were maintained in the same room so as to equalise the moisture content in the grains. Then the whole grains of rice samples were finely ground to make powder (100 mm mesh size). 100 mg of the freshly ground powder was transferred into test tubes. 0.2 ml of 95 per cent ethanol and 2 ml of 0.2 M KOH was added and mixed well. The test tubes were covered with glass lids. The samples were cooked in a vigorously boiling water bath for eight minutes. This was done until the contents in the tube raised to about $2/3^{rd}$ height of the test tube. The test tubes were removed from the water bath and kept in room temperature for five minutes, following which the tubes were cooled in an ice water bath for 20 minutes. The tubes were laid horizontally on a graph sheet placed on the table. The length (mm) of the gel was measured from the bottom of the tube to the gel front. The gel consistency was classified as follows :

Hard	:	< 26-35 mm
Medium hard	:	36-40 mm
Medium	:	41-60 mm
Soft	:	> 61-100 mm

3.2.2.3 Sensory evaluation by DUS (Distinctness, Uniformity, Stability) test

The procedure mentioned by DRR, Hyderabad (Rani *et al.*, 2006) was followed for sensory evaluation. Five gram of decorticated rice seeds from all the genotypes were added into individual test tubes containing 15 ml of water and closed with tight caps. The samples were soaked for 10 minutes and later cooked in hot water bath for 15 minutes. The cooked rice was then transferred into a petri plate. The petri plates were named with English alphabet (A to G) to avoid psychological preference and kept for cooling after sealing the lids with parafilm. After the petri plates were cooled, it was refrigerated for 20 minutes. Then the petri plates were opened and the contents were allowed to be smelled by ten individuals. The observed aroma was scored by each individual into different classes as mentioned below.

No Aroma	Slight Aroma	Medium Aroma	Good Aroma	Strong Aroma
1	3	5	7	9

3.2.3 Molecular characterisation

3.2.3.1 DNA isolation

Genomic DNA was isolated from young leaves of rice plants grown in pots. Fresh and green leaves yielded good quality of DNA in required quantity. The leaves were collected early in the morning from all the three pots (Plate 2). The collected leaves were immediately covered in aluminium foil and carried to the laboratory. The extraction of genomic DNA from the leaves was performed through CTAB method (Dellaporta *et al.*

Rice plants at Booting stage

Triveni, BC- *Biriyanicheera*, GS- Gandhakasala

Plate 2. Rice plants grown for DNA isolation

1983). The concentration of extraction buffer and reagents used in the experiment are listed below. Composition of reagents used for the work is mentioned in Appendix- I.

3.2.3.1.1 Reagents

I. CTAB Buffer (2X)

a) 4.0 per cent CTAB (w/v)
b) 100 mM Tris HCl (pH: 8)
c) 20 mM EDTA (pH: 8.8)
d) 1.4 M NaCl
e) 2.0 per cent Polyvinyl pyrrolidine (PVP)
f) 0.2 per cent β-mercaptoethanol

II. Choloroform: Isoamyl alcohol (24: 1 v/v)

III. Chilled isopropanol

IV. 70 and 100 per cent ethanol

V. Sterile distilled water

Freshly prepared reagents I and III were autoclaved separately and stored at room temperature.

3.2.3.1.2 Procedure for DNA isolation

Total genomic DNA of genotypes *Biriyanicheera*, Gandhakasala and Triveni was isolated by following protocol described by CTAB method (Dellaporta *et al.*, 1983).

1. 0.1 gram of fresh leaves of rice was obtained using sterilized scissor, weighed and transferred to a pre-chilled mortar and pestle.
2. The leaves were powdered by adding 50µl of β-mercaptoethanol and pinch of polyvinyl pyrrolidine (PVP) along with liquid nitrogen and turned it into a fine powder.
3. The homogenized liquid was transferred to a sterile tube (2 ml) containing 1 ml of pre-warmed CTAB extraction buffer and mixed well by inverting the tube.

4. The mixture was incubated at 65°C for 30 min with intermittent mixing by gentle inversion.
5. After incubation, 1 ml of Choloroform: isoamyl alcohol (24: 1) was added and mixed thoroughly until the formation of emulsion.
6. The tubes were centrifuged at 15,000 rpm for 15 min at 4°C.
7. The clear aqueous phase was transferred to a clean tube.
8. 2 µl of RNase solution was added to the tube and kept for incubation at room temperature for 30 min.
9. An equal volume of Choloroform : isoamyl alcohol was added into the tube.
10. The tube was gently inverted and centrifuged at 10,000 rpm for 5 min. At 4°C.
11. The aqueous phase was removed with a pipette into a clean tube (1.5 ml), the 0.6^{th} volume of ice cold isopropanol was added and mixed thoroughly until the DNA was precipitated and the tube was kept at 4°C for 2 hours.
12. The tube was centrifuged at 15,000 rpm for 20 min. and carefully poured the supernatant out by inverting tube.
13. The pellet was then washed with 70 per cent ethanol and dried.
14. After drying, the DNA was dissolved in the sterile distilled water (100 ml) and stored at -20°C.

3.2.3.2 Assessing the quality and quantity of DNA by spectrophotometer

The purity of DNA was analysed using NanoDrop ND-100 spectrophotometer. Nucleic acids and proteins show absorption maxima at 260 nm and 280 nm respectively. NanoDrop recorded the absorbance at both wavelengths and purity was determined by the ratio OD ($A_{260/280}$). The OD values obtained between 1.8 and 2.0 shows the purity of the DNA, which is free from proteins. The quantity of DNA in the sample was mentioned as ng/µl.

Procedure for quantity detection using NanoDrop :

1. NanoDrop spectrophotometer was connected to the system and the software ND-1000 was opened.
2. The option, nucleic acid was selected.

3. The sampling arm was opened and 1 µl distilled water pippetted on measurement pedestral.
4. Spectral measurements were started after closing sampling arm.
5. The reading was set to zero with the blank sample.
6. 1 µl of the sample was poured onto the measurement pedestral and measure was selected.
7. After each reading, a soft laboratory wipe was used to clean the upper and lower pedestrals of sampling.
8. OD_{260}/OD_{280} ratio and DNA quantity were recorded by the above procedure.

3.2.3.3 Assessing the quality of DNA by electrophoresis

The quantity of isolated DNA was evaluated through Agarose gel electrophoresis (Sambrook *et al.,* 1989).

3.2.3.3.1 Reagents and equipments

1. Agarose
2. 50X TAE buffer (pH 8.0)
3. 6X loading/ tracking buffer
4. Ethidium bromide (10 mg/ml)
5. Electrophoresis unit, power pack (BIO-RAD)
6. Gel documentation and analysis system

Procedure for Agarose gel electrophoresis of DNA

1. The gel casting tray was cleaned with distilled water and open ends were sealed with a tape.
2. The comb was then positioned parallel to open edges about 2 mm above the surface of tray.
3. Agarose (1.2 g) was added to 150 ml 1X TAE buffer and dissolved by melting. The solution was then allowed to cool.
4. After cooling 7.5 µl of ethidium bromide (1 mg per ml) was added as a staining agent.
5. Then the solution was poured into the gel casting tray and allowed to solidify.

6. After setting, the gel was placed in the electrophoresis unit with wells towards the cathode and tank was filled with 1X TAE buffer just enough to cover the surface of the gel.
7. The DNA sample was pipetted onto a parafilm and mixed well with 2 µl of 6X loading dye.
8. DNA samples were loaded in individual wells. The electrodes were connected to power supply and electrophoresis was carried out at 60 volts for 1-1.5 hours till the dye migrates to the end of the gel.
9. The DNA was visualized and documented using the gel documentation system.

3.2.3.4 Gel documentation

The gel image was documented in gel documentation system (BioRad Gel DocTM XR+). The gel profile was examined for intactness, clarity of DNA band, the presence of contaminants such as RNA and proteins. An image of a gel was captured using the controls in the imaging device window and displayed on the computer screen.

3.2.3.5 Normalisation of DNA concentration for PCR

Normalisation of DNA was done to bring all DNA concentrations to relatively equal level (25 ng/ µl) *via* appropriate dilutions. The dilutions were done with distilled water.

Designing of primers for PCR

The *BADH2* gene sequence (size = 6.1 Kb) was retrieved from the Rice Genome Annotation Project. The gene was suitably divided into seven distinct regions based on the position of exons for the convenience of PCR amplification. The primers were designed so as to amplify the exonic regions on the *BADH2* gene using Primer3 software.

Details of primers for *BADH2* gene:

Primers	Sequence	Product size	Exons covered
Primer 1	5' CACTCCACACCTGACACCAC 3'	980	1 & 2
	5'CTGCCAACTAACGAAGCACA3'		
Primer 2	5'AGCAGCATGGGACATGGTAT3'	741	4
	5'ACCGGGACAGAACAAATGAG3'		
Primer 3	5'GCTTTCAGCTTCTTGCTCCT3'	1002	10, 11 & 12
	5'GGTCACCGGAAAGCACAG3'		
Primer 4	5'TTGGCCAACGATACTCAGTG3'	944	13, 14 & 15
	5'ATGCACAAATTGTCGCGTAA3'		
Primer 5	5'CAGTGACCAGTGCACACTTT 3'	509	3
	5'ACCCAACTACACCGATAGGC3'		
Primer 6	5'CCTCTCCTCCGTGTTAATGC3'	1093	5, 6, 7 & 8
	5' GGTCTAGCATCCAGCTCAG 3'		
Primer 7	5' GTGGCAAGGAAGGCAGTTAG 3'	707	9
	5' GGTCAGGAGCAAGAAGCTGA 3'		
Primer 1 (new)	5' ACAGAACAGAGCACTCCCTCTC 3'	846	1 & 2
	5' CCAGATACACAATCAGCCATGC 3'		

3.2.3.6 Polymerase Chain Reaction (PCR)

PCR reaction mixture was prepared in 0.2 ml thin walled flat cap PCR tubes containing the following components. The total volume of each reaction was 10 µl.

Reagents	Volume (µl)
Taq assay buffer (contains $MgCl_2$)(10X)	1.5
dNTPs (10 mM)	0.5
Forward primer (10 µM)	1.0
Reverse primer (10 µM)	1.0
Taq DNA Polymerase (3U/ µl)	0.4
Template (DNA 50 ng)	2.0
Sterile distilled water	3.6
Total	**10.0**

Amplification was carried out on Applied Biosystems PCR machine. The amplification profile was as follows :

1. Initial denaturation at 95˚C temperature for 3 min.
2. Denaturation at 94˚C for 50 sec.
3. Primer annealing at around ~57˚C for 30 sec.
4. Primer extension at 72˚C for 1 min.

   Later steps 2 to 4 were repeated for 35 times.
5. Complete primer extension at 72 ˚C for 10 min
6. Hold at 4 ˚C until removal

3.2.3.6.1 Modified PCR protocol by using Dimethyl Sulphoxide (DMSO)

PCR reaction mixture was prepared in 0.2 ml thin walled flat cap PCR tubes containing the following components. The total volume of each reaction was 50 µl. Three per cent of DMSO (1.5 µl for 50 µl reaction) was used for the amplification.

Reagents	Volume (µl)
Taq assay buffer (contains $MgCl_2$)(10X)	7.5
dNTPs (10 mM)	2.5
Forward primer (10 µM)	5.0
Reverse primer (10 µM)	5.0
Taq DNA Polymerase (3U/ µl)	2.0
Template (DNA 50 ng)	10.0
Sterile distilled water	16.5
DMSO	1.5
Total	**50.0**

Amplification was carried out on Applied Biosystems PCR machine. The amplification profile was as follows.

1. Initial denaturation at 95°C temperature for 8 min.
2. Denaturation at 94°C for 3 min.
3. Primer annealing at around 55°C for 30 sec.
4. Primer extension at 72°C for 1 min.

   Later steps 2 to 4 were repeated 35 times
5. Complete primer extension at 72°C for 10 min.
6. Hold at 4 °C until removal

3.2.3.7 Resolving the amplified products by Agarose gel electrophoresis

1.2 per cent agarose gel was prepared using electrophoresis grade agarose (Sigma Aldrich) of the volume of 120 ml 1X TAE (Electrophoresis buffer). 5 µl of Ethidium bromide was added at a concentration of 0.5 µl per 10 ml (10 mg/ml). The gel was poured into gel casting tray fitted with comb and allowed to solidify fully before removing the comb from the gel. The gel tray was placed in the electrophoresis tank filled with 1X TAE buffer. 2 µl of loading dye was added to 10 µl of PCR products and mixed well before loading into the wells. A voltage of 80 volt was set for 90 min to separate the PCR products. After the gel run, the gel was taken from the buffer and examined under gel doc equipment and observed for the band. The gel was photographed directly from the gel doc.

3.2.3.8 Elution of bands from gel using commercial gel elution kit

The gel elution was performed using QIAquick® Gel extraction kit obtained from QIAGEN Pvt. Ltd.

Procedure:

- The PCR product band in the agarose gel was excised using a clean and sharp scalpel.
- The weight of the gel was measured using a colourless tube. Three volumes of buffer QG was added to one volume of gel weight (100 mg gel ~ 100 µl).
- The tube was incubated in dry bath at 50°C for 10 minutes until the gel is completely dissolved.

- The tube was vortexed after every 3 minutes to help in dissolving gel.
- After the appearance of yellow colour in tube, 1 gel volume of isopropanol was added and mixed well.
- QIAquick spin was placed in a 2 ml collection tube and the sample was pippetted into the spin tube.
- The tube was centrifuged at 13000 rpm for 1 min allow the passage of sample through the column. The flow-through obtained in collection tube was discarded and the column was placed in same tube.
- 500 µl of Buffer QG was added to the QIAquick column and centrifuged for 1 min. The flow-through was discarded and the column was placed in same tube.
- 750 µl of Buffer PE was added to the QIAquick column and centrifuged for 1 min at 13000 rpm.
- The tube was again centrifuged for 1 min to remove the residual wash buffer in the column. The QIAquick column was placed in a fresh 1.5 ml tube.
- For eluting the DNA, 50 µl of Buffer EB was added to the centre of the column and centrifuged for 1 min.

The eluted PCR products were sent for the Sequencing.

3.2.3.9 Sequencing of the PCR products

The gel eluted PCR samples/ PCR products were sent to Agrigenome Labs Pvt. Ltd., Kochi for sequencing. The samples loaded in properly labelled 0.5 ml tubes, along with the Forward and Reverse primers packed in a zip lock cover was sent for sequencing.

3.2.3.10 Analysis of sequence data

The forward and Reverse DNA sequence obtained from sequencing results were used to construct contigs using CAP3 software. The contigs were then aligned with the reference *BADH2* sequence retrieved from the Rice Genome Annotation Project using CLUSTAL OMEGA software to analyze the possible mutations in the exonic regions of genomic DNA.

3.2.3.11 Validation of specific primers of *BADH2* gene

The specific primers designed for *BADH2* gene was evaluated for region 1 covering the exons 1 and 2 in the three aromatic rice genotypes *Biriyanicheera*, Gandhakasala and Pusa basmati 1 along with the non-aromatic rice genotype Triveni. The genomic DNA was used for the amplification of PCR products.

3.2.4 Collection of meteorological observations of all the locations

The meteorological observations from the three districts Palakkad, Thrissur and Ernakulam were collected from Agromet observatory available at RARS Pattambi, Department of Agricultural Meteorology, College of Horticulture, Vellanikkara and Rice Research Station, Vytilla respectively.

Results

4. RESULTS

The results of the study on 'Molecular and biochemical characterisation of aroma in *Biriyanicheera* rice genotype' was undertaken during the period 2018-2019 at Centre of Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur, are described in this chapter.

4.1 Raising of rice plants

The rice grains of aromatic genotypes *Biriyanicheera* and Gandhakasala, obtained from three locations (Palakkad, Thrissur and Ernakulam districts) and non-aromatic genotype Triveni (from Thrissur), were sown in mud pots kept in the Poly house of CPBMB, College of Horticulture. The young leaves from these rice plants were used for DNA isolation during the study.

4.2 BIOCHEMICAL CHARACTERISATION

GC-MS analysis of volatile compounds present in the dehusked kernels of three rice genotypes, *viz.*, *Biriyanicheera* (BC), Gandhakasala (GS), and Triveni, was carried out at TNAU. The detected compounds were scored based on the probability of similarity of the individual compound with the standard chemical structures. The NIST Library Database was used for the comparison of compounds.

