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ORIGINAL ARTICLE

Morphological, Biochemical, Molecular Marker, Gas Chromatography-Tandem Mass Spectrometer Analysis of Garlic (*Allium sativum* L.) Landraces in the Rain Shadow High Hills of Kerala, India

Jalaja S. Menon[1], Berin Pathrose[2], A. C. Asna[1], Rukam Singh Tomer[3], B. Dineshkumar[4]

[1]Cashew Research Station, [2]Pesticide Residue Lab, Kerala Agricultural University, Thrissur, Kerala, [3]Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, [4]Department of Pharmaceutics, St. James' College of Pharmaceutical Sciences, Thrissur, Kerala, India

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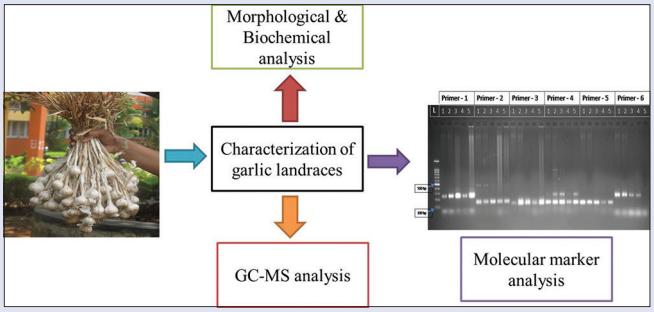
ABSTRACT

Background: In Kerala, dry hill agro ecological zone is famous for the commercial cultivation of hill garlic. *Vattavada* and *Kanthalloor panchayaths* in Devikulam block of Idukki district, India, are famous for the cultivation of hill garlic. The traditional land races a being cultivated by *Muthuva* tribes has role in traditional home remedies and fetches better price in market Therefore, we investigated local landraces of garlic (MLPD garlic, MPD garlic, and SGPR garlic) along with two released varieties (Ooty 1 garlic, Yamuna safed 3-garlic) for their chemical composition, bulb morphological traits, and diversity at molecular level. **Materials and Methods:** Morphological characterization, biochemical parameters analysis, molecular marker analysis using simple sequence repeats, and gas chromatography-tandem mass spectrometer (GC-MS) analysis were performed for five different genotypes of garlic. **Results:** Significant difference was observed in morphological characterization, biochemical parameters analysis of five different genotypes of garlic. Molecular marker anlalysis results indicated DNA level variation in traditional garlic genotypes and found these garlic having high storability at field level and used in traditional folk medicines preparations. GC-MS analysis results showed MLPD garlic as having 10 total number of active constituents. MPD garlic as having 14 total number of active constituents. SGPR garlic as having six total number of active constituents. Ooty 1 garlic as having 13 total number of active constituents and Yamuna safed 3 garlic having 7 total number of active constituents. **Conclusion:** This study showed the uniqueness of MLPD garlic and SGPR garlic used in traditional home remedies with high storability at the field level with the presence of high percent of total sulphides and a significant variation at the molecular level.

Key words: Gas chromatography-tandem mass spectrometer constituents, hill garlic, Kerala, molecular markers

SUMMARY

- Local landraces of garlic (MLPD garlic, MPD garlic, and SGPR garlic) along with two released varieties (Ooty 1 garlic, Yamuna safed 3-garlic) were investigated for their chemical composition, bulb morphological traits, and diversity at the molecular level
- Significant difference was observed in morphological characterization, biochemical parameters analysis in different traditional genotypes of garlic
- Gas chromatography-tandem mass spectrometer analysis of constituents, hill garlic, Kerala, molecular markers analysis was performed to know the bioactive compounds present in the different traditional garlic genotypes
- Molecular marker analysis results indicated that DNA level variation in traditional garlic genotypes and found this garlic having high storability at the field level.



Abbreviations used: MLPD: Malappondu variety; MPD: Mettupalayam variety; SGPR: Singappupoondu variety; SSRs: Simple Sequence Repeats; GC-MS: Gas chromatography-tandem mass spectrometer; SSR-PCR: Simple sequence repeats-Polymerase chain reaction; NTSYS-pc: numerical taxonomy and multivariate analysis system; EI: Electron impact

Correspondence:

Dr. Jalaja S. Menon,
Kerala Agricultural University, Thrissur - 680 656, Kerala, India.
E-mail: jalaja.menon@kau.in
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INTRODUCTION