The list of all prominent compounds having higher probability is presented in Table1. A total of 65 compounds were found important in the volatile profile of rice grains of all genotypes together (Table 1). The contaminants and column material compounds were not taken into considerations. A very diverse composition of volatile data was obtained from rice grains.

The compound Ethyl acetate (fruity odour) was having maximum probability range of 83-97.1 in Non-aromatic genotype Triveni, *Biriyanicheera* from Palakkad and Gandhakasala from Ernakulam districts. An aromatic volatile compound Benzothiazole was detected in all the three genotypes from different locations. However, the lowest value was detected in Gandhakasala rice from Ernakulam district. The compounds such as 2-ethyl-1-Hexanol, Decanal, 4-Nitrobenzylamine, Dimethyl disulfide, 2-Undecanone, Dodecane, 4-Methyl-undecane, Tetradecane, and Benzene were detected

Table 1: Volatile compounds of *Biriyanicheera*, Gandhakasala and Triveni rice varieties from different locations (probability value)

Sl.No.	Compound	Nature	NA	*Biriyanicheera*			Gandhakasala		
				BCP	BCT	BCE	GSP	GST	GSE
1	3-Cyclopentene-1,2-diol, cis-			45.1					
2	Ethyl Acetate	fruity odor	97.1	83.0					96.1
3	Benzene		31.8	61.8	63.1		67.0		66.6
4	Styrene	Sweet odor		40.1					
5	Benzaldehyde, 2-hydroxy-	bitter almond odor		68.3	24.7				
6	Nonanal	floral, fruity		69.3			45.5		
7	Benzothiazole	slightly sweet	78.3	71.9	78.0	68.2	76.8	21.0	78.6
8	2-Undecanone	fruity, floral	61.7	89.1	74.9				86.8
9	Hexadecane	odorless		35.5					
10	Disulfide, dimethyl	garlic-like odor	79.9		75.0	49.0		75.7	82.1
11	Heptane, 2,4-dimethyl-				43.7				
12	(1S,2S,3R,5S)-(+)-Pinanediol				35.9				
13	Furan, 2-pentyl-	floral, fruit, nutty, bean			73.0				68.9
14	1-Hexanol, 2-ethyl-		52.3		64.7	42.1	45.8		31.8
15	Undecane, 4-methyl-		22.0		30.4	30.0			29.3
16	4-Nitrobenzylamine				89.3	93.2	86.6		
17	Dodecane	gasoline-like to odorless	35.1		26.8	25.7			25.6

18	Decanal	sweet, waxy, floral	43.9		45.0	42.8	53.5	27.6	45.7
19	Clobetasol propionate				37.8				
20	Rhodamine 6G cation				34.9				
21	Tetradecane		30.4		31.9		28.7		
22	Allomatrine					35.6			
23	1-Butanol, 2-methyl-, acetate					48.1			
24	Octane, 4-methyl-					61.9			35.3
25	p-Xylene	medicinal, solvent	38.8			30.0			32.4
26	5-Hexen-2-one, 5-methyl-3-methylene-					35.7			
27	Ethanone, 1-(1-cyclohexen-1-yl)-					24.5			
28	Lincomycin					71.3			
29	Azetidine, 1,1'-methylenebis[2-methyl-					33.5			
30	Decane					38.2			
31	Cyclohexane, butyl-					52.8			
32	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-					89.5			
33	Cyclohexane, hexyl-					32.6			
34	Furan, 2-methyl-						80.4	39.4	
35	Ethanol, 2-nitro-, propionate (ester)						52.4		

36	Propanoic acid						38.0		
37	1,3-Dioxolane, 2-(1-phenylethyl)-						49.9		
38	1,3-Dioxolane, 2-(phenylmethyl)-						31.2		
39	2-Heptanone	fruity, floral					62.2		
40	Heptanal	fatty, rancid , fruity					70.7		
41	N,N-Dimethylacetamide						46.3		
42	2 (R)-3-Hydroxybutyric acid						35.5		
43	Benzaldehyde	nutty, bitter					37.0		
44	ethanone, 2,2-dichloro-2-fluoro-1-phenyl-						28.4		
45	Dimethyl trisulfide						84.0		
46	Octanal	citrus, fruity, floral, fatty					73.1		
47	2-Ethyl-1-hexanol						28.6		
48	Mefluidide		43.0				59.8		
49	Acetophenone	sweet, floral					68.9	30.3	44.2
50	1,3-Diazacyclooctane-2-thione						53.3	40.8	
51	Tetrasulfide, dimethyl						58.2		
52	Pentasulfide, dimethyl						41.1		
53	Tetradecane						28.7		
54	2-Butanol, 1-benzyloxy-3-methyl-							31.3	

55	Hexanal	green, grass like						59.3	
56	5-Nitro-2-toluidine							38.4	
57	Arsenous acid, tris(trimethylsilyl) ester							60.3	
58	á-Myrcene							40.2	
59	Linalool							65.6	
60	3,5-Dibutoxy-1,1,1,7,7,7-hexamethyl-3,5-							48.3	
61	Dimethyl sulfide		44.1						47.3
62	Guanidine, methyl-		53.2						31.4
63	Borane-methyl sulfide complex		30.2						
64	4-Methoxy-4'-(methylthio)chalcone		55.2						
65	Diethylphenylamine		33.0						

NA – Triveni

BCP - *Biriyanicheera* (Palakkad)

BCT – *Biriyanicheera* (Thrissur)

BCE – *Biriyanicheera* (Ernakulam)

GSP – Gandhakasala (Palakkad)

GST – Gandhakasala (Thrissur)

GSE – Gandhakasala (Ernakulam)

in both aromatic and non-aromatic cultivars. *Biriyanicheera* rice genotype from different locations was found to possess 33 compounds in total, varying across locations as mentioned in Table 2. Among these, Benzothiazole was detected in *Biriyanicheera* from all locations, and the highest value was shown in Thrissur district. Two compounds, Benzene and 2-hydroxy Benzaldehyde, were present in *Biriyanicheera* from Palakkad and Thrissur whereas absent in grains from Ernakulam location.

Gandhakasala rice genotype was observed to contain 44 compounds from rice grown in three different locations (Table 3). The compounds Benzothiazole, Decanal and Acetophenone were found in all locations with lower quantity in Thrissur. Dimethyl disulfide was higher in both Thrissur and Ernakulam, where as it was absent in Gandhakasala from Palakkad district. The volatiles nonanal, 2-ethyl-1-Hexanol were present in Gandhakasala from Palakkad and Ernakulam regions but absent in Thrissur district.

The nature of volatile compounds present in the Non-aromatic genotype (Triveni) grown in Thrissur district are detailed in Table 1. A total of eighteen compounds were found to be present in Triveni genotype (Table 4). The GC-MS results identified several aromatic compounds such as Benzothiazole (78.3), 2-Undecanone (61.7), Dimethyl disulfide (79.9) to be present with higher probability values in Triveni cultivar.

The comparison of volatile compounds present in *Biriyanicheera*, and absent in Triveni are mentioned in Table 5. *Biriyanicheera* grown in Palakkad, Thrissur and Ernakulam contained five, seven and twelve compounds unique to respective locations. The compounds Nonanal and 2-hydroxy Benzaldehyde were found to be the major volatile compounds for the aroma in *Biriyanicheera* from Palakkad region. Similarly, 4 nitrobenzylamine and 2-pentyl furan in Thrissur location and 4 nitrobenzylamine, 2(3H)-Furanone, 5-ethenyldihydro-5-methyl-, and Lincomycin were identified as the major compounds responsible for their aroma strength.

Twenty one, ten and five volatiles which are uniquely present in Gandhakasala but absent in Triveni were identified (Table 6). 2-methyl furan, 4-nitrobenzylamine, Acetophenone, Heptanal, 2-Heptanone and Octanal were the major volatile principles

Table 2: Total volatile compounds present in *Biriyanicheera* from different locations (probability value)

Sl.No.	Compound	Nature	*Biriyanicheera*		
			Palakkad	Thrissur	Ernakulam
1	3-Cyclopentene-1,2-diol, cis-		45.1		
2	Ethyl Acetate	fruity odor	83.0		
3	Benzene		61.8	63.1	
4	Styrene	Sweet odor	40.1		
5	Benzaldehyde, 2-hydroxy-	bitter almond odor	68.3	24.7	
6	Nonanal	floral, fruity	69.3		
7	Benzothiazole	slightly sweet	71.9	78.0	68.2
8	2-Undecanone	fruity, floral	89.1	74.9	
9	Hexadecane	odorless	35.5		
10	Disulfide, dimethyl	garlic-like odor		75.0	49.0
11	Heptane, 2,4-dimethyl-			43.7	
12	(1S,2S,3R,5S)-(+)-Pinanediol			35.9	
13	Furan, 2-pentyl-	floral, fruit, nutty, bean		73.0	
14	1-Hexanol, 2-ethyl-			64.7	42.1
15	Undecane, 4-methyl-			30.4	30.0
16	4-Nitrobenzylamine			89.3	93.2

17	Dodecane	gasoline-like to odorless		26.8	25.7
18	Decanal	sweet, waxy, floral		45.0	42.8
19	Clobetasol propionate			37.8	
20	Rhodamine 6G cation			34.9	
21	Tetradecane			31.9	
22	Allomatrine				35.6
23	1-Butanol, 2-methyl-, acetate				48.1
24	Octane, 4-methyl-				61.9
25	p-Xylene	medicinal, solvent			30.0
26	5-Hexen-2-one, 5-methyl-3-methylene-				35.7
27	Ethanone, 1-(1-cyclohexen-1-yl)-				24.5
28	Lincomycin				71.3
29	Azetidine, 1,1'-methylenebis[2-methyl-				33.5
30	Decane				38.2
31	Cyclohexane, butyl-				52.8
32	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-				89.5
33	Cyclohexane, hexyl-				32.6

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Table 3: Total volatile compounds present in Gandhakasala from different locations (probability value)

Sl.No.	Compound	Nature	Gandhakasala		
			Palakkad	Thrissur	Ernakulam
1	Ethyl Acetate	fruity odor			96.1
2	Benzene		67.0		66.6
3	Nonanal	floral, fruity	45.5		
4	Benzothiazole	slightly sweet	76.8	21.0	78.6
5	2-Undecanone	fruity, floral			86.8
6	Disulfide, dimethyl	garlic-like odor		75.7	82.1
7	Furan, 2-pentyl-	floral, fruit, nutty, bean			68.9
8	1-Hexanol, 2-ethyl-		45.8		31.8
9	Undecane, 4-methyl-				29.3
10	4-Nitrobenzylamine		86.6		
11	Dodecane	gasoline-like to odorless			25.6
12	Decanal	sweet, waxy, floral	53.5	27.6	45.7
13	Tetradecane		28.7		
14	Octane, 4-methyl-				35.3
15	p-Xylene	medicinal, solvent			32.4
16	Furan, 2-methyl-		80.4	39.4	

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17	Ethanol, 2-nitro-, propionate (ester)		52.4		
18	Propanoic acid		38.0		
19	1,3-Dioxolane, 2-(1-phenylethyl)-		49.9		
20	1,3-Dioxolane, 2-(phenylmethyl)-		31.2		
21	2-Heptanone	fruity, floral	62.2		
22	Heptanal	fatty, rancid , fruity	70.7		
23	N,N-Dimethylacetamide		46.3		
24	2 (R)-3-Hydroxybutyric acid		35.5		
25	Benzaldehyde	nutty, bitter	37.0		
26	ethanone, 2,2-dichloro-2-fluoro-1-phenyl-		28.4		
27	Dimethyl trisulfide		84.0		
28	Octanal	citrus, fruity, floral, fatty	73.1		
29	2-Ethyl-1-hexanol		28.6		
30	Mefluidide		59.8		
31	Acetophenone	sweet, floral	68.9	30.3	44.2
32	1,3-Diazacyclooctane-2-thione		53.3	40.8	
33	Tetrasulfide, dimethyl		58.2		
34	Pentasulfide, dimethyl		41.1		
35	Tetradecane		28.7		
36	2-Butanol, 1-benzyloxy-3-methyl-			31.3	
37	Hexanal	green, grass like		59.3	

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38	5-Nitro-2-toluidine			38.4	
39	Arsenous acid, tris(trimethylsilyl) ester			60.3	
40	á-Myrcene			40.2	
41	Linalool			65.6	
42	3,5-Dibutoxy-1,1,1,7,7,7-hexamethyl-3,5-			48.3	
43	Dimethyl sulfide				47.3
44	Guanidine, methyl-				31.4

Table 4. Volatile compounds present in Triveni (probability value)

Sl.No.	Compound	Nature	Triveni
1	Ethyl Acetate	fruity odor	97.1
2	Trichloromethane	Solvent	65.6
3	Benzene		31.8
4	Benzothiazole	slightly sweet	78.3
5	2-Undecanone	fruity, floral	61.7
6	Disulfide, dimethyl	garlic-like odor	79.9
7	1-Hexanol, 2-ethyl-		52.3
8	Undecane, 4-methyl-		22
9	Dodecane	gasoline-like to odorless	35.1
10	Decanal	sweet, waxy, floral	43.9
11	Tetradecane		30.4
12	p-Xylene	medicinal, solvent	38.8
13	Mefluidide		43
14	Dimethyl sulfide		44.1
15	Guanidine, methyl-		53.2
16	Borane-methyl sulfide complex		30.2
17	4-Methoxy-4'-(methylthio)chalcone		55.2
18	Diethylphenylamine		33

Table 5: Volatiles in *Biriyanicheera* from different locations (absent in non-aromatic rice)

	Palakkad		Thrissur		Ernakulam	
	Compounds	**prob.**	**Compounds**	**prob.**	**Compounds**	**prob.**
1	3-Cyclopentene-1,2-diol, cis-	45.1	Benzaldehyde, 2-hydroxy-	24.7	4-Nitrobenzylamine	93.2
2	Styrene	40.1	Heptane, 2,4-dimethyl-	43.7	Allomatrine	35.6
3	Benzaldehyde, 2-hydroxy-	68.3	(1S,2S,3R,5S)-(+)-Pinanediol	35.9	1-Butanol, 2-methyl-, acetate	48.1
4	Nonanal	69.3	Furan, 2-pentyl-	73.0	Octane, 4-methyl-	61.9
5	Hexadecane	35.5	4-Nitrobenzylamine	89.3	5-Hexen-2-one, 5-methyl-3-methylene-	35.7
6			Clobetasol propionate	37.8	Ethanone, 1-(1-cyclohexen-1-yl)-	24.5
7			Rhodamine 6G cation	34.9	Lincomycin	71.3
8					Azetidine, 1,1'-methylenebis[2-methyl-	33.5
9					Decane	38.2
10					Cyclohexane, butyl-	52.8
11					2(3H)-Furanone, 5-ethenyldihydro-5-methyl-	89.5
12					Cyclohexane, hexyl-	32.6

Table 6: Volatiles in Gandhakasala from different locations (which are absent in non-aromatic rice)

Palakkad			Thrissur		Ernakulam	
Sl.no.	**Compounds**	**prob.**	**Compounds**	**prob.**	**Compounds**	**prob.**
1	Nonanal	45.5	Furan, 2-methyl-	39.4	Furan, 2-pentyl-	68.9
2	4-Nitrobenzylamine	86.6	Acetophenone	30.3	Octane, 4-methyl-	35.3
3	Furan, 2-methyl-	80.4	1,3-Diazacyclooctane-2-thione	40.8	Acetophenone	44.2
4	Ethanol, 2-nitro-, propionate (ester)	52.4	2-Butanol, 1-benzyloxy-3-methyl-	31.3	Dimethyl sulfide	47.3
5	Propanoic acid	38.0	Hexanal	59.3	Guanidine, methyl-	31.4
6	1,3-Dioxolane, 2-(1-phenylethyl)-	49.9	5-Nitro-2-toluidine	38.4		
7	1,3-Dioxolane, 2-(phenylmethyl)-	31.2	Arsenous acid, tris(trimethylsilyl) ester	60.3		
8	2-Heptanone	62.2	á-Myrcene	40.2		
9	Heptanal	70.7	Linalool	65.6		
10	N,N-Dimethylacetamide	46.3	3,5-Dibutoxy-1,1,1,7,7,7-hexamethyl-3,5-	48.3		
11	2 (R)-3-Hydroxybutyric acid	35.5				
12	Benzaldehyde	37.0				