Kerala, a tropical sub-humid region in Southern India, is regarded as the spice bowl of the world. Kanthalloor and Vattavada regions in the hill agro ecosystems in the rain shadow region of Idukki district of Kerala are known for the commercial cultivation of winter garlic. Garlic (*Allium sativum* L.), a vegetable cum spice crop, is primarily grown for its cloves and highly valued for its flavoring and medicinal properties including antifungal, antibacterial, antioxidant, and anticancer effects. Biological activities of garlic are partially ascribed to its volatile oil consisting of sulfur compounds.[1,2] Although propagation in garlic is asexual, wide diversity exists in morphological, reproductive, and quality traits,

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which may be due to natural mutation through its long cultivation history.[3] Natural selection and preferencs of human for adaptation in growing regions led to a large number of garlic cultivars.[4] The high hill traditional garlic landraces of Kerala are almost at the verge of extinction as high yielding bold clove varieties are becoming popular. As suggested by Shaaf *et al.*, emphasis should be made on collecting and preserving wild landraces that could help to identify novel genes suitable for local acclimatization and introgression into new cultivars.[5] Hence, these accessions with unique characters need to be preserved as they have some adaptiveness to a particular geographic region and high storability. The survey conducted in the Kanthalloor and Vattavada villages of Kerala has revealed the prevalence of local landraces of garlic with specific properties. The strong aroma and high storability of these landraces were derived from the unique climatic condition of the high hills and the peculiar post harvest curing and storage techniques. Since the availability of space for seed storage is limited, the villagers used to procure seeds from the nereby local market (Mettupalayam landraces) for the commercial cultivation of garlic. However, the traditional landraces distinctly named as *Malappondu* (Accession MLPD) and *Singappupoondu* (Accession SGPR) are being preserved by the tribal group called "*Muthuva*" and the storability of traditional landraces were found to be higher (7–9 months) than the sample collected from the local market (2–3 months). The tribal people usually cultivate the landrace MLPD during the first crop season and SGPR as the second crop. Due to excellent medicinal properties and high storage life, traditional landrace fetches the highest price in the market and widely used in the preparation of Ayurveda medicines. Accession MLPD is also used to prepare garlands by braiding the fresh bulb with the aerial part to adorn diety at home and to honour dignitaries in functions. Traditional landraces SGPR and MLPD grown in the high hill of Kerala are well adapted to the climatic conditions of high hills of Kerala.[6] Although various diversity studies have been reported in garlic worldwide, the information on the performance of region-specific genotypes of garlic in Kerala is lacking. This is the first study on the molecular characterization and chemo profiling of essential oil of unique garlic landraces in Kerala's high ranges. The present study aimed to assess the traditional landraces of garlic (MLPD garlic, MPD garlic, and SGPR garlic) along with two released varieties (Ooty 1 garlic, Yamuna safed 3-garlic) cultivated in the *Vattavada* and *Kanthalloor* areas of Kerala, for their chemical composition, bulb morphological traits, and diversity at molecular level.

MATERIALS AND METHODS

Instruments and chemicals

In this study, we used simple sequence repeats-polymerase chain reaction (SSR-PCR (Eppendorf Vepoprotect Thermo cycle, Chennai, India)) (Veriti ABI thermal cycler), NTSYS pc version 2.2 software, R-based software, Agarose Gel Electrophoresis (GENESYS gel documentation system, Syngene, UK) unit (BioGene), and Gas chromatography-tandem mass spectrometer (GC-MS GC–MS (gas chromatography–mass spectrometry, Shimadzu QP2010Plus, Japan)) (Shimadzu). Agarose Gel DNA Extraction Kit was procured from Merck Limited, Mumbai, India

Plant materials

The seed cloves of traditional landraces Malappondu (Accession MLPD) and Singappupoondu (Accession SGPR) collected from the tribal hamlets. Ooty 1 seed cloves-(released from Tamil Nadu Agricultural University) and Yamuna Safed 3 seed cloves-(a variety released by National Horticultural Research and Development Foundation, Delhi) collected and then commercial seed cloves of Metupalayam type (MPD) were collected. They were dibbled in beds of 2 m^2 size at a spacing of 15 cm × 8 cm in randomized block design with four replications. The recommended package of practices of Kerala Agricultural University was followed for raising the crop during May–September 2020. The plant samples were harvested and cured as per the traditional practices of farmers where the bulbs were heaped immediately after harvest along with the leaves in a circular manner with bulb inside and leaves toward periphery. After 48 h, the outer leaves were stripped off. The present study was conducted at Kanthalloor and Vattavada panchayats of Idukki district, Kerala, India (10°C 13′ N latitude and 77°C 11′ E longitude, 5800 feet above Mean Sea Level). The area represents a rain shadow region with a tropical sub-humid monsoon climate having an annual temperature of 27.73°C and a rainfall of 1703.71 mm. Soil texture of the experimental site was loam to clay loam.

Analysis of morphological characters and biochemical parameters

The morphological characters of traditional landraces of Malappondu (Accession MLPD), Singappupoondu (Accession SGPR), Ooty 1, Yamuna Safed 3, Mettupalayam varitety (MPD) were analyzed and recorded as per IBPGR descriptor for leaf shape, leaf habit, bulb shape in cross section, clove distribution, and bulb color. The yield parameters, namely cured bulb weight (g), number of cloves per bulb, and clove weight (cm) were observed. Biochemical parameters of bulbs harvested at maturity were also recorded. TSS (brix), total sugar (%), ascorbic acid (mg/100 g), and essential oil (%) were estimated. All the data were statistically analyzed to test the significance of difference among the genotypes using the R based software.

Molecular marker analysis using simple sequence repeats

Molecular marker of traditional landraces of Malappondu (Accession MLPD), Singappupoondu (Accession SGPR), Ooty 1, Yamuna Safed 3, Mettupalayam type (MPD) was analyzed using SSRs. The molecular analysis was done at the biotechnology laboratory of Junagadh Agricultural University, Gujarat, India. The DNA was extracted by using Doyle and Doyle method with minor modifications.[7] The three landraces MLPD, MPD, and SGPR along with the two promising garlic varieties, Ooty-1 and Yamuna Safed -3 were analyzed for genetic diversity using SSR markers. DNA quantification was done using a Pico drop spectrophotometer, and DNA sample was diluted using TE buffer up to 50 ng/µl. Quality of sample DNA was checked by 0.8% Agarose gel electrophoresis. SSR-PCR was carried out in Veriti ABI thermal cycler. Initially, a set of 36 SSR primers was used for screening the five genotypes of garlic. Among the 36 SSR primers, only 18 primers were able to amplify the garlic DNA. Hence, only 18 SSR primers were further used for studying genetic variation among the garlic genotypes. After the PCR amplification of all the garlic genotypes by SSR primers, the PCR product was loaded on the 2.5% agarose gel. The resolved amplification products were visualized by illumination under UV light in the gel documentation system. Data generated using SSR primers were scored in binary format and processed using NTSYS pc version 2.2 software.

Gas chromatography-tandem mass spectrometer analysis

Traditional landraces of Malappondu (Accession MLPD), Singappupoondu (Accession SGPR), Ooty 1, Yamuna Safed 3, Mettupalayam varitety (MPD) were subjected to GC-MS analysis to identify chemo-profiling variations. Chemoprofiling of various garlic samples was done by GC-MS analysis. Chromatographic columns used were HP5MS (30 m × 0.25 mm × 0.25 µm) with Helium as carrier gas at a flow of 2.97 $ml \cdot min^{-1}$. The column temperature was maintained at

50°C for 1 min, programmed at 8°C/min to 90°C, then 4°C/min to 120°C and then at 10°C/min to 200°C, which was held for 5 min. The mass spectrometer was operated in the electron impact ionization mode with electron energy of 70 eV and temperature of 230°C. The transfer line temperature was 200°C. Scan range was 30–800 m/z. The components of volatile compounds were identified using NIST14 library.

RESULTS AND DISCUSSION

Morphological characterization

The results of number of cloves per bulb in garlic genotypes indicated significant difference and Yamuna safed 3 showed the highest number (15) followed by Ooty 1 (12), MPD garlic (11.5), MLPD garlic (9.5), and SGPR garlic (8.5), as shown in Figure 1. The clove weight of garlic genotypes was found to be 1.82 g in MPD garlic, 1.76 g in Ooty 1 garlic, 1.71 g in MLPD garlic 1.66 g in SGPR garlic, 1.36 g in Yamuna safed 3 garlic and is shown in Figure 2. There was no significant variation in the bulb weight of garlic genotypes, and it was found to be 14.5 g in MLPD garlic, 16.2 g in MPD garlic, 14.7 g in SGPR garlic, 15.4 g in Ooty 1 garlic, and 14.8 g in Yamuna safed 3 garlic, respectively, and shown in Figure 3.