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13	ethanone, 2,2-dichloro-2-fluoro-1-phenyl-	28.4				
14	Dimethyl trisulfide	84.0				
15	Octanal	73.1				
16	2-Ethyl-1-hexanol	28.6				
17	Acetophenone	68.9				
18	1,3-Diazacyclooctane-2-thione	53.3				
19	Tetrasulfide, dimethyl	58.2				
20	Pentasulfide, dimethyl	41.1				
21	Tetradecane	28.7				

Table 7: Unique compounds present in aromatic rice varieties (absent in Triveni)

Biriyanicheera			**Gandhakasala**		
Reference	**Compounds**	**Odour**	**Reference**	**Compounds**	**Odour**
	3-Cyclopentene-1,2-diol, cis-		Choi *et al.*, 2018	Nonanal	
Choi *et al.*, 2018	Styrene	sweet, balsam, floral, plastic		4-Nitrobenzylamine	
	Benzaldehyde, 2-hydroxy-		Xia *et al.*, 2018	Furan, 2-methyl-	
Choi *et al.*, 2018	Nonanal	aldehydic, waxy, citrus, tart, sweet		Ethanol, 2-nitro-, propionate (ester)	
Choi *et al.*, 2018	Hexadecane		Sanseya *et al.*, 2018	Propanoic acid	

	Heptane, 2,4-dimethyl-			1,3-Dioxolane, 2-(1-phenylethyl)-	
	(1S,2S,3R,5S)-(+)-Pinanediol			1,3-Dioxolane, 2-(phenylmethyl)-	
Choi *et al.*, 2018	Furan, 2-pentyl-	floral, fruit	Choi *et al.*, 2018	2-Heptanone	
	4-Nitrobenzylamine		Hinge *et al.*, 2016	Heptanal	grass,fresh
	Clobetasol propionate			N,N-Dimethylacetamide	
	Rhodamine 6G cation			2 (R)-3-Hydroxybutyric acid	
	Allomatrine		Choi *et al.*, 2018	Benzaldehyde	almond
	1-Butanol, 2-methyl-, acetate			ethanone, 2,2-dichloro-2-fluoro-1-phenyl-	
	Octane, 4-methyl-		Chen *et al.*, 2018	Dimethyl trisulfide	
	5-Hexen-2-one, 5-methyl-3-methylene-		Choi *et al.*, 2018	Octanal	
	Ethanone, 1-(1-cyclohexen-1-yl)-		Sanseya 2018	2-Ethyl-1-hexanol	
	Lincomycin		Sanseya 2018	Acetophenone	
	Azetidine, 1,1'-methylenebis[2-methyl-			1,3-Diazacyclooctane-2-thione	
Choi *et al.*, 2018	Decane			Tetrasulfide, dimethyl	
	Cyclohexane, butyl-			Pentasulfide, dimethyl	
	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-		Choi *et al.*, 2018	Tetradecane	
	Cyclohexane, hexyl-			1,3-Diazacyclooctane-2-thione	
				2-Butanol, 1-benzyloxy-3-methyl-	
			Choi *et al.*, 2018	Hexanal	

			Choi *et al.*, 2018	5-Nitro-2-toluidine	green tomato, greeen, grassy
				Arsenous acid, tris(trimethylsilyl) ester	
				á-Myrcene	
			Hinge *et al.*, 2016	Linalool	
				3,5-Dibutoxy-1,1,1,7,7,7-hexamethyl-3,5-	sweet floral
			Choi *et al.*, 2018	Furan, 2-pentyl-	
				Octane, 4-methyl-	floral, fruit
				Dimethyl sulfide	
				Guanidine, methyl-	

Table 8a: Total no. of compounds obtained from different varieties

Varieties	Triveni	*Biriyanicheera*			Gandhakasala		
		Palakkad	Thrissur	Ernakulam	Palakkad	Thrissur	Ernakulam
No. of compounds	18	9	16	19	27	13	15

Table 8b: Total no. of aromatic compounds obtained from different varieties

Varieties	Triveni	*Biriyanicheera*			Gandhakasala		
		Palakkad	Thrissur	Palakkad	Thrissur	Palakkad	Thrissur
No. of compounds	10	7	11	8	16	6	11

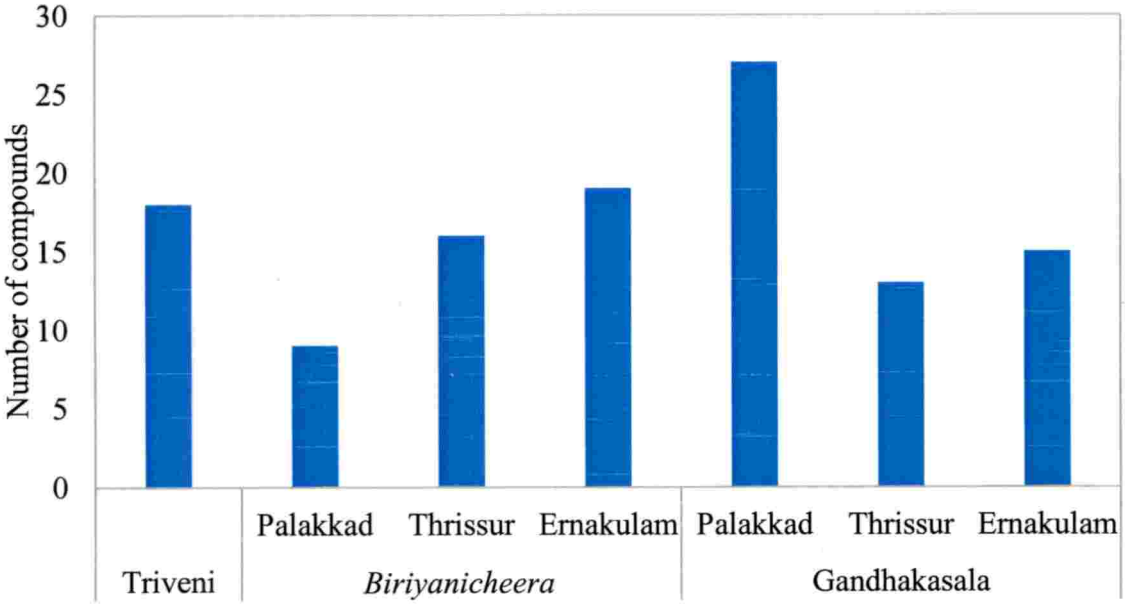


Fig. 1A Number of compounds present in Triveni, *Biriyanicheera* and Gandhakasala from different locations

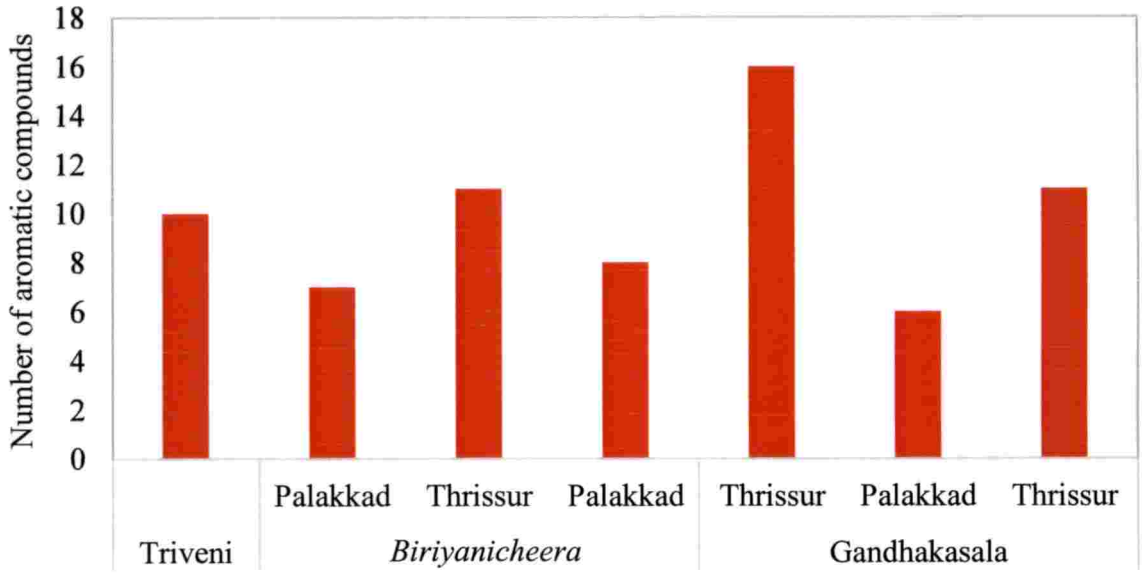


Fig. 1B Number of aromatic compounds present in Triveni, *Biriyanicheera* and Gandhakasala from different locations

Table 9: Comparison of all the volatile compounds present in rice varieties

Sl.No.	Compound	Nature	NA	BC			GS		
				BCP	BCT	BCE	GSP	GST	GSE
1	3-Cyclopentene-1,2-diol, cis-		–	✓	–	–	–	–	–
2	Ethyl Acetate	fruity odor	✓	✓	–	–	–	–	✓
3	Benzene		✓	✓	✓	–	✓	–	✓
4	Styrene	Sweet odor	–	✓	–	–	–	–	–
5	Benzaldehyde, 2-hydroxy-	bitter almond odor	–	✓	✓	–	–	–	–
6	Nonanal	floral, fruity	–	✓	–	–	✓	–	–
7	Benzothiazole	slightly sweet	✓	✓	✓	✓	✓	✓	✓
8	2-Undecanone	fruity, floral	✓	✓	✓	–	–	–	✓
9	Hexadecane	odorless	–	✓	–	–	–	–	–
10	Disulfide, dimethyl	garlic-like odor	✓	–	✓	✓		✓	✓
11	Heptane, 2,4-dimethyl-		–	–	✓	–	–	–	–
12	(1S,2S,3R,5S)-(+)-Pinanediol		–	–	✓	–	–	–	–
13	Furan, 2-pentyl-	floral, fruit, nutty, bean	–	–	✓	–	–	–	✓
14	1-Hexanol, 2-ethyl-		✓	–	✓	✓	✓	–	✓

15	Undecane, 4-methyl-		✓	–	✓	✓	–	–	✓
16	4-Nitrobenzylamine		–	–	✓	✓	✓	–	–
17	Dodecane	gasoline-like to odorless	✓	–	✓	✓	–	–	✓
18	Decanal	sweet, waxy, floral	✓	–	✓	✓	✓	✓	✓
19	Clobetasol propionate		–	–	✓	–	–	–	–
20	Rhodamine 6G cation		–	–	✓	–	–	–	–
21	Tetradecane		✓	–	✓	–	✓	–	–
22	Allomatrine		–	–	–	✓	–	–	–
23	1-Butanol, 2-methyl-, acetate		–	–	–	✓	–	–	–
24	Octane, 4-methyl-		–	–	–	✓	–	–	✓
25	p-Xylene	medicinal, solvent	✓	–	–	✓	–	–	✓
26	5-Hexen-2-one, 5-methyl-3-methylene-		–	–	–	✓	–	–	–
27	Ethanone, 1-(1-cyclohexen-1-yl)-		–	–	–	✓	–	–	–
28	Lincomycin		–	–	–	✓	–	–	–
29	Azetidine, 1,1'-methylenebis[2-methyl-		–	–	–	✓	–	–	–
30	Decane		–	–	–	✓	–	–	–

31	Cyclohexane, butyl-		–	–	–	✓	–	–	–
32	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-		–	–	–	✓	–	–	–
33	Cyclohexane, hexyl-		–	–	–	✓	–	–	–
34	Furan, 2-methyl-		–	–	–	–	✓	✓	–
35	Ethanol, 2-nitro-, propionate (ester)		–	–	–	–	✓	–	–
36	Propanoic acid		–	–	–	–	✓	–	–
37	1,3-Dioxolane, 2-(1-phenylethyl)-		–	–	–	–	✓	–	–
38	1,3-Dioxolane, 2-(phenylmethyl)-		–	–	–	–	✓	–	–
39	2-Heptanone	fruity, floral	–	–	–	–	✓	–	–
40	Heptanal	fatty, rancid , fruity	–	–	–	–	✓	–	–
41	N,N-Dimethylacetamide		–	–	–	–	✓	–	–
42	2 (R)-3-Hydroxybutyric acid		–	–	–	–	✓	–	–
43	Benzaldehyde	nutty, bitter	–	–	–	–	✓	–	–
44	ethanone, 2,2-dichloro-2-fluoro-1-phenyl-		–	–	–	–	✓	–	–
45	Dimethyl trisulfide		–	–	–	–	✓	–	–
46	Octanal	citrus, fruity, floral, fatty	–	–	–	–	✓	–	–

47	2-Ethyl-1-hexanol		–	–	–	–	✓	–	–
48	Mefluidide		✓	–	–	–	✓	–	–
49	Acetophenone	sweet, floral	–	–	–	–	✓	✓	✓
50	1,3-Diazacyclooctane-2-thione		–	–	–	–	✓	✓	–
51	Tetrasulfide, dimethyl		–	–	–	–	✓	–	–
52	Pentasulfide, dimethyl		–	–	–	–	✓	–	–
53	Tetradecane		–	–	–	–	✓	–	–
54	2-Butanol, 1-benzyloxy-3-methyl-		–	–	–	–	–	✓	–
55	Hexanal	green, grass like	–	–	–	–	–	✓	–
56	5-Nitro-2-toluidine		–	–	–	–	–	✓	–
57	Arsenous acid, tris(trimethylsilyl) ester		–	–	–	–	–	✓	–
58	á-Myrcene		–	–	–	–	–	✓	–
59	Linalool		–	–	–	–	–	✓	–
60	3,5-Dibutoxy-1,1,1,7,7,7-hexamethyl-3,5-		–	–	–	–	–	✓	–
61	Dimethyl sulfide		✓	–	–	–	–	–	✓
62	Guanidine, methyl-		✓	–	–	–	–	–	✓

63	Borane-methyl sulfide complex		✓	–	–	–	–	–	–
64	4-Methoxy-4'-(methylthio)chalcone		✓	–	–	–	–	–	–
65	Diethylphenylamine		✓	–	–	–	–	–	–

NA – Triveni

BCP - Biriyanicheera (Palakkad)

BCT – Biriyanicheera (Thrissur)

BCE – Biriyanicheera (Ernakulam)

GSP – Gandhakasala (Palakkad)

GST – Gandhakasala (Thrissur)

GSE – Gandhakasala (Ernakulam)

responsible for aroma in Gandhakasala grown in Palakkad. Hexanal, Linalool in Thrissur and 2-pentyl furan, Dimethyl sulphide from Ernakulam were involved in imparting aroma in Gandhakasala.

The unique compounds identified both in *Biriyanicheera* and Gandhakasala are listed in Table 7. A total of 22 compounds were identified to be unique in *Biriyanicheera* genotype across all locations (Table 7). The compounds such as Styrene, Nonanal, Hexadecane, 2-pentyl furan, and decane detected in *Biriyanicheera* were previously reported in other rice genotypes. Nine, sixteen, and nineteen volatile compounds were obtained from *Biriyanicheera* grown in Palakkad, Thrissur and Ernakulam respectively (Table 8a and 8b) and is graphically represented (Fig. 1A and 1B). The compounds 2-Undecanone (89.1) in BC from Palakkad, 4-Nitrobenzylamine (89.3) from Thrissur and Palakkad were found to be in higher probability range. The compounds Dimethyl Disulfide, 2-ethyl-1-Hexanol, Decanal, 4-nitrobenzylamine, 2-Undecanone, Dodecane, 4-Methyl-Undecane were present in BC grown in both Thrissur and Ernakulam districts whereas absent in Palakkad district. The comparison of the compounds present in all genotypes is mentioned in Table 9.

4.2.2 Cooking quality parameters

4.2.2.1 Gelatinisation temperature analysis by Alkali spreading test

Rice grains of genotypes *Biriyanicheera*, Gandhakasala, and Triveni from different locations were subjected to the alkali spreading test. The visual observation was taken to evaluate the degree of disintegration in alkali. After the prescribed incubation, very little changes were observed in the grain expansion. The gelatinisation temperature index of all the genotypes is displayed in plate 3. All the rice genotypes were found to be of medium gelatinisation index.