Biochemical parameters

Profound differences were observed in the biochemical characters of garlic genotypes. The essential oil content of garlic genotypes was found to be 0.325% in MLPD garlic, 0.22% in MPD garlic, 0.2% in SGPR garlic, 0.125% in Ooty 1 garlic, 0.22% in Yamuna safed 3-garlic respectively and shown in Figure 4. The ascorbic acid content of garlic genotypes was found to be 7.64 mg/100 g of MLPD garlic, 8.8 mg/100 g of MPD garlic, 15.2 mg/100 g of SGPR garlic, 16.50 mg/100 g of Ooty 1 garlic, 12.6 mg/100 g of Yamuna safed 3-garlic, respectively, and shown in Figure 5. The oleoresin content of garlic genotypes was found to be 0.67% in MLPD garlic, 0.90% in MPD garlic, 0.64% in SGPR garlic, 0.86% in Ooty 1 garlic, 0.64% in Yamuna safed 3-garlic, respectively, and shown in Figure 6. The total sugar content of garlic genotypes was found to be 29.4% in MLPD garlic, 17.2% in MPD garlic, 33.5% in SGPR garlic, 31.41% in Ooty 1 garlic, and 28.3% in Yamuna safed 3-garlic, as shown in Figure 7. Significant difference was observed in bulb and clove morphological characters of garlic genotypes as reported by Petropoulos *et al.*[8] An essential oil content 1.1 percent (v/w) was reported in Kerala garlic by Antony *et al.*[9] Our study, clearly indicated that MLPD garlic had high essential oil content. SGPR garlic and of Ooty 1 garlic having high content of ascorbic acid than MLPD or MPD garlic.

Molecular marker analysis

The genetic diversity of local landraces of Kerala was assessed using 18 SSR markers and depicted in Figures 8-10 as vertical columns with horizontal light bands on a dark background. The molecular analysis detected 45 bands and two unique bands. The unique band for Acc. SGPR garlic and Yamuna Safed 3 garlic was generated by the primer 11 at around 500 bp and 1100 bp, respectively.

The dendrogram generated from the Jaccard's similarity values using NTSYS software based on 18 SSR primers is presented as Figure 11. The dendrogram of genetic distances among five tested local accessions

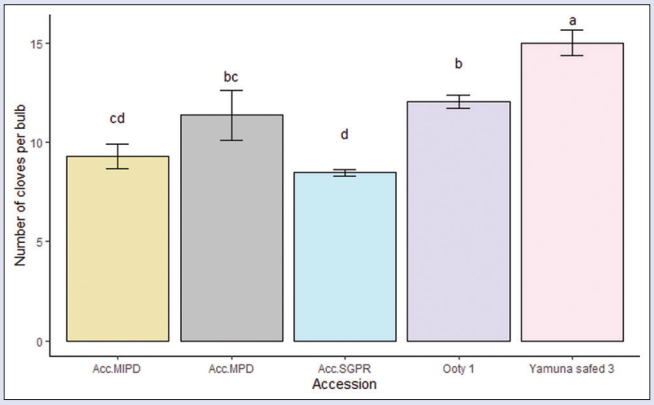

Figure 1: Number of cloves per bulb of garlic genotypes

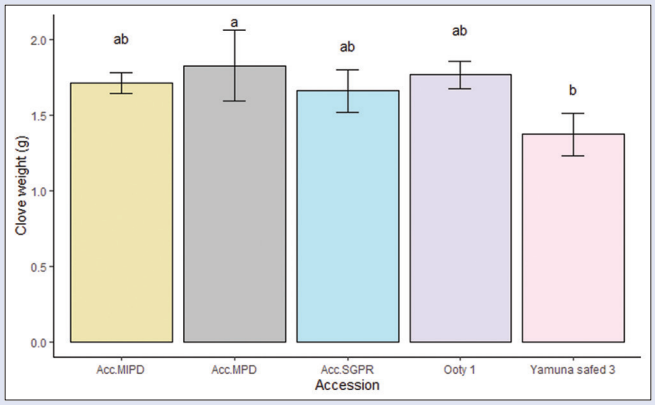

Figure 2: Clove weight of garlic genotypes

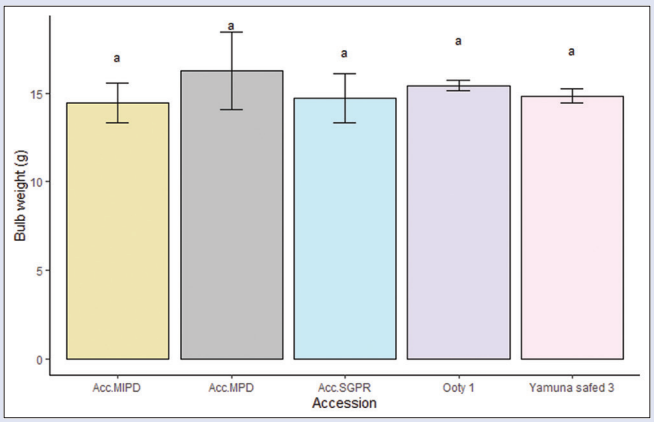

Figure 3: Bulb weight of garlic genotypes

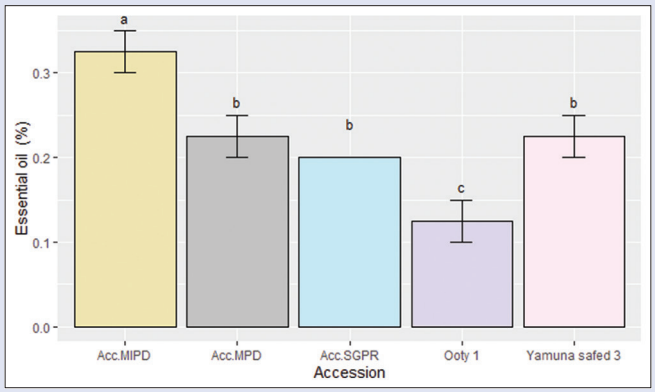

Figure 4: Essential oil content of garlic genotypes

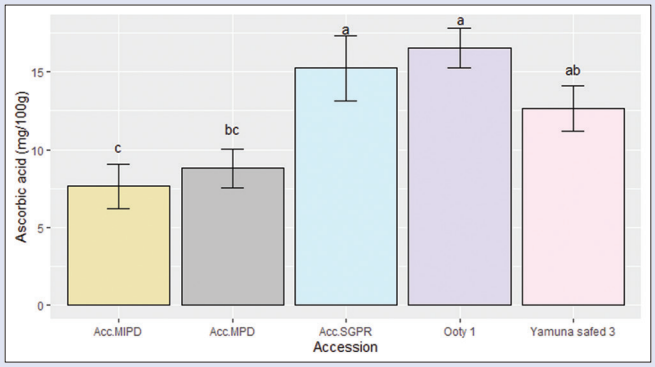

Figure 5: Ascorbic acid content of garlic genotypes

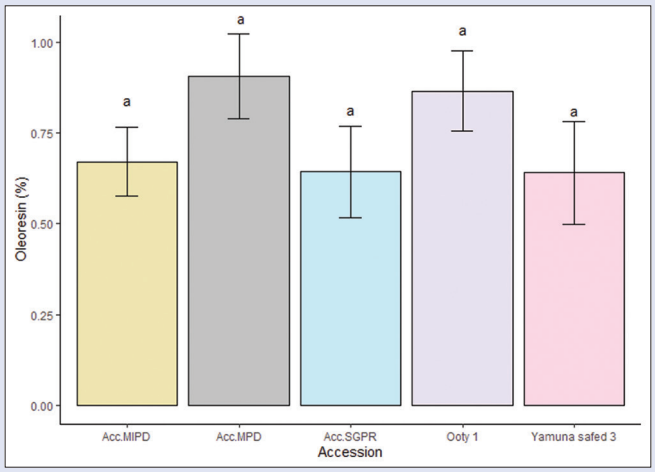

Figure 6: Oleoresin content of garlic genotypes

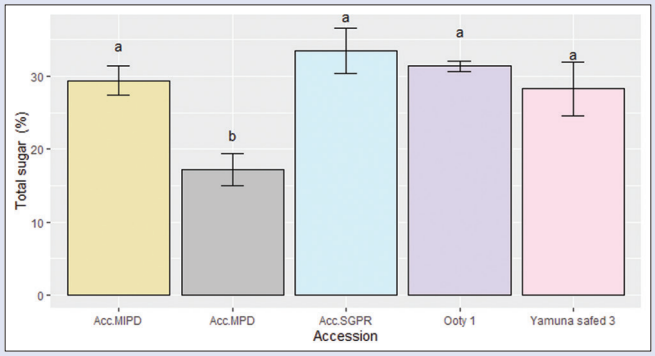

Figure 7: Total sugar content of garlic genotypes

showed three clusters at 92% similarity level. A level of polymorphism was found among the clones with SSR markers. The local accession SGPR garlic and the released variety Yamuna Safed 3 garlic were included in cluster 1 and MPD garlic and Ooty 1 garlic in cluster 2 with 94% similarity. The garlic genotypes in cluster 1 shared 84% similarity with accessions in cluster 2 and the landrace MLPD.

The difference in adaptation of garlic to diverse ecogeographic conditions and local selection pressure may be involved in the molecular diversity using SSR markers within a particular geographical region.[10] In our study, the hill garlic accession MLPD falls independent and showed its uniqueness at the molecular level. This variation at DNA level was also reflected by its peculiar properties, especially the high storability at field level and its use in traditional folk medicines.