4.2.2.2 Amylose content

The amylose content of all rice genotypes were determined and presented in and Table 10 and Plate 4. The highest amylose content was observed in *Biriyanicheera* genotype (24.62 per cent) whereas the lowest amylose per cent (13.85) was observed in Gandhakasala.

Table 10. Amylose content in rice grains

Variety	Amylose content (%)
Biriyanicheera	24.62
Gandhakasala	13.85
Triveni	23.85

Table 11. Gel consistency analysis of rice grains

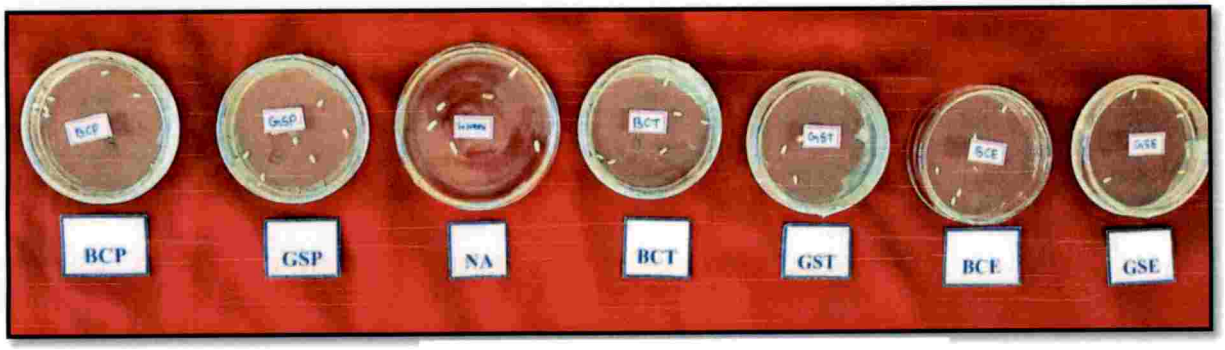
Variety	Gel length (in cm)
Biriyanicheera	10.5
Gandhakasala	10.73
Triveni	9.5

Table 12. DUS test for analysis of aroma in rice varieties

Scores/ Samples	No aroma (1)	Slight aroma (3)	Medium aroma (5)	Good aroma (7)	Strong aroma (9)
Triveni	8	2			
***Biriyanicheera* (Palakkad)**			2	4	4
Gandhakasala (Palakkad)			4	6	
***Biriyanicheera* (Thrissur)**		1	6	3	
Gandhakasala (Thrissur)		5	3	2	
***Biriyanicheera* (Ernakulam)**		7	3		
Gandhakasala (Ernakulam)		7	2	1	

(No. of candidates per individual score class)

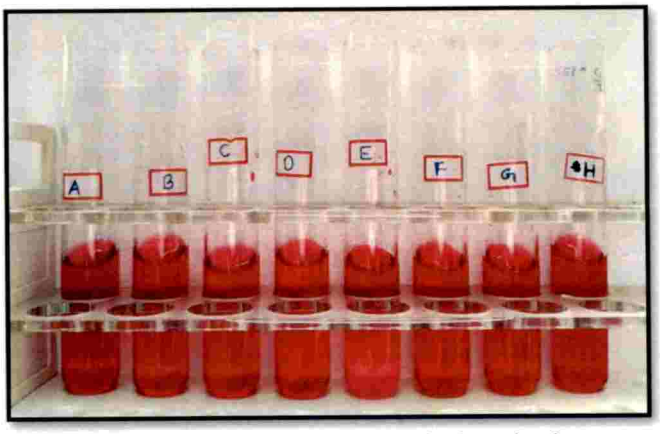
Before 23 hours of incubation



After 23 hours of incubation

Plate 3. Gelatinisation temperature analysis by Alkali spreading test

Test tubes after the addition of phenolphthalein



Test tubes kept for overnight incubation

Plate 4. Determination of amylose content in rice grains

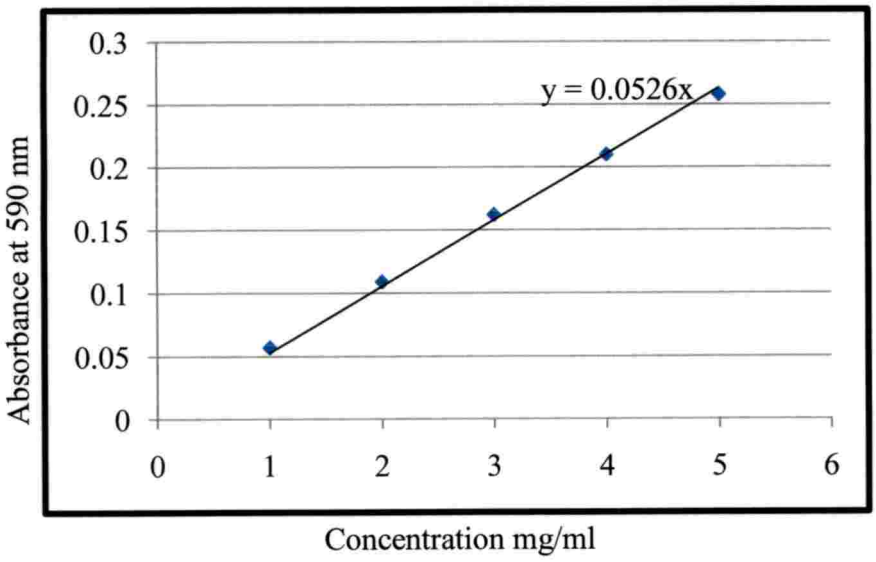
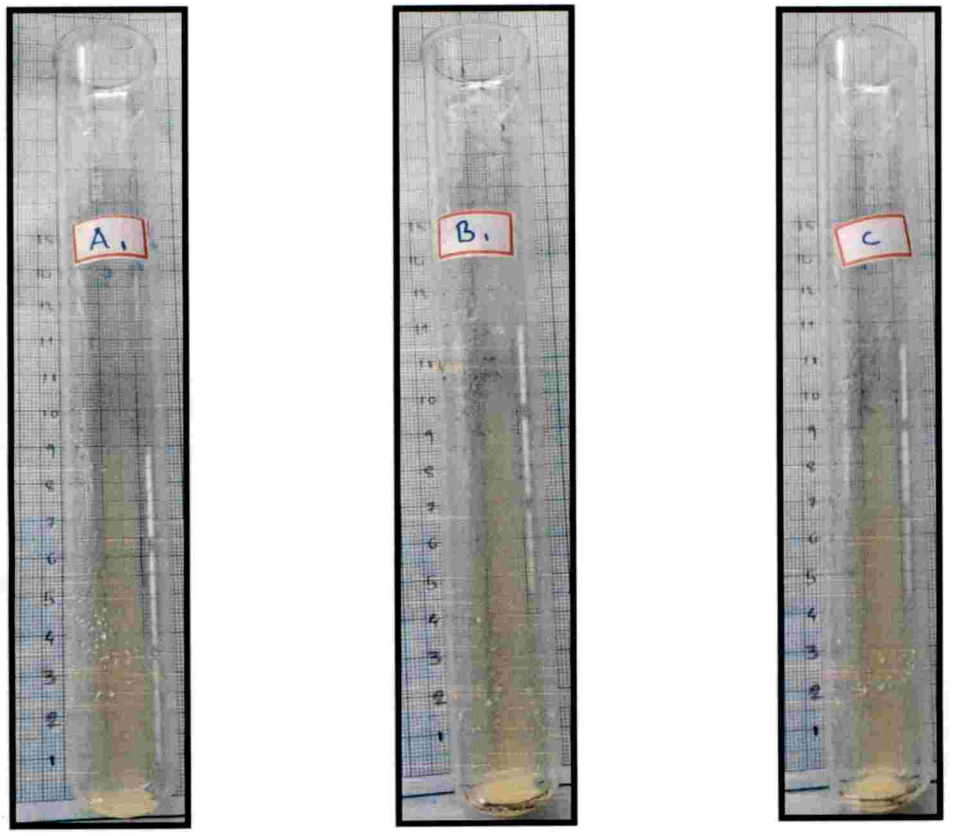


Fig 2. Standard curve for Amylose content in rice grains



A1- Triveni **B1- *Biriyanicheera*** **C- Gandhakasala**

Plate 5. Gel consistency analysis of rice grains

A-*Biriyanicheera* (Pattambi)
B-Gandhakasala (Pattambi)
C-*Biriyanicheera* (Thrissur)
D- Triveni
E- Gandhakasala (Thrissur)
F-*Biriyanicheera* (Ernakulam)
G- Gandhakasala (Ernakulam)

Plate 6. Sensory evaluation of rice grains by DUS test

4.2.2.3 Gel consistency

Gel consistency of rice genotypes was analysed by measuring the gel length obtained from powdered rice grains. The details of the results are mentioned in plate 5 and Table 11. The higher gel length was found in *Biriyanicheera* (10.5 cm) and lower gel length was found in Triveni genotype (9.5 cm).

4.2.3 Sensory evaluation

Aroma detection of rice grains was performed by Sensory evaluation test by DUS (Distinctness, Uniformity, Stability) protocol prescribed by Rani *et. al.* (2006). The results of the analysis are mentioned in Table 12 and Plate 6. The scores given by ten candidates showed that aroma in *Biriyanicheera* was the highest in Palakkad district and the lowest in Ernakulam district. Similarly, the Gandhakasala grown in Palakkad region showed higher aroma than the other two locations, whereas the Triveni was rated as non-aromatic. Inference from the aroma test is mentioned below:

Genotype	Nature
Triveni	No aroma
Biriyanicheera (Palakkad)	Good to strong aroma
Biriyanicheera (Thrissur)	Medium to good aroma
Biriyanicheera (Ernakulam)	Slight to medium aroma
Gandhakasala (Palakkad)	Medium to good aroma
Gandhakasala (Thrissur)	Slight to medium aroma
Gandhakasala (Ernakulam)	Slight to medium aroma

4.3 MOLECULAR CHARACTERISATION

4.3.1 DNA isolation

Fresh and young leaves of *Biriyanicheera*, Gandhakasala, and Triveni were collected during early morning for DNA isolation. The genomic DNA was isolated through CTAB method (Dellaporta *et al.*, 1983) with slight modifications. RNase treatment was done during isolation procedure to eliminate all RNA contamination.

4.3.2 Quality of DNA by agarose gel electrophoresis

The quality of DNA was assessed using agarose gel electrophoresis. After gel documentation, a clear and single intact prominent band of intensity was obtained indicating the non-degraded good quality of isolated DNA. The DNA was free from RNA and protein contamination (Plate 7).

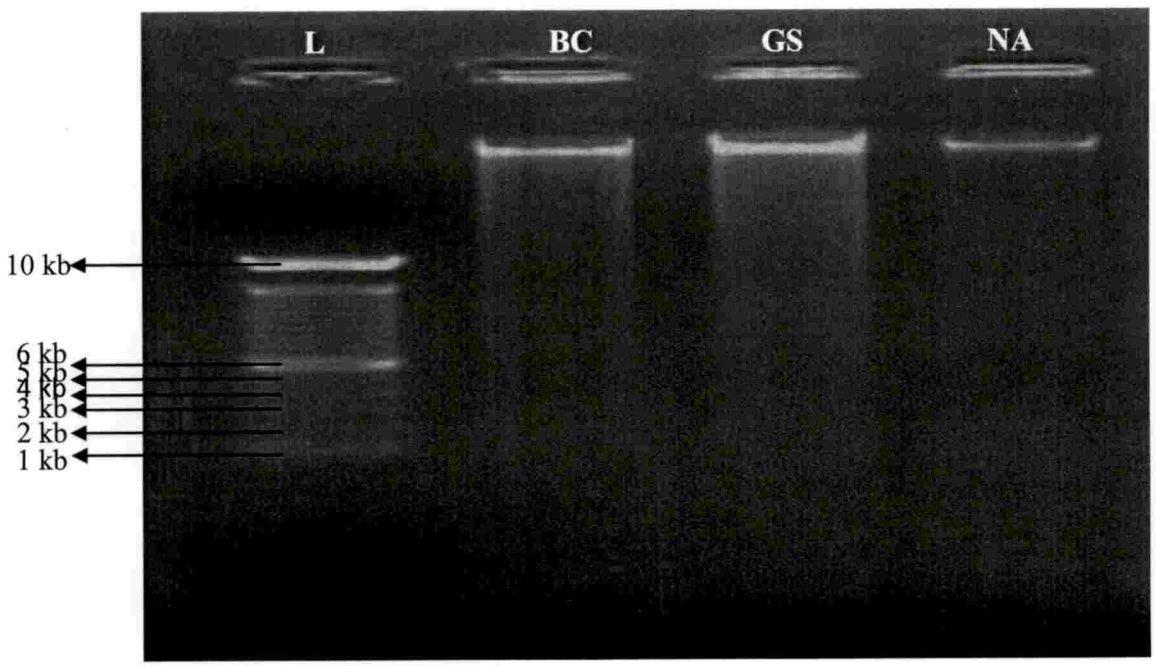
4.3.3 Quantity of DNA using NanoDrop method

The total genomic DNA isolated was analysed by spectrometer using the instrument NanoDrop® ND-1000. The result of the analysis is presented below.

Genotype	A260/A280	Conc. (ng/µl)	Quality
Biriyanicheera	1.93	760.00	Good
Gandhakasala	1.86	518.07	Good
Triveni	1.98	246.80	Good

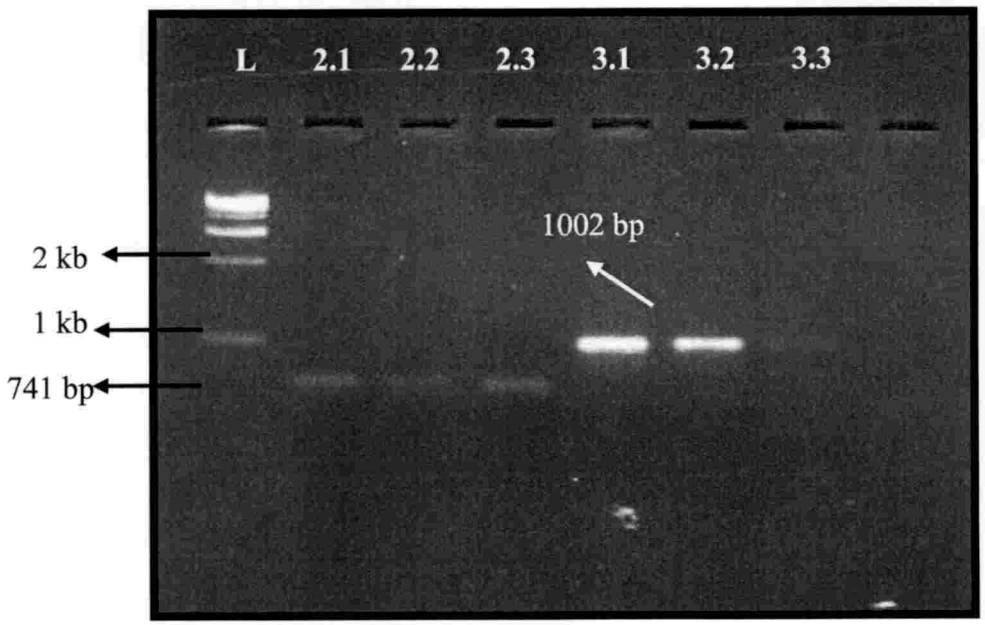
4.3.4 Polymerase Chain Reaction (PCR)

PCR reaction was performed using the seven different primers amplifying the regions in genomic DNA containing the exonic sequences. PCR standardization was done at varying temperatures to determine the proper annealing temperatures for amplifying the DNA. The annealing temperatures for standardization were in the range