Gas chromatography-tandem mass spectrometer analysis

The principal local landraces SGPR and MLPD are well adapted to the climatic conditions of high hills of Kerala. The seed materials of these local land races with high storability are being conservd by few farmers and tribes of the locality, while the commercial growers depend on MPD and the seeds are being regularly purchased from the open market in the neighboring state. The seeds of MPD cannot be stored up to the next cropping season as done in the cases of local accession SGPR and MLPD. The variability among these accessions were also identified at the molecular level. Therefore, it is interesting to reveal the differences in the chemical composition of essential oils from these unique land races, commercial cultivars and released varieties of garlic, which may also add to its potential pharmacological differences. GC-MS analysis of essential oils (total number of active constituents) from MLPD garlic, MPD garlic, SGPR garlic, Ooty 1 garlic, Yamuna safed 3 garlic was performed and variations were found in the concentrations of active constituents [Table 1]. MLPD garlic had 10 total number of active constituents. MPD garlic having 14 total number of active constituents. MLPD garlic had 14 total number of active constituents. SGPR garlic had 6 total number of active constituents. Ooty 1 garlic had 13 total number of active constituents. Yamuna safed 3 garlic had 7 total number of active constituents. Lai *et al*. reported that higher content of sulfur atoms can be correlated to stronger biological activity.[11] The increased activity of sulfur compounds may be due to increased allinase acivity.[12] Our study showed that highest total sulphides content was found in Yamuna Safed 3 garlic (96.99%), SGPR garlic (96.87%), MLPD garlic (90.99%), MPD garlic (82.24%), and Ooty1 garlic (64.96%) [Table 2]. Several studies reported that trisulfide, di-2-propenyl, and diallyl disulphide (DDS) were the major components in garlic and found to have therapeutic activites such as anti-platelet aggregation, anticancer, antimicrobial, and hypolipidemic activities.[13,14] In the present study, 2.2% of disulfide, 1-(1-propenylthio) propylpropyl was noticed only in SGPR. Local accession MLPD also recorded a higher concentration of trisulfide, methyl 2-propenyl (9.34%). Two compounds dimethyl trisulfide (1.42%) and 1,3,2-Oxathioborolane, 2-ethyl-5-methyl-(1.12%) were only noticed only in local accession MLPD. The DDS content was 37.16% in SGPR and 34.25% in MLPD. Whereas, the garlic varities, Ooty 1 and Yamuna Safeed 3 exhibited 25.13% and 36.23% of DDS**.** In our study, it is interestingly noted that the ascorbic acid content was the highest in Ooty 1 garlic (16.50 mg/100 g) and in SGPR garlic (15 mg/100 g), whereas it was lowest in MLPD garlic (7.64 mg/100 g).

Singh *et al*. reported that octa atomic sulfur was formed by L-cysteine degradation and rest by L-ascorbic acid degradation. Cyclic octa atomic sulfur is having medicinal properties like action against common cold, hay fever, dandruff, acne, scaly and red skin patches (seborrheic dermatitis), control of blood glucose levels, modulation of the immune system, hepatoprotection, and bacteriostasis.[15] In our study, GC-MS/MS analysis showed presence of cyclic octa atomic sulfur in garlic samples. Highest concentration of cyclic octa atomic sulfur (3.94%) was recorded in the traditional accession MLPD. Rahman reported that organosulfur compounds showed cancer-preventive agents by modulating the acivity of enzymes. Allicin (diallyl-thiosulfinate) is one of the major organosulfur compounds in garlic considered to be biologically active.[16] Al-Taai *et al*. reported that appearance of 3-vinyl-1,2-dithiacyclohex-5-ene content in the garlic was due to

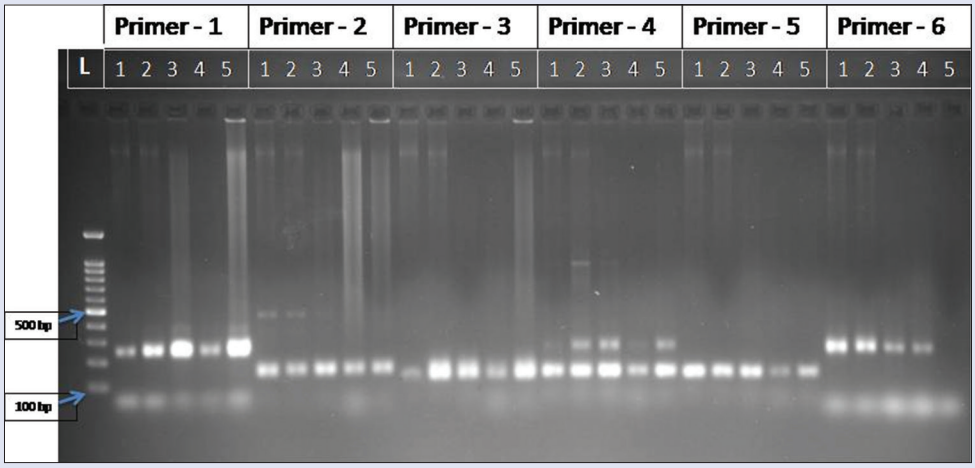

Figure 8: Simple sequence repeat primer 1 to 6 banding pattern on agarose gel for sample 1–5

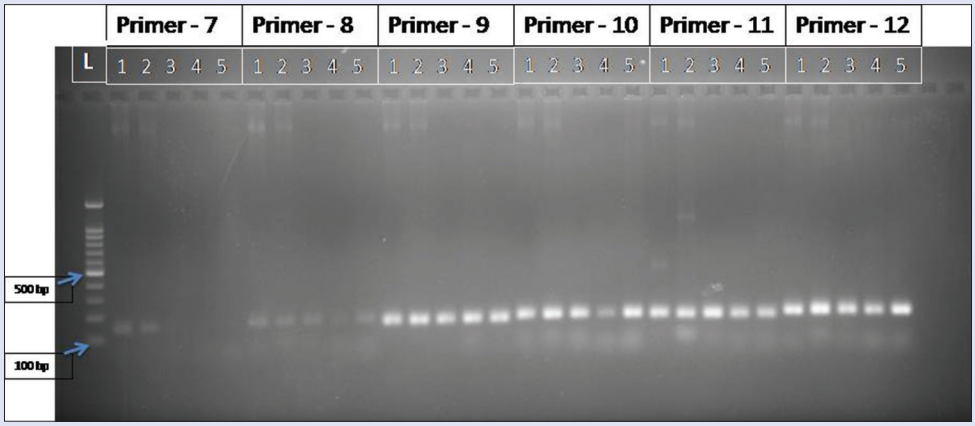

Figure 9: Simple sequence repeat primer 7 to 12 banding pattern on agarose gel for sample 1–5

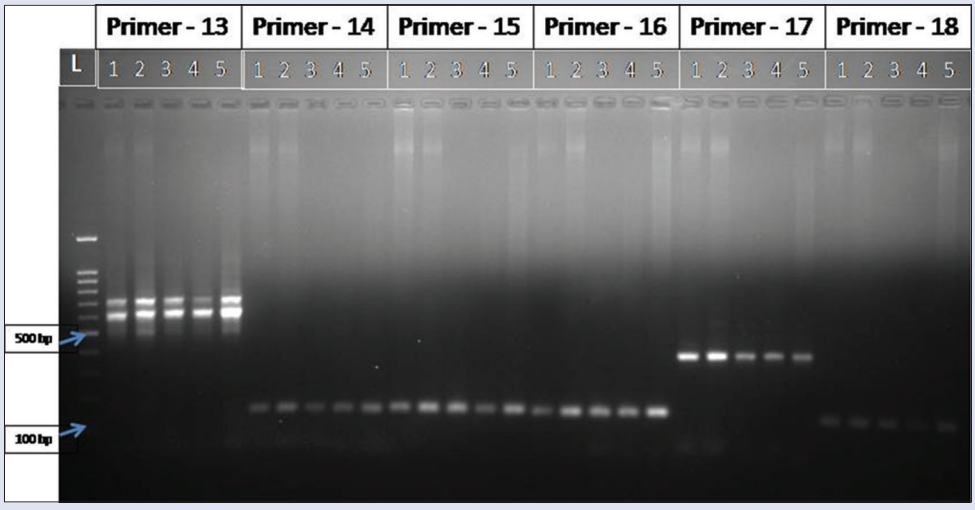

Figure 10: Simple Sequence Repeat primer 13–18 banding pattern on agarose gel for sample 1–5

enzyme hydrolysis of allicin.[17] In our study, it was observed that 3-vinyl-1,2-dithiacyclohex-5-ene (1.89%) was present only in Ooty-1 garlic. The unique chemical composition in local accessions may be the reason behind their use in traditional folk medicines.