L-1 kb Ladder BC- *Biriyanicheera* GS- Gandhakasala, NA- Triveni

Plate 7. Quality analysis of the isolated total genomic DNA

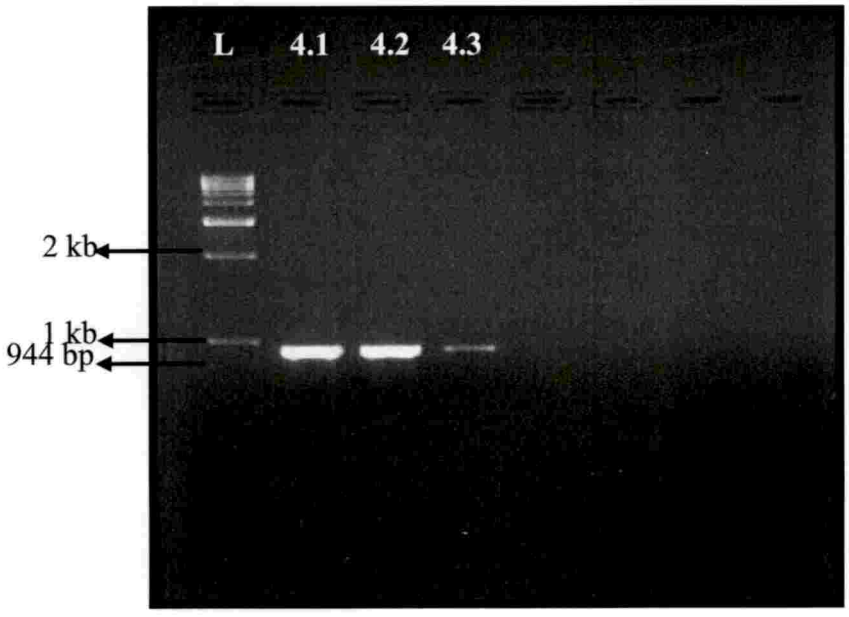


2.1 - *Biriyanicheera* (primer 2) 3.1 – *Biriyanicheera* (primer 3)

2.2 - Gandhakasaala (primer 2) 3.2 – Gandhakasaala (primer 3)

2.3 - Non-aromatic (primer 2) 3.3 - Non-aromatic (primer 3)

Plate 8 (A). *BADH2* gene specific PCR bands



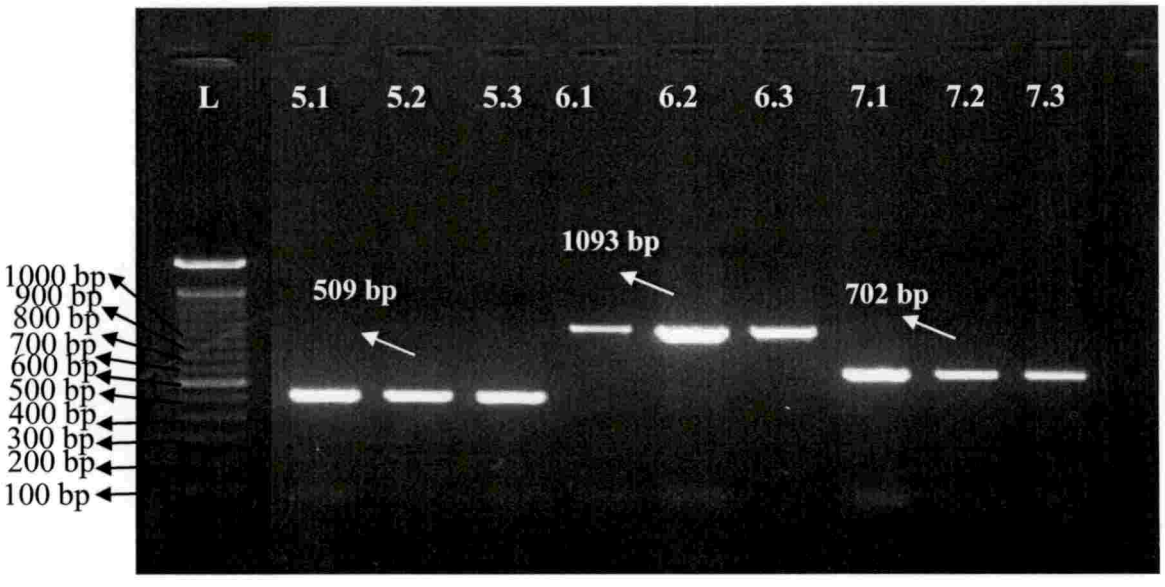
L- 1 kb ladder

4.1 - *Biriyanicheera* (primer 4)

4.2 – Gandhakasaala (primer 4)

4.3 - Non-aromatic (primer 4)

Plate 8 (B). *BADH2* gene specific PCR bands



L- 100bp ladder

5.1 - *Biriyanicheera* (primer 5)

5.2 – Gandhakasaala (primer 5)

5.3 - Non-aromatic (primer 5)

6.1 – *Biriyanicheera* (primer 6)

6.2 – Gandhakasaala (primer 6)

6.3 - Non-aromatic (primer 6)

7.1 – *Biriyanicheera* (primer 7)

7.2- Gandhakasaala (primer 7)

7.3- Non-aromatic (primer 7)

Plate 8 (C). *BADH2* gene specific PCR bands

of 55 to 63°C. PCR products obtained were run in 1.2 per cent gel prepared using 1X TAE buffer.

The suitable annealing temperatures determined for PCR amplification are mentioned below.

Primers	**Annealing temperature**
Primer set 1	no amplification
Primer set 2	60°C
Primer set 3	58°C
Primer set 4	55°C
Primer set 5	57°C
Primer set 6	59°C
Primer set 7	59°C

PCR amplification was performed in all the genotypes by using annealing temperatures mentioned above. The PCR products were subjected for separation using 1.2 per cent agarose gel, the required specified bands were obtained as mentioned in plate 8(A), 8(B), 8(C).

There was no amplification for primer 1 designed for amplifying exons 1 and 2. The amplification of the above region was attempted in different annealing temperatures of range 55°C to 65°C (Plate 9(A)).

Since there was no amplification of DNA with the specified primer, a new set of primer was designed to amplify the genomic DNA (exons 1 and 2). The new set of primer is mentioned below.

Forward primer : 5' ACAGAACAGAGCACTCCCTCTC 3'

Reverse primer : 5' CCAGATACACAATCAGCCATGC 3'

The PCR reaction was performed using the new set of primers and it showed negative results. The product amplification was attempted by making several changes such as, altered primers concentration, change of PCR Buffer *etc*. PCR reaction of Triveni genotype produced a non-specific amplification (Plate 9(B) and 9(C)), whereas the genotypes *Biriyanicheera* and Gandhakasala showed absence of bands in Gel separation of PCR products (Plate 9(D)).

The reason for non-amplification was assumed to be due to the presence of GC rich regions in the genomic DNA sequence lying between exons 1 and 2.

Modified PCR protocol

In order to amplify the GC region of Genomic region carrying exons 1 and 2, a modified PCR reaction was performed by using Dimethyl Sulphoxide (DMSO). The amplification was done using 3 per cent of DMSO by keeping annealing temperature at 55°C.

The use of DMSO in PCR was found to be useful in amplifying Genomic DNA in genotype Triveni (non-aromatic) (Plate 10(A)). But there was no amplification in both *Biriyanicheera* and Gandhakasala (Plate 10(B)).

PCR reaction using Phusion enzyme (High fidelity *Taq* Polymerase)

High fidelity *Taq* polymerase enzyme from Phusion, capable of amplifying GC rich genomic regions, was used to amplify the exons 1 and 2.

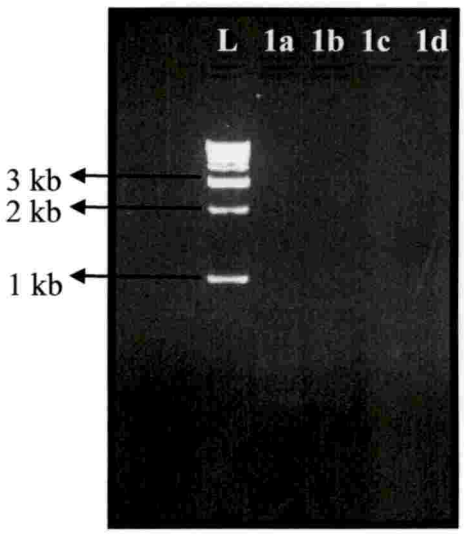
The gel separation results showed the presence of band of desired size in Triveni genotype, whereas non-specific amplification in both *Biriyanicheera* and Gandhakasala genotypes (Plate 11(A)). The secondary amplification of above PCR products produced non-specific amplification (Plate 11(B)).

4.3.5 Amplification of PCR products and Gel Elution

The regions in genomic DNA of *BADH2* gene covering exonic sequence were amplified using PCR machine ProFlex by Life Technologies. The gel elution was performed using QIAquick® Gel extraction kit obtained from QIAGEN Pvt. Ltd. Since the eluted PCR products were found to be low in concentration, sequencing of the

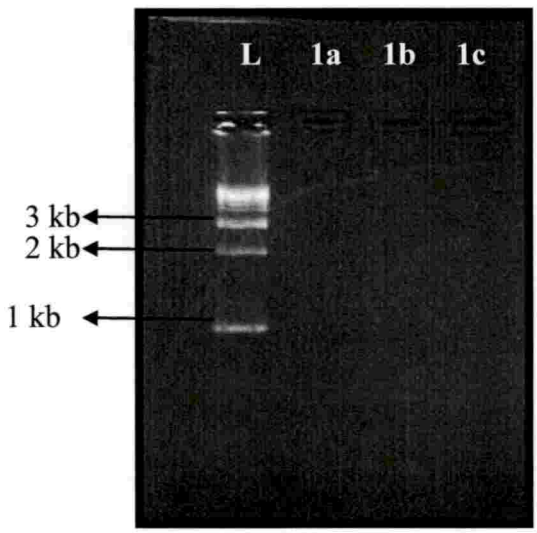
Primer 1

P1- 55°C



Primer 1

1a- 56°C 1b- 57°C 1c-58°C 1d-59°C



Primer 1

1a- 60°C 1b- 61°C 1c-62°C

Plate 9(A). Agarose gel showing no amplification by primer 1 at different annealing temperature in PCR

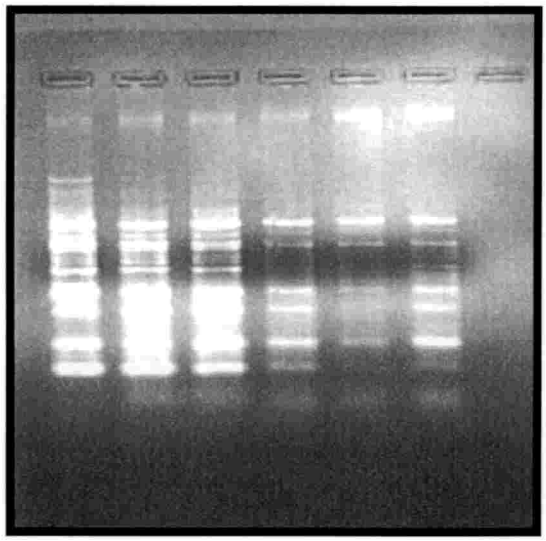


Plate 9 (B). Gradient PCR showing non-specific amplification using Primer 1 (new)

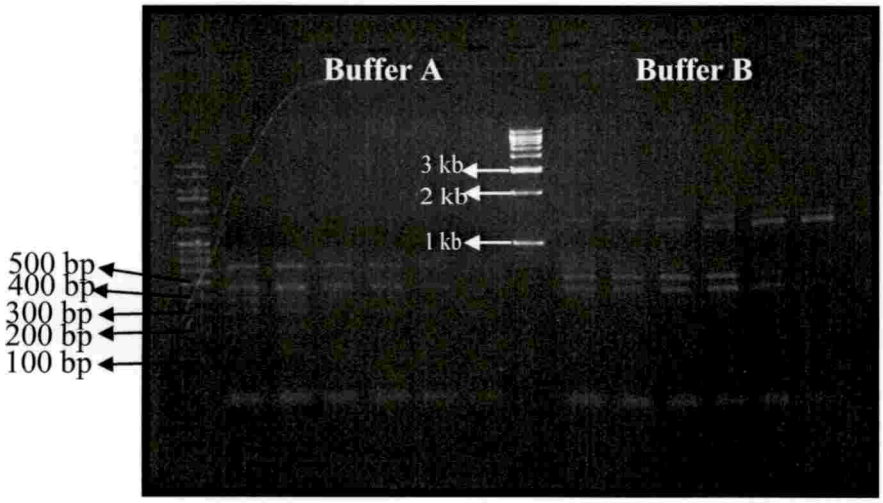


Plate 9 (C). Gradient PCR for Primer 1 (new) using Buffer A and Buffer B in PCR showing non-specific amplification

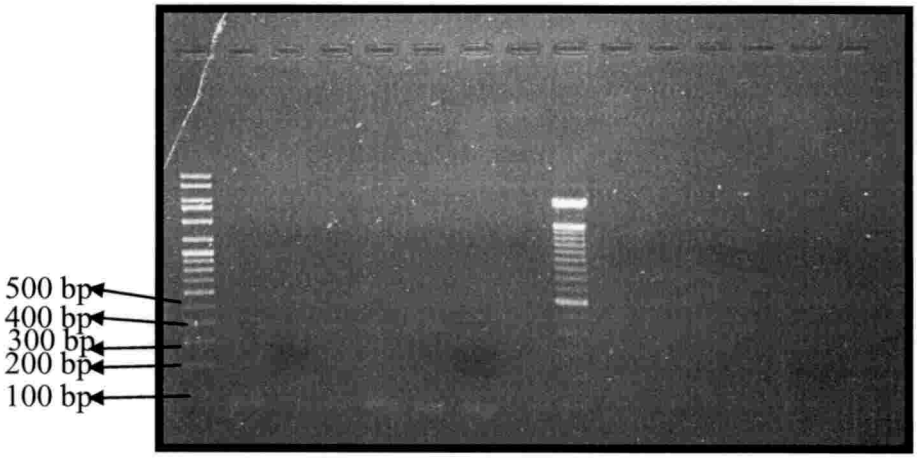


Plate 9 (D). Gradient PCR for Primer 1 (new) showing no amplification in *Biriyanicheera* and Gandhakasala