CONCLUSION

The traditional landraces investigated in this study was well adapted to the peculiar agro climatic situation in the hill agriculture system of

Table 1: Active constituents in the essential oil of garlic genotypes

Component name Name of compound	CAS ID	Peak area percentage				
		MPD garlic	SGPR garlic	Ooty 1 garlic	MLPD garlic	Yamuna safed 3 garlic
Disulfide, methyl 2-propenyl (C4H8S2)	2179-58-0	2.74	0.57	5.91	6.9	5.22
2- Vinyl-4H-1,3-dithiine (C6H8S2)	80028-57-5	1.1			0.86	
Diallyl disulphide (C6H10S2)	2179-57-9	24.28	37.16	25.12	34.25	36.23
(E)-1-Allyl-2-(prop-1-en-1-yl) disulfane (C6H10S2)	122156-02-9	2.33				
Trisulfide, methyl 2-propenyl (C4H8S3)	34135-85-8	7.24	2.94	6.19	9.34	7.34
2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-(C7H10O2)	21835-01-8	1.02				
Trisulfide, di-2-propenyl (C6H10S3)	2050-87-5	47.98	53.98	27.74	39.08	48.2
1-Methyl-1-(2-buty) oxy)-1-silacyclobutane (C8H18OSi)	1000216-97-5	2.41		5.52		0.55
1,2-Benzisothiazole, 3-methoxy-, 1,1-dioxide (C8H7NO3S)	18712-14-6	1.87				
9,10-anthracenedione, 1-amino-4-[(4-methylphenyl) amino]- (C21H16N2O2)	1000402-38-7	1				
Benzofuran-5,6-diol-3-one, 2-benzylidine-(C15H10O4)	1000128-65-2	2.92				
Cyclic octa atomic sulphur (S8)	10544-50-0		1.38	1.04	3.94	1.53
Disulfide, 1-(1-propenylthio) propylpropyl (C9H18S3)	143193-11-7		2.22			
3-Vinyl-1,2-dithiacyclohex-5-ene (C6H8S2)	62488-53-3			1.89		
3H-1,2-Dithiole (C3H4S2)	288-26-6			1.03		
Ethanone, 1-cyclohexyl- (C8H14O)	823-76-7			4.03		
1-Butanol, 3,3-dimethyl- (C6H14O)	624-95-3			17.1		
(E)-sec-Butyl propenyldisulfide (C7H14S2)	24351-71-1	0.48		1.84		0.93
2-Methyl-3-(methylthio) furan (C6H8OS)	1000360-40-6	0.69		1	0.82	
(E)-Allyl (prop-1-en-1-yl) sulfane (C6H10S)	104324-36-9	0.97		0.36	1.38	
Dimethyl trisulfide (C2H6S3)	3658-80-8				1.42	
1,3,2-Oxathioborolane, 2-ethyl-5-methyl-(C5H11BOS)	1000163-05-5				1.12	
Total number of compounds identified		14	6	13	10	7

MPD: Mettupalayam variety; SGPR: Singappupoondu variety; MLPD: Malappondu variety

Table 2: Total sulphides concentration in the essential oil of garlic genotypes

Name of Compound	Peak area percentage				
	MPD garlic	SGPR garlic	Ooty 1 garlic	MLPD garlic	Yamuna safed 3 garlic
Total disulphides	27.02	39.95	31.03	41.15	41.45
Total trisulphides	55.22	56.92	33.93	49.84	55.54
Total sulphides	82.24	96.87	64.96	90.99	96.99

MPD: Mettupalayam variety; SGPR: Singappupoondu variety; MLPD: Malappondu variety

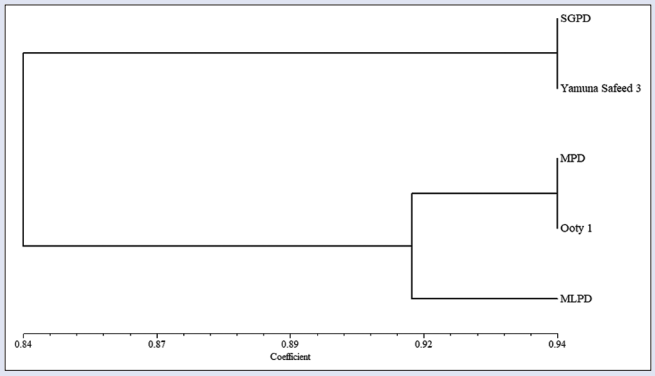

Figure 11: Dendrogram of five garlic genotypes using simple sequence repeat combination markers

Kerala and are being regularly cultivated and conserved, especially by *Muthuva* tribal community. Accessions MLPD garlic and SGPR garlic are being conserved by the tribes due to its good shelf life, used in the Ayurveda preparation and good price in the local market. The accessions performed as good as released varieties and need to be conserved to sustain the gene pool. They have unique chemical composition which could have been the reason for its use in the traditional home remedies and health-care systems. These land races were also elucidated at molecular level and found different from the popular released varities in South India. This study concluded that morphological, biochemical, molecular, and GC-MS analysis has proved the uniqueness of traditional landraces of MLPD garlic and SGPR garlic with high storability at field level and its use in traditional home remedies.

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Conflicts of interest

There are no conflicts of interest.

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