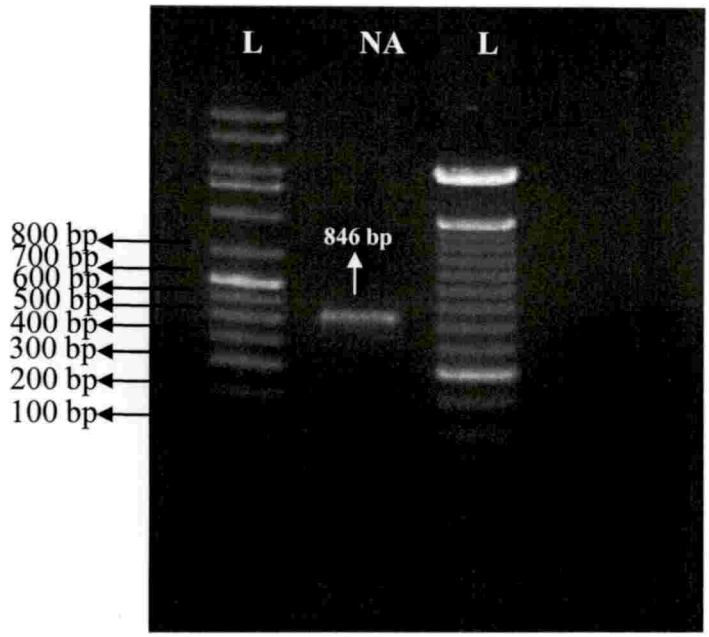


Plate 10 (A). Modified PCR (DMSO) for Triveni rice variety

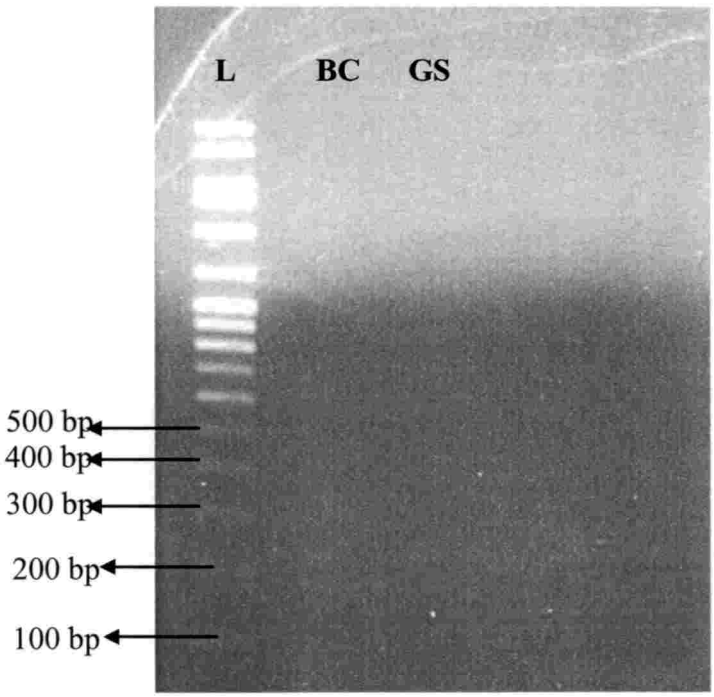


Plate 10 (B). Modified PCR (DMSO) for *Biriyanicheera* and Gandhakasala

BC- *Biriyanicheera* and GS- Gandhakasala

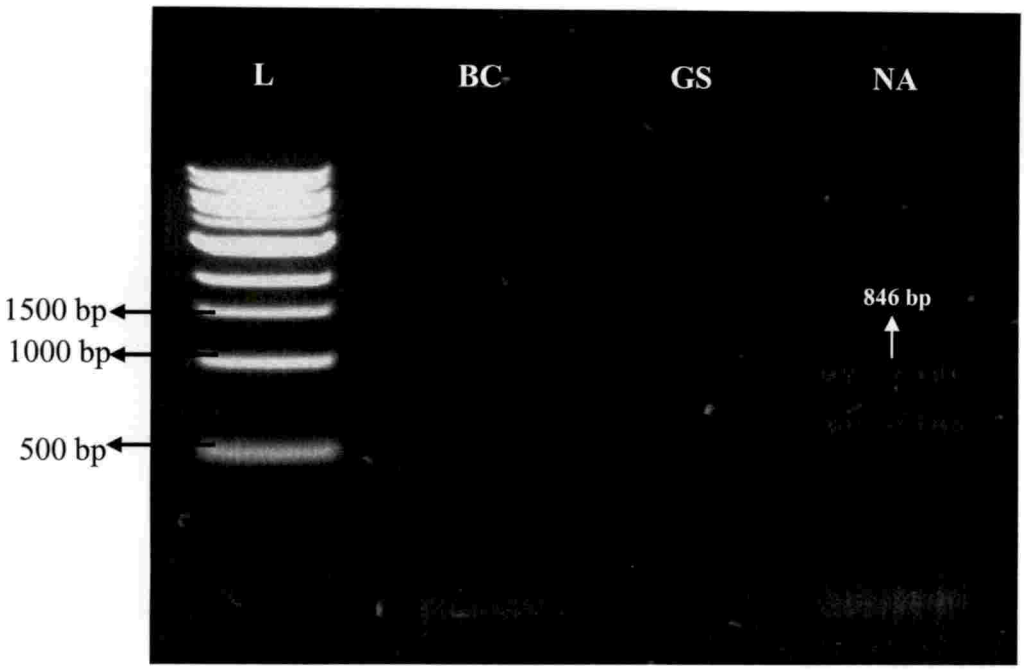


Plate 11 (A). PCR amplification using Phusion enzyme

NA-Triveni, BC– *Biriyanicheera*, GS- Gandhakasala

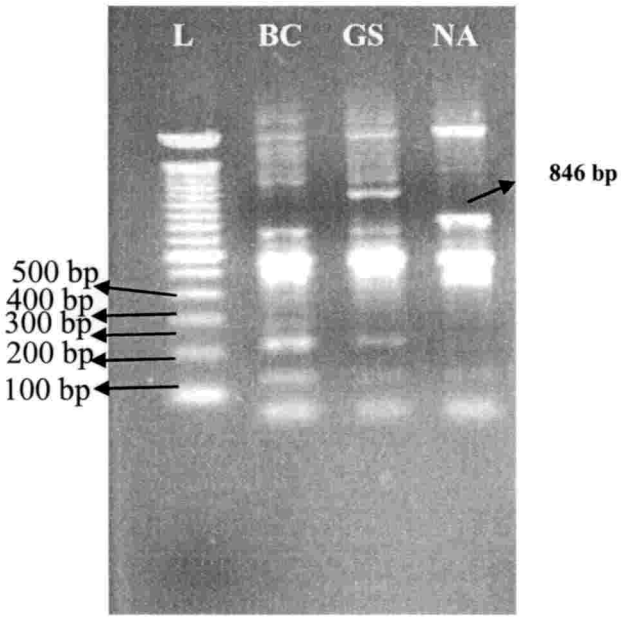


Plate 11 (B). Secondary PCR amplification of PCR products produced by Phusion enzyme

NA-Triveni, BC– *Biriyanicheera*, GS- Gandhakasala

products was not possible. The PCR products were sent directly without any purification to Agrigenome Labs Pvt. Ltd., Kochi for sequencing.

4.3.6 Sequencing results of *BADH2* gene

The genomic DNA of *BADH2* gene present in genotypes *Biriyanicheera*, Gandhakasala and Triveni amplified through PCR were sent for sequencing.

badh2 gene sequence present in both the aromatic genotypes was obtained through sequencing and the contigs were generated from the forward and reverse sequences of sequencing results using CAP3 software. The *badh2* gene sequence of the rice genotypes was submitted to NCBI Gen bank.

***badh2* gene in *Biriyanicheera* rice genotype accession number: MN337026**

***badh2* gene in Gandhakasala rice genotype accession number: MN337027**

4.3.7 Sequence analysis of *BADH2* gene

The genomic DNA sequence of *BADH2* gene in present in all genotypes was aligned through Multiple Sequence Alignment (MSA) by using CLUSTAL OMEGA software. The reference sequence, Locus Id: LOC_Os08g32870, obtained from Rice Genome Annotation Project, was used for the comparison of sequences. MSA identified the presence of 8 base pair mutation and 3 SNPs in the exon 7 of both the genotypes *Biriyanicheera* and Gandhakasala. The mutation was absent in Non-aromatic genotype Triveni which is used as the control. The sequences were further compared with the reported exon 7 sequence of Basmati genotype available in NCBI. The MSA showed that mutation in both *Biriyanicheera* and Gandhakasala were similar to that of the reported mutation in Basmati cultivars as mentioned below.

Exon 7 mutation

```
Reference seq     GGTAAAAAGATTATGGCTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
Basmati           GGTATAT--------ATTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
Triveni           GGTAAAAAGATTATGGCTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
Biriyanicheera    GGTATAT--------ATTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
Gandhakasala      GGTATAT--------ATTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
                  **** *          ****************************************
```

4.3.8 Validation of specific primers of *BADH2* gene

The specific primers designed for region 1 covering the exons 1 and 2 of *BADH2* gene was used for the amplification of genomic DNA. The amplification was successful only in non-aromatic genotype Triveni, whereas aromatic genotypes *Biriyanicheera*, Gandhakasala and Pusa basmati 1 showed no amplification (Plate 12). The primer 1 produced PCR band with desired band size of 846 bp in Triveni genotype.

4.4 Meteorological observations from three different locations

The weather parameters such as maximum and minimum temperatures, relative humidity, sunshine hours, wind speed and rainfall of Palakkad, Thrissur and Ernakulam locations over the period September (2017) to January (2018) were collected and tabulated (Table 13). These micro-meteorological parameters were compared and is graphically represented (Fig. 3). Maximum temperature showed an increasing trend for all the locations. Thrissur district has recorded the highest maximum temperature and ranged between 31.5°C to 33.5°C as compared to Palakkad and Ernakulam. Minimum temperature of the three districts did not show considerable difference. In all the three locations, the relative humidity was found to decrease over the months. There was no much difference with respect to relative humidity during the months in all the three locations. Sunshine hours recorded a gradual increase in all the three locations over the period September (2017) to January (2018). Wind speed recorded a similar trend in both Palakkad and Thrissur districts. The Ernakulam district showed a different pattern with respect to sunshine hours as compared to the other two locations. The rainfall also did not record a greater difference over the period between the locations.

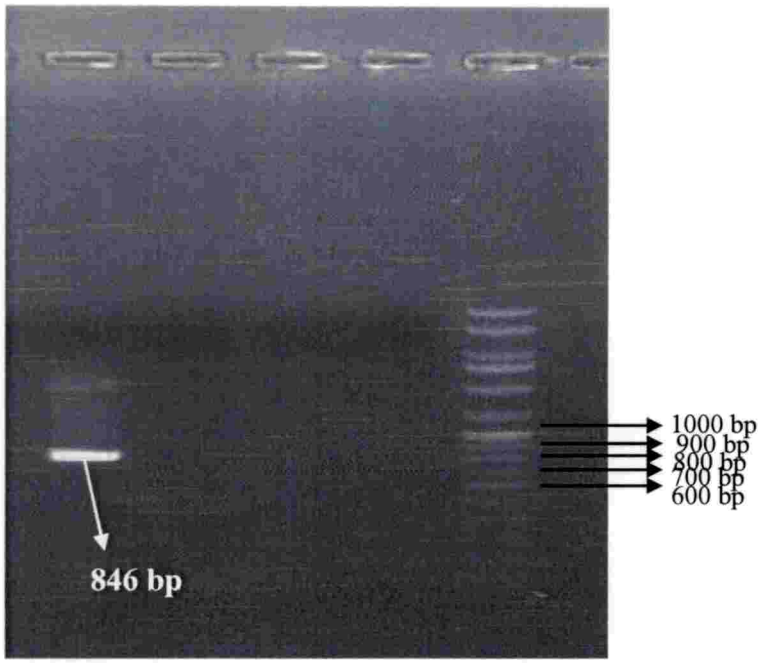
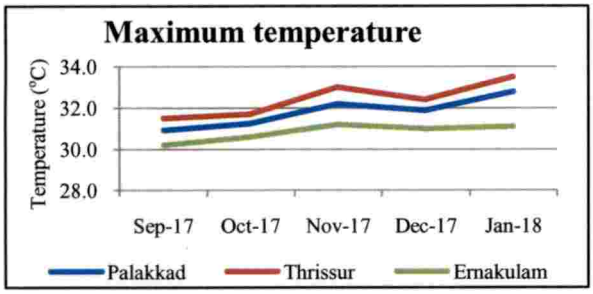
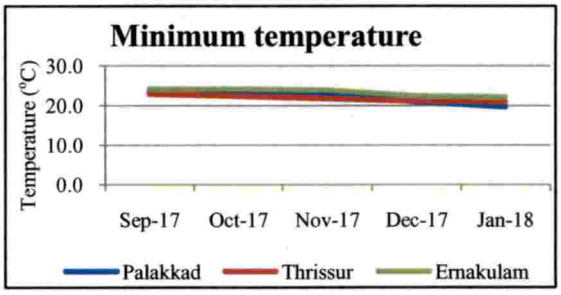
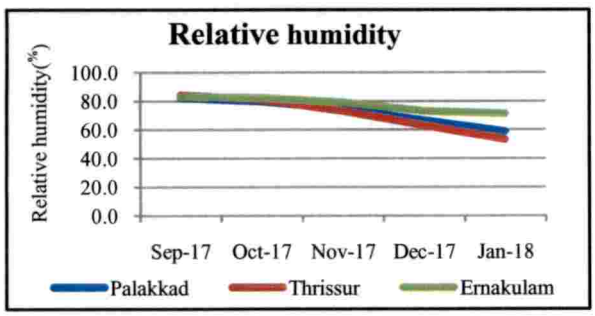
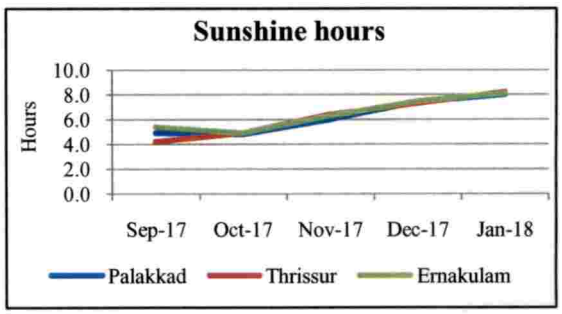


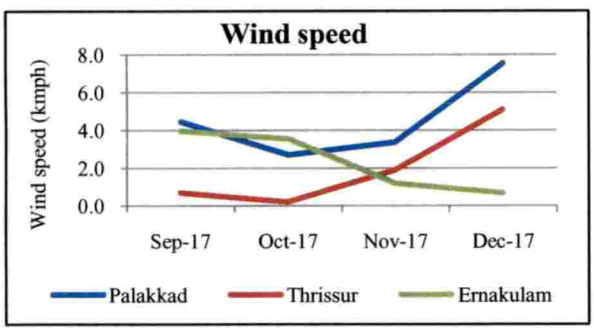
Plate 12. Validation of specific primers of *BADH2* gene











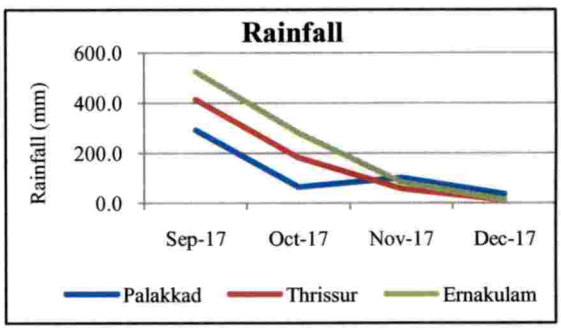


Fig 3. Meteorological data from different locations for the period September (2017) to January (2018)

Table 13. Meteorological observations from three different locations

A. Meteorological observations recorded in Palakkad district						
Month/ Parameters	Temperature in °C		Relative humidity in %	Sunshine hrs.	Wind speed (kmph)	Rain fall (mm)
	Maximum	Minimum				
Sep-17	30.9	23.6	82.1	4.9	4.5	291.2
Oct-17	31.2	23.4	80.0	4.8	2.7	64.2
Nov-17	32.2	22.7	74.7	6.0	3.4	101.7
Dec-17	31.9	21.0	66.2	7.4	7.5	35.4
Jan-18	32.8	19.7	58.9	8.0	7.7	0

B. Meteorological observations recorded in Thrissur district						
Month/ Parameters	Temperature		Relative humidity in %	Sunshine hrs.	Wind speed (kmph)	Rain fall (mm)
	Maximum	Minimum				
Sep-17	31.5	22.9	84.0	4.2	0.7	413.9
Oct-17	31.7	22.3	81.0	4.9	0.2	183.4
Nov-17	33.0	21.8	73.0	6.4	1.9	58.3
Dec-17	32.4	21.1	63.0	7.3	5.1	11.5
Jan-18	33.5	20.9	53.0	8.2	5.4	0

C. Meteorological observations recorded in Ernakulam district						
Month/ Parameters	Temperature		Relative humidity in %	Sunshine hrs.	Wind speed (kmph)	Rain fall (mm)
	Maximum	Minimum				
Sep-17	30.2	24.1	83.1	5.4	3.9	525.0
Oct-17	30.6	24.1	82.4	4.9	3.5	281.4
Nov-17	31.2	23.9	79.0	6.3	1.2	83.3
Dec-17	31.0	22.4	73.0	7.5	0.7	14.4
Jan-18	31.1	22.1	71.4	8.1	0.6	0

Discussion

5. DISCUSSION

Rice is a major food crop in terms of both production and consumption. The importance of the rice is attributed to its special characters of digestibility, taste, texture and aroma in some special cultivars called aromatic rice genotypes. The aromatic rice is regarded as an important commodity in rice export in India. The production of aromatic rice has been greatly influenced by environmental factors such as cool climate, which makes it difficult to have sufficiently strong aroma when it is grown in normal rice growing tracts, especially in tropical regions (Hashemi *et al.*, 2015). There has been no much efforts to develop an elite aromatic cultivar possessing good aroma and to popularise them in normal rice growing areas of Kerala.

The early maturing selection from a local landrace named *Biriyanicheera* was found to have sufficient aroma in normal rice growing tracts of Central Kerala, especially, Palakkad, Thrissur, and Ernakulam districts. Hence the present study focuses on the analysis of aroma in *Biriyanicheera* rice genotype through molecular and biochemical methods. The present investigation 'Molecular and biochemical characterisation of aroma in *Biriyanicheera* rice genotype' was carried out on rice grains of *Biriyanicheera* and Gandhakasala grown in three different districts of Kerala during the period 2017-2018, along with a non-aromatic check genotype Triveni. The details of the findings are discussed below.

5.1 Biochemical characterisation

5.1.1 Gas Chromatography - Mass Spectroscopy (GC-MS)

The analysis of volatile compounds in rice grains of genotypes *Biriyanicheera*, Gandhakasala and Triveni through Gas Chromatography- Mass Spectroscopy revealed the presence of several volatile compounds. The list of all volatile principles detected is listed in Table 1. When the three genotypes are considered together, a total of 65 compounds which belong to very wide classes of volatiles such as, Alkanes (Dodecane, 4-methyl-undecane, Hexadecane, Tetradecane, Decane), Alkenes (Styrene, a-Myrcene), Ketones (2-Undecanone, 2-Heptanone, Acetophenone), Aromatic hydrocarbons (Benzene, p-Xylene), Alcohols (Linalool, 2-Ethyl-1-hexanol), Aldehydes (Nonanal, Decanal, Heptanal, Octanal, Hexanal, 2-hydroxy-Benzaldehyde), and Furans (2-

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Pentylfuran) have been detected in the study. In aromatic rice genotypes, more volatile compounds (33-44 nos.) were observed than the non-aromatic genotype (18).

2-Acetyl-1-pyrroline, is largely believed to be the prominent aromatic component of aromatic rice genotypes such Basmati and Jasmine genotypes and has been detected in many rice cultivars worldwide (Buttery *et al.*, 1983; Adams and De Kimpe, 2006; Mahatheeranont *et al.*, 2001). In the present study 2-AP was not detected in the aromatic genotypes from any location. The absence of 2-AP can be attributed to the selectivity of the column which was used in the analysis. The column materials which are designed to absorb non-polar compounds show effective detection of non-polar substances rather than heavy molecular compounds like 2-AP (Zeng *et al.*, 2008). By the volatile analysis, the genotype *Biriyanicheera* is unlikely to possess 2-AP as a major compound. There have been many reports showing 2-AP as not so important component of aroma in rice (Adekoyeni *et al.*, 2018). Moreover, Hien *et al.* (2006) stated the possibility of production of rice aroma from integrated effects of several compounds in the endosperm of rice.

However, of all the detected aromatic compounds from both the scented genotypes, 21 specific compounds had earlier been reported in several diverse scented rice cultivars, which includes Nonanal, Hexadecane, 2-pentylfuran, Decane, Octanal, Tetradecane (Widjaja *et al.*, 1996a; Jezussek *et al.*, 2002), Styrene, Acetophenone, Linalool, 2-Nitro-toluidine, Benzothiazole, *etc.* (Buttery *et al.*, 1988;; Yang *et al.*, 2008a). The normal compounds detected in rice such as 1-octen-3-ol, 3-octen-2-one, (E)-2-octenal, (E,E)-2-decadienal, and hexanoic acid were not detected in the present study and is likely to be due to lower concentration of the above compounds. The present study identified three compounds *viz.*, 2-Methyl-butanol, 4-Methyl-octane and 5-Ethylenyl dihydro-5-methyl-2(3H)-furanone in *Biriyanicheera* from Ernakulam, Six compounds such as, 2-(1-phenylethyl)-1,3-Dioxolane, 2-(phenylethyl)-1,3-Dioxolane, N,N-Dimethylacetamide, Mefluidide, 1,3-Diazacyclooctane-2-thione, Dimethyl-tetrasulfide and Dimethyl-pentasulfide in Gandhakasala from Palakkad, and One compound 3,5-Dibutoxy-1,1,1,7,7,7,-hexamethyl in Gandhakasala from Thrissur as new compounds which are not previously reported by other researchers.

The aroma profile of *Biriyanicheera* grown in three districts showed variation in the diversity of components and their prevalence. The highest number of volatile

compounds (19) was observed in sample from Ernakulam district whereas Palakkad district sample showed very few compounds. However, the Palakkad sample was more aromatic than Ernakulam sample. By the above observations, it is evident that the strength of aroma does not depend on the volatile diversity but on their quantity. Similarly, the comparative volatile analysis by Widjaja *et al.* (1996a) revealed no difference in the presence of the compounds but only with their quantity. The mechanism of production of volatiles in rice varies with the location, period, cultivation conditions, soil characteristics, pH and also weather conditions (Itani *et al.*, 2004; Gay *et al.,* 2010; Monggoot *et al.*, 2014; Mo *et al.*, 2015).

The compound Hexanal has been detected only in Gandhakasala grown in Thrissur location. Since hexanal is identified as off odour producing volatile (Widjaja *et al.*, 1996b), its absence in Gandhakasala and *Biriyanicheera* grown in other locations may be a valid reason for significant aroma production in rice, which is an appreciable result in this study.

Decanal, Benzothiazole, 2-Undecanone, Dimethyl disulfide, 1-Hexanol are found in majority of rice samples along with the non-aromatic rice genotype Triveni. Similar results have been reported by Widjaja *et al.* (1996b), Yang *et al.* (2010) and Jezussek *et al.* (2002). Nonanal had been detected in both *Biriyanicheera* (Higher) and Gandhakasala (Lower) grown only in Palakkad district and not in other locations. The detection of Nonanal is in agreement with the findings of Tava and Bocchi (1999) in Basmati genotype, and in other aromatic genotypes by Maraval *et al.* (2008) and Yang *et al.* (2008).

Gandhakasala from Palakkad had higher contents of Octanal, Heptanal, 2-methyl furan and Acetophenone, whereas it was absent in *Biriyanicheera* from all locations. Mathure *et al.* (2014) reported higher Octanal and Heptanal contents in Basmati rice genotype. The compounds Heptanal, Octanal, Hexanal, Nonanal, Decanal are said to be derived from either degradation or oxidation of lipids (Mildner-Szkudlarz *et al.*, 2003). Hence it can be the reason for the aroma specific to Gandhakasala. 1-Hexanol has been identified as the distinguishing factor between scented and non-scented genotypes by Yang *et al.* (2008b). The study identified 1-Hexanol to be present in both non-aromatic and aromatic rice genotypes. Mathure *et al.* (2014) identified

similar pattern of presence for the same compound between non-basmati and non-scented cultivars.

The aroma strength of *Biriyanicheera* has been mainly contributed by Benzaldehyde (Almond odour), 2-Undecanone (fruity, floral), Benzothiazole (sweet), Nonanal (foral, fruity), 4- nitrobenzalamine, 4-methyl octane and Dimethyl disulfide. Several researchers have reported these compounds as major contributors of the scent in rice grains (Xia *et al.*, 2016; Hinge *et al.*, 2016; Adekoyeni *et al.*, 2018). The results are in contrary with respect to the presence of 2-AP in the volatilome of aromatic rice genotypes as reported by Buttery *et al.* (1983), Widjaja *et al.* (1996b), and Nadaf *et al.* (2014).

5.1.2 Cooking qualities

Consumer preference towards quality rice is decided based on many parameters, and cooking quality index is the major one among them. In the present study, quality attributes *viz.,* Gelatinisation temperature, Gel consistency and Amylose content test had been carried out.

Gelatinisation temperature of a particular genotype denotes the time taken for the cooking of rice grains. In the present study, both the aromatic genotypes along with Triveni were found to be of medium gelatinization index. The rice genotype Jyothi was reported to be possessing intermediate gelatinization temperature index by Lakshmy (2011) and Sathyan (2012).

High gelatinisation temperature or lower alkali spreading value denotes the presence of crystalline structure and increased resistance to water penetration and also swelling of rice kernels (Yadav and Jindal, 2007).

The amylose content is an important aspect in influencing the consumer preference. Similar to many other nutrient components present in rice, amylose content of rice kernels varies with the respective rice genotype (Unnevehr *et al.*, 1992). According to Juliano *et al.* (1985), the amylose content present in rice grains is classified as waxy (1-2%), low (7-20%), intermediate (20-25%) and high (>25%).

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In the present study, genotypes *Biriyanicheera* and Triveni showed amylose content of intermediate range (20-24%), whereas Gandhakasala had lower amylose content (13.85%) (Table No. 10). Rice grains with intermediate amylose content had been reported to produce moist and soft kernels after cooking and it is preferred mostly by Asian population (IRRI, 1985). Rice grains with lower amylose content are found to be sticky, glossy and soft, whereas increase in amylose content makes the grains firmer (Ashraf, 2012).

The decrease in the amylose content in Gandhakasala may be due to the effect of hot conditions during growing period. Since Gandhakasala is usually grown in cooler regions, the change in environment leads to reduction in amylose content. Ram (2012) has reported low aroma and low amylose content for Gandhakasala and Jeerakasala grown in the Palakkad region, which was proposed to be due to high temperature during grain filling stage of the rice crop. Here it is true that *Biriyanicheera*, unlike Gandhakasala, is possessing unique nature to produce grains with optimum amylose content. This gives additional fame for the genotype to be suitable to the conditions of Palakkad region (hot).

Gel consistency is an indicative of the hardening of the cooked rice grains, upon gradual cooling. It is one of the attributes of eating quality of rice and usually soft to medium gel consistency is preferred for consumption than hard gel.

IRRI (2002) classified the gel consistency in rice grains into classes such as hard (40mm or less), medium (41-60mm) and soft (61mm and more) based on the gel length. Maximum gel length was observed in Gandhakasala genotype (107.3mm) and minimum gel length in Triveni (95mm). However, the gel length of rice grains of all these three genotypes falls under soft category.

Biriyanicheera genotype was found to possess a gel length of 105mm (10.5cm) and it similar to that of IR-64 (103mm), as reported by Singh (2005). The soft gel consistency value of *Biriyanicheera* obtained in the study makes it possible for better adaptability of the genotype in rice market.

5.1.3 Sensory evaluation of aroma by DUS test

In the present work, *Biriyanicheera* rice genotype was observed to be superior to Gandhakasala in aroma. *Biriyanicheera* has shown the highest aroma in Palakkad sample than others, which indicates the better suitability of the genotype for normal tropical conditions for its cultivation. The reduction in the aroma in Gandhakasala can be due to its growing conditions. Normally, cooler conditions are preferred for the effective aroma production in Gandhakasala genotype, which is generally grown in Waynad district of Kerala. Ram (2012) also identified lower aroma and amylose content for Gandhakasala grown in Palakkad region than those plants grown in Waynad district.

It had been reported that strength of aroma is affected by the variation in the environmental conditions and cultivation practices (Hien *et al.*, 2006; Itani *et al.*, 2004; Yoshihashi *et al.*, 2002). Tava and Bocchi (1999) stated that the aroma in Italian and Basmati genotypes was due to genotype, environment and genotype × environment interactions. The aroma is said to be varying under many conditions such as : a) rainy or summer season (Nadaf *et al.*, 2006), b) water stress (Bradburry *et al.*, 2005a), c) extraction procedure of volatiles (Yang *et al.*, 2008), d) soil type and salt stress (Yoshihashi *et al.*, 2002; Itani *et al.*, 2004), e) environmental conditions such as, drought and temperature (Itani *et al.*, 2004), f) cultural practices (Bhattacharjee *et al.*, 2002), g) fertilizer application (Bradburry 2005b), h) flowering time (Yi *et al.*, 2009) i) level of milling of grains (Yang *et al.*, 2008) j) shading during grain filling stage (Mo *et al.*, 2015) *etc.*

In the present work, since the crop was grown in the same period of time, the influence of the factors such as, season, flowering time and cultural practices can be neglected. The extraction procedure of volatile compounds and milling of grains was also followed in a similar manner for all the samples. The impact of fertilizers can be ruled out as the crop was grown as per KAU PoP (40:20:20). There are probable chances for the influence of water stress during the crop growth, soil type, salt stress, shading of the rice crop, which can vary from location to location, to a greater extent. The micro-meteorological parameters of the three locations did not record much variation except for the wind speed. Even though Palakkad district is said to be hotter than the remaining two districts, similar temperature range has been recorded in other

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locations also during the period of crop growth. It is believed that there might be some unknown factors, which is influencing the increased aroma production of *Biriyanicheera* grown in Palakkad region. Further investigations may help in uncovering the truth behind the anomaly of *Biriyanicheera* in aroma production.

Even though tropical conditions with higher temperature and lower relative humidity are said to be negatively affecting the aroma production, *Biriyanicheera* rice genotype is a clear exception in its nature.

5.2 *Fgr* gene characterisation

The genetic basis of rice aroma trait (*fgr*) explored by many researchers in early 1992, revealed the recessive nature of the gene and it was located at 4.5 cM distance from the marker RG28 (Ahn *et al.*, 1992). Later, the aroma gene *fgr* was located to be between markers RM223 and RM342 (Wanchana *et al.*, 2005). The map-based cloning and sequencing of the *fgr* region identified significant difference in the *Badh2* gene between scented and non-scented cultivars. The mutation in 7^{th} exon of *Badh2* gene, which led to loss of *Badh2* protein, was thought to be controlling the aroma in rice.

Similarly, the amplification of *BADH2* (or *fgr*) gene was attempted to understand the actual molecular reasons for the special nature of *Biriyanicheera* genotype. The sequencing of the genomic DNA consisting of exons of *BADH2* gene helped to unravel the molecular nature in *Biriyanicheera*. The occurrence of previously reported 8bp mutation (TATAT--------ATTT) in the exon 7 of *BADH2* gene in both the *Biriyanicheera* and Gandhakasala genotypes suggests that the aroma gene present in them must have followed similar path of evolution and inheritance as that of Basmati cultivars. The occurrence of 8 bp deletion in the *BADH2* gene is also reported by many researchers in various rice genotypes of the world (Bradburry *et al.*, 2005; Chen *et al.*, 2008). The results showed negative for the occurrence of mutations in other exons of *BADH2* gene, whereas there have been reports of presence of mutations in exons 1, 2, 10, 13, 14 and also regions between exon 4 and 5 (Shi *et al.*, 2008; Amarawathi *et al.*, 2008; Kovach *et al.*, 2009; Shao *et al.*, 2011).

The non-amplification of genomic DNA coding for exon 1 and 2 in both the aromatic genotypes was not clear. Two primers designed to amplify the exons along with the promoter regions were lying in different regions. The amplification of same

region in Triveni genotype shows the functionality of the primers designed. Hence, the problem can be attributed to the major difference in genome sequence of the region, which makes it difficult to amplify the regions by the primers used. The molecular complexity of the above region can be solved through advanced methods like, 5' - Genome walking or 5' - RACE (Rapid Amplification of cDNA Ends).

The production of aroma by *Biriyanicheera* might not be precisely due to mutation in chromosome 8. The presence of novel gene in other chromosomes can be a factor for the differential aroma production in the genotype. Amarawathi *et al.* (2008) has reported similar example about *BADH1* gene present in chromosome 3 and chromosome 4 as a cause for aroma production. The *BADH1* gene is involved in salt stress response in rice crop and the mechanism in unknown (Bradburry *et al.*, 2005) Two additional genes similar to that of *BADH* has been reported on chromosome 7 (Chen *et al.*, 2008). The other contradicting reports have suggested the presence of several genes determining the aroma in rice. For example, a major QTL on chromosome 8 and two minor QTL on chromosome 3 and 4 (Amaravathi *et al.*, 2008), two recessive genes (Hien *et al.*, 2006), one major QTL on chromosome 8 and two minor QTL on chromosome 4 and 12 (Lorieux *et al.*, 1996) are some of the reported locations aroma trait. Based on the above reports, it is difficult to draw the exact conclusion for the molecular basis of *Biriyanicheera* genotype. However, future experiments with transcriptomics can crack the mistery of the aroma production in *Biriyanicheera* and other similar aromatic rice genotypes.

The primers designed for amplifying the promoter region, exons 1 and 2 was used to differentiate the aromatic and non-aromatic rice cultivars. The presence of clear band from PCR reaction of Triveni (non-aromatic) rice variety shows the usefulness of the primer as a marker for scentedness. Similarly Bradburry *et al.* (2005b) has used allele specific primers for discriminating the aromatic rice varieties from the non-aromatic.

From the present study, it is evident that the genotype *Biriyanicheera* can be recommended as a suitable aromatic rice genotype for the tropical conditions of Kerala and other parts.

Summary

6. SUMMARY

The study on 'Molecular and biochemical characterisation of aroma in *Biriyanicheera* rice genotype' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Thrissur during the period 2017-2019. The main objective of the study was to analyse the volatile compounds present in the rice grains and to elucidate molecular nature of aroma trait in *Biriyanicheera* rice genotype. The plant materials used in the study were two aromatic rice genotypes *viz.*, *Biriyanicheera*, Gandhakasala and one non-aromatic control genotype, Triveni.

The salient findings of the study are summarised below:

I. The rice grains of all the genotypes grown during the period September 2017 to January 2018 were used to analyse the volatiles present in them. Gas Chromatography and Mass Spectroscopy (GC-MS) method were employed to study the compounds present in the powdered rice sample. A total of 65 compounds were identified as important volatiles in rice grains.

II. A diverse profile of volatile compounds was obtained from GC-MS analysis. The aromatic volatile compound Benzothiazole was detected in all the genotypes from different locations including Triveni. The compound Ethyl acetate (fruity odour) was having maximum probability range of 83 - 97.1 in Non-aromatic genotype Triveni, *Biriyanicheera* from Palakkad and Gandhakasala from Ernakulam districts. The compounds such as 2-ethyl-1-Hexanol, Decanal, 4-Nitrobenzylamine, Dimethyl disulfide, 2-Undecanone, Dodecane, 4-Methyl-undecane, Tetradecane, and Benzene were detected in both aromatic and non-aromatic types.

III. *Biriyanicheera* rice genotype from different locations was found to possess 33 compounds in total. The compounds, Nonanal and 2-hydroxy Benzaldehyde were found to be the major volatile compounds for aroma of *Biriyanicheera* in Palakkad region. Similarly, 4 nitrobenzylamine and 2-pentyl furan in Thrissur location and 4

nitrobenzylamine, 2(3H)-Furanone, 5-ethenyldihydro-5-methyl- and Lincomycin were identified as major compounds present in the aroma profile of the rice grains.

IV. Gandhakasala rice genotype was observed to contain 44 compounds from rice grown in three different locations. 2-methyl furan, 4-nitrobenzylamine, Acetophenone, Heptanal, 2-Heptanone, and Octanal were the major volatile principles responsible for aroma in Gandhakasala grown in Palakkad. Hexanal, and Linalool in Thrissur, and 2-pentyl furan, and Dimethyl sulphide from Ernakulam, were involved in imparting aroma in Gandhakasala.

V. Rice grains of all the three genotypes, *Biriyanicheera*, Gandhakasala and Triveni, from different locations were found to have medium gelatinisation index, as determined by the alkali spreading test.

VI. The highest amylose content was observed in *Biriyanicheera* genotype (24.62 per cent), whereas the lowest amylose content was observed in Gandhakasala (13.85 per cent). Triveni was found to be possessing 23.85 per cent amylose content.

VII. Gel consistency of rice genotypes was analysed by measuring the gel length obtained from powdered rice grains. The higher Gel length was found in *Biriyanicheera* (10.5 cm) and lower gel length was found in Triveni genotype (9.5 cm).

VIII. Aroma detection of rice grains was performed by Sensory evaluation test by DUS protocol prescribed by DRR (Hyderabad). The aroma scores obtained showed that aroma in *Biriyanicheera* was the highest in Palakkad district and the lowest in Ernakulam district. Similarly, the Gandhakasala grown in Palakkad region showed higher aroma than the other two locations, whereas the Triveni was rated as non-aromatic. The aroma strength in *Biriyanicheera* genotype was rated higher than the Gandhakasala genotype.

IX. The rice plants of *Biriyanicheera*, Gandhakasala and Triveni were raised during the period 2018 to assess the molecular nature of *BADH2* gene. Tender leaves were used for DNA isolation through CTAB method. PCR reaction was performed with seven designed primers amplifying the regions in genomic DNA of *BADH2* gene.

Standardisation of annealing temperature for all the primers was performed at varying gradient temperatures. The amplification of the specific bands was performed for all the primers except for the primer 1 covering exons 1 and 2. The amplified PCR product was sent for sequencing analysis.

X. The genomic DNA sequence of *BADH2* gene present in all genotypes was aligned through Multiple Sequence Alignment (MSA) by using CLUSTAL OMEGA software. The MSA identified the presence of 8 base pair mutation and 3 SNPs in the exon 7 of both the genotypes *Biriyanicheera* and Gandhakasala similar to that of Basmati genotype. The mutation was absent in Non-aromatic genotype Triveni which is used as control. The mutation in the exon 7 can be the driving factor for the aroma production in *Biriyanicheera* rice genotype.

XI. The sequence alignment showed the absence of other mutations in the remaining exonic regions. However, amplification of the genomic sequence coding for exons 1 and 2 was not possible. The non-amplification of promoter region of *BADH2* gene is due to sequence difference in the genomic DNA of both the aromatic genotypes.

From the present study it is evident that aroma in *Biriyanicheera* is produced by the combination of several volatiles present in the rice kernels. The aroma strength is also greater than Gandhakasala genotype, when both the genotypes are grown in Palakkad region. The molecular analysis shows the similar mutations in both the aromatic rice genotypes to cause the aroma production. However, the exact reason for differential aroma production by *Biriyanicheera* in tropical conditions is yet to be analysed.

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Appendices

APPENDIX I

Composition of buffers and dyes used for agarose gel electrophoresis

1. 50X TAE buffer 50X (for 1 L)

242 g tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

1000 ml distilled water

The solution was autoclaved and stored at room temperature.

2. Loading dye/tracking dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

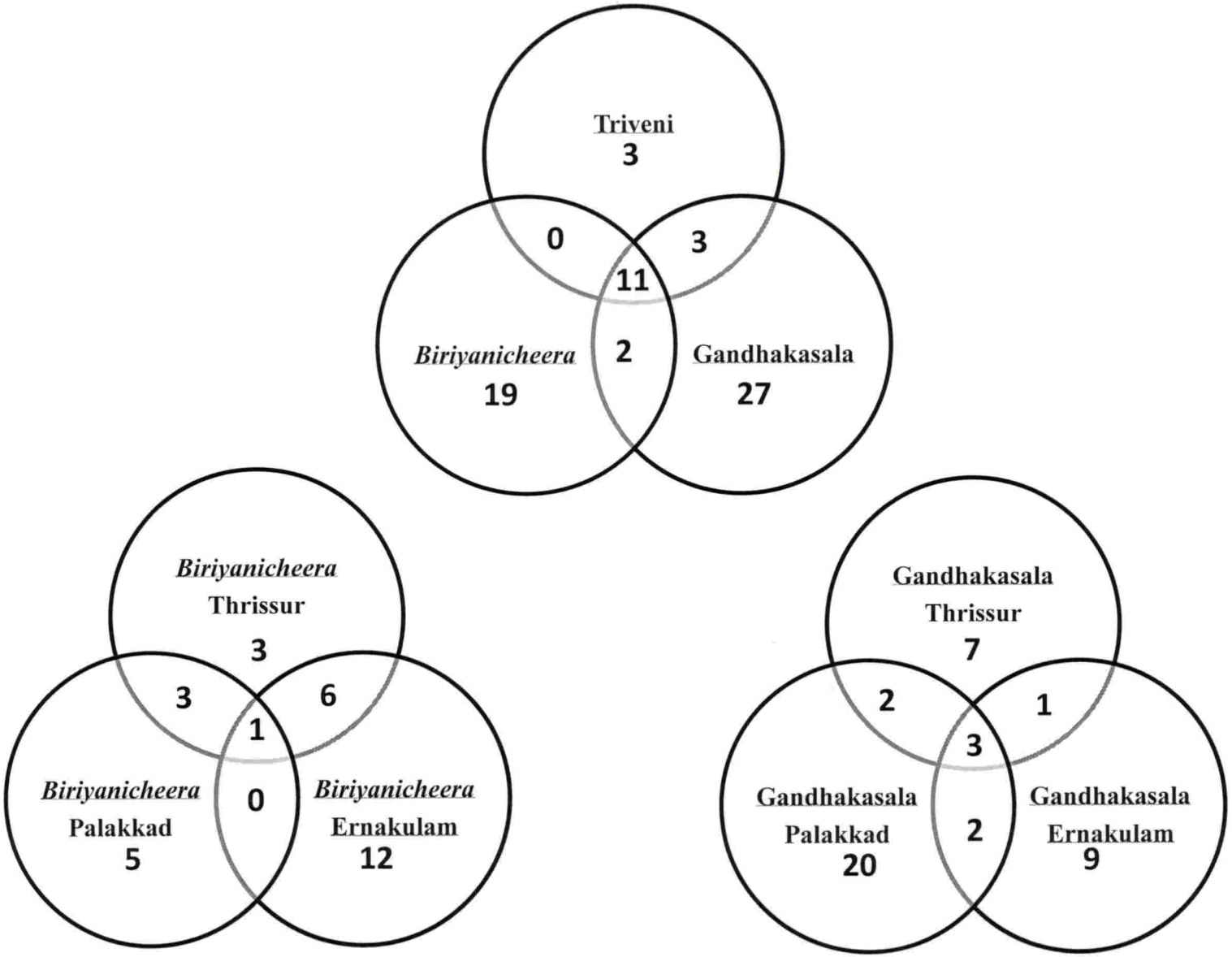
30% glycerol in water

The dye was stored at 4°C

3. Ethidium bromide (intercalating dye)

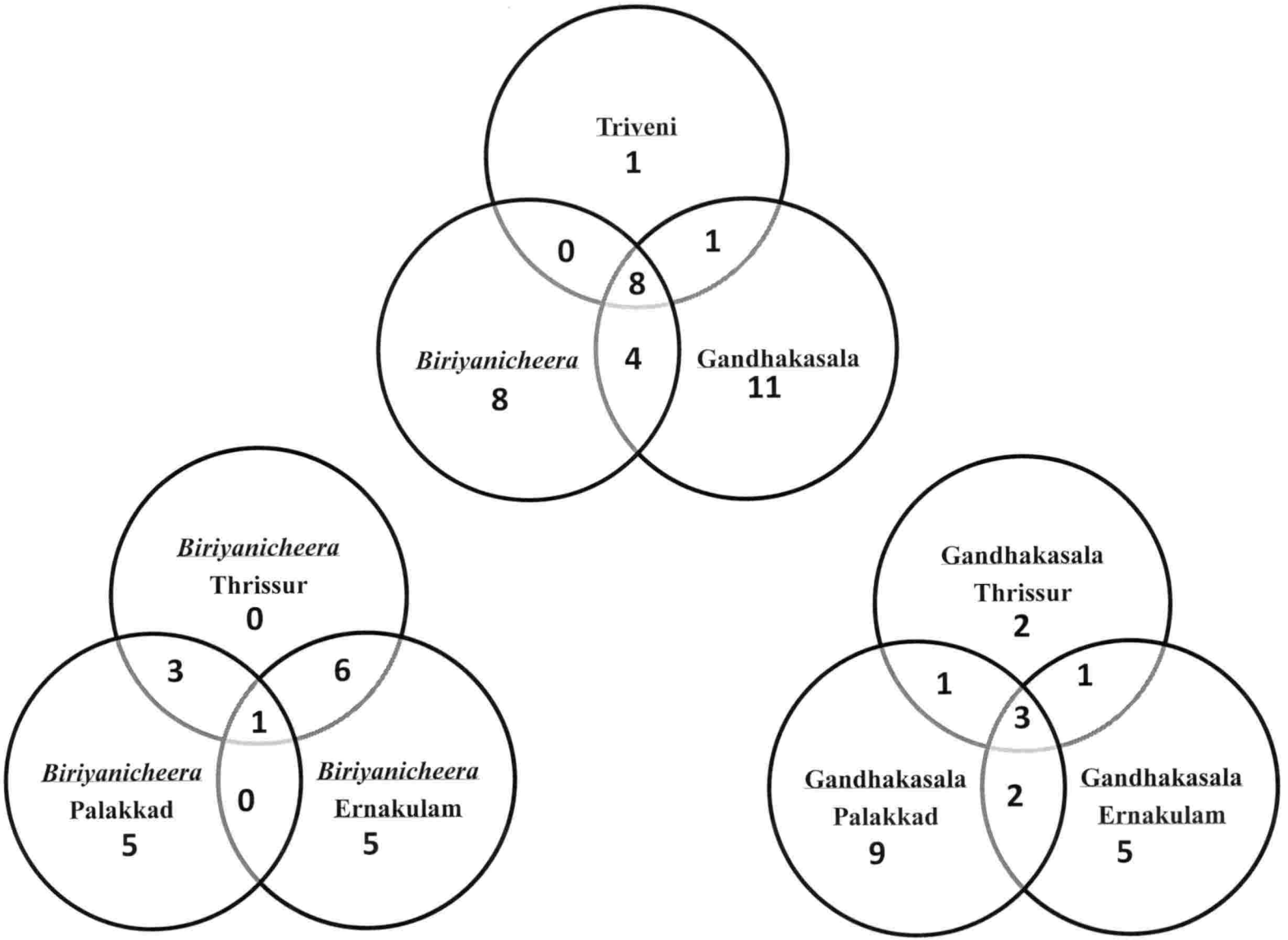
The dye was prepared as a stock solution of 10mg/ml in water and was stored at room temperature in a dark bottle.

APPENDIX II



Volatile compounds present in *Biriyanicheera*, Gandhakasala and Triveni

APPENDIX III



Aromatic compounds present in *Biriyanicheera*, Gandhakasala and Triveni

Molecular and biochemical characterisation of aroma in *Biriyanicheera* rice genotype

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Rice is the dietary staple food for as high as 62.8 per cent of world's population and it contributes to 20 per cent of the total calorific intake of the people. Rice possesses many unique qualities which determine its value and preference in the market. The fragrance of rice has been one such special character that provides an extra preference to scented rice and fetches premium price in rice market.

The aromatic rice cultivars are found to possess higher fragrance, when grown in relatively cooler conditions in hilly regions. Hence the production of such plant types is confined to only a few locations of India. The early maturing selection from a Kerala local landrace with short grains, named '*Biriyanicheera*', when grown in normal tropical conditions was observed to have sufficient aroma. In this context, the present study focuses on the analysis of aroma in '*Biriyanicheera*' rice genotype through molecular and biochemical methods. The research programme was carried out at CPBMB during 2017-2019.

The seeds of aromatic rice varieties *Biriyanicheera*, Gandhakasala (from Palakkad, Thrissur and Ernakulam districts) and non-aromatic rice variety Triveni were used for the study. The biochemical characterisation was performed using finely powdered rice grains of all varieties through Gas Chromatography and Mass Spectroscopy (GC-MS). The GC-MS analysis revealed the presence of a diverse profile of volatiles present in the rice grains. The volatile compound Benzothiazole was detected in all the varieties, where as Ethyl acetate was the highest in Triveni, *Biriyanicheera* (Palakkad) and Gandhakasala (Ernakulam). The compounds, Nonanal and 2-hydroxy Benzaldehyde were found to be the major volatile compounds for aroma of *Biriyanicheera* in Palakkad region. Similarly, 4 nitrobenzylamine and 2-pentyl furan in Thrissur location and 4 nitrobenzylamine, 5-ethenyldihydro-5-methyl-2(3H)-Furanone, and Lincomycin in Ernakulam

location were identified as the major compounds present in the aroma profile of the rice grains.

The cooking quality parameters such as, Gelatinisation temperature, Amylose content and Gel consistency were evaluated for all the rice varieties. Alkali spreading test showed medium gelatinisation index for all three varieties which is optimum for the rice quality. Amylose content in rice grains was determined by treating the fine powder of rice with 0.1 N NaOH. The *Biriyanicheera* rice variety was found to contain 24.62 per cent of amylose content whereas, Gandhakasala and Triveni varieties showed 13.85 and 23.85 per cent respectively. Gel consistency of the rice varieties was analysed by measuring the gel length produced by boiling the rice powder. The gel length for *Biriyanicheera* (105mm), Gandhakasala (107.3mm) and Triveni (95mm) falls under soft category.

Aroma strength detection by sensory evaluation was performed using the DUS (Distinctness, Uniformity and Stability) protocol. The *Biriyanicheera* variety grown in Palakkad district showed exceptionally higher aroma than the other locations. Similarly, Gandhakasala variety produced maximum aroma in Palakkad region. From the above results Palakkad district was found to influence in aroma production of aromatic varieties.

Molecular characterisation was performed by amplifying the *BADH2* gene present in all the three rice varieties. The amplified PCR products of genomic DNA were sequenced to analyse the presence of any mutations in *BADH2* gene. The sequencing results of aromatic varieties *Biriyanicheera* and Gandhakasala revealed the presence of an 8 base pair mutation in exon 7 in both the varieties, whereas this mutation was absent in the non-aromatic variety Triveni. Hence it can be inferred that the aroma production in *Biriyanicheera* variety is due to similar mutation as that of the Basmati rice variety.

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