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Genetic Differentiation of *Syzygium aromaticum* Among the Altitudes Of Nilgiris

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ABSTRACT

Clove (*Syzygium aromaticum*) is one of the most economically significant oil producing trees in India, whose importance is mainly due to its medicinal properties. Unfortunately, importing rate of the spice is increasing which would be an economic drawback for the country. Therefore, information regarding genetic diversity and the oil quality of the tree is necessary to increase the cultivation. Thus the present work was conducted to identify the genetic differences between the collected samples growing at different altitudes of Nilgiris in comparison to the extracted oil quality to select high quality oil yielding variety for cropping. RAPD and GC-MS techniques were used for analyzing genetic diversity and oil extraction, respectively. The phylogeny constructed revealed that the samples S1& S6 are genetically more similar, whereas S2 is far distinct from all other samples. S3 shared a close relation with S1 & S6. Samples S4, S5 were separated out but are within the same node. The eugenol percentage of the samples S1, S2, S3, S4, S5 and S6 were 42.96%, 68.78%, 55.95%, 71.17%, 74.04% and 73.47%, respectively. From the collected data it is inferred that though the samples shared genetic similarity there observed a considerable difference in the eugenol content. This information needs to be correlated with the cultivation practices and soil nutrition based on which commercial cropping could be done to improvize the clove sector of the country.

Key Words:

Clove oil, GC-MS, Genetic differentiation , RAPD, Syzygium

INTRODUCTION

Clove [*Syzygium aromaticum* (Myrtaceae)], already well known as a spice, a food preservative is becoming increasingly important for its medicinal values such as its anti-oxidant, anti-septic, local anesthetic, anti-inflammatory, rubefacient (warming and soothing), carminative and anti-flatulent properties. They can be used to stimulate the mind as well as prevent nausea, diarrhoea, ease coughs, aid in digestion, and even treat conditions like malaria and cholera (Bhowmik et al. 2012). It represents one of the richest sources of phenolic compounds such as eugenol, eugenol acetate and gallic acid. Eugenol is a phenyl-propanoids class of chemical compound which gives pleasant sweet aromatic fragrances to

the clove bud. Eugenol has local anesthetic and anti-septic properties, hence useful in dental care essentials as well as in the treatment procedures (Shrivastava 2006; Dhiman 2006). In an earlier report, seven compounds have been isolated and characterized from *Syzygium aromaticum* namely oleanolic acid lactone , -sitosterol , nigricin , flavaellagic acid , 2 hydroxyoleanolic acid, 3 -hydroxy-11-oxo-olean-12-en-28-oic acid and -sitosterol 3-O- -D-glucopyranoside(Begum et al. 2014).

India is one of the producer, consumer, and exporter of clove, which is traded mainly in the form of wholedried flower buds, as powder, as stem and in oil forms. Approximately, 72-90% of the essential oil extracted from cloves has Eugenol. Other essential oil ingredients reported by (Bowmik et al. 2012) areAcetyl Eugenol, Beta-Caryophyllene, Vanillin, Crategolic acid, tannins, Gallotannicacid, Methyl Salicylate (painkiller), Flavanoids, Eugenin, Kaempferol, Rhamnetin, Eugenitin, triterpenoids like Oleanolicacid. The dried buds of cloves contain about 15-20 percent of essential oil, and the bulk of this is Eugenol. A kilogram of dried buds provides about 150 ml. of Eugenol. Recent reports on the medicinal value of cloves in treating a variety of ailments have further increased the demand for cloves all over the world. Consumer preference for organic products has also spurred the demand for clove at the same time, as with other organic products, especially those that are sold in oil form.

As part of thework, Random Amplification Polymorphic DNA(RAPD) was used to investigate the genetic diversity of *S. aromaticum,* since morphological and morphometric study is time-consuming, laborious and consequently not suitable for large-scale application. DNA polymerase assay based on RAPD-PCR has been proved useful for analyzing the inter and intra specific genetic variations and phylogenetic relationships. RAPD technique is based on the amplification of a random DNA segment with a single primer of arbitrary nucleotide sequence using polymerase chain reaction. This technique is very rapid, simple and generates a reproducible fingerprint of the PCR products.

In addition, GC-MS technique is used to analyze the Eugenolcontent of *S. aromaticum.* Gas Chromatography-Mass Spectrometry(GC-MS) is a hyphenated analytical technique that combines the separation properties of gas-liquid chromatography with the detection feature of mass spectrometry to identify different substances within a test sample. GC-MS has become a highly recommended tool for monitoring and tracking organic compounds in the test sample (Choi and Chung 2014).

In India clove is grown in the hilly areas of Tamilnadu, Kerala and Karnataka. The important clove growing districts in India are Nilgiris, Tirunelveli, Kanyakumari, Nagercoil and Ramanathapuram districts of TamilNadu: Kozhikode, Kottayam, Kollam and Thiruvananthapuram districts of Kerala and South kanara district of Karnataka. Eventhough, Nilgiris is one of the producers of *S. aromaticum*, the genomic based research works to identify the genetic difference.works on high quality oil yielding varieties, etc. is limited but is very much required for development of the species. This could be possible by phylogenetic analysis.

The present research work describes the use of RAPDto identify the genetic difference between the collected samples in comparison to the extracted oil quality using GC-MS technique to select high quality oil yielding variety for cropping.

MATERIALS AND METHODS

Six clove leaf samples and flower buds from six different sampling points namely S1 (Athipali, Gudalur), S2 (Baaram, Gudalur), S3 (Maamaram, Kotagiri), S4 (Kallar, Coonoor), S5 (Barliar, Coonoor) and S6 (Muloor, Kotagiri) were used for Genetic Diversity analysis using RAPD markers. Oil quality analysis was performed using GC-MS technique.

DNA isolation protocol

Total genomic DNA from the plants was

isolated by N- Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method. 2gm of the sample was weighed and transferred to a sterile mortar and pestle; 50mg of Polyvinylpyrolidone was added to the sample and ground well. 750 l of extraction buffer was added and incubated at 65°C for 2 hours. Centrifuged at 10000 rpm for 10 min at 4°C.Clear solution was collected in a sterile microcentrifuge tube. Equal volumes of Chloroform: Isoamyl alcohol (24:1) was added. Centrifuged at 10000 rpm for 10 min. at 4°C.The aqueous phase was removed and taken in a sterile microcentrifuge tube. 0.6 volumes of isopropyl alcohol was added and incubated at room temperature for 1hour. Centrifuged at 10000 rpm for 10 min. Pellet was washed in 500 l of 70% ethanol. Centrifuged at 10000 rpm for 10 min at room temperature. Pellet was dried and dissolved in 20 l sterile distilled water.

Lithium Chloride Precipitation:

DNA sample of 10μL was taken in a sterile 1.5ml tube and 90μl of sterile water was added and mixed well. Equal volume of Chloroform: isoamyl alcohol (24:1) was added to the tube and centrifuged at 10,000rpm for 10 minutes. The aqueous layer was transferred to a new 1.5 ml tube and 200μl of 0.5M lithium chloride was added and incubated at C overnight. The tube was centrifuged at 10000 rpm for 10 min, the supernatant was discarded. To the pellet 500 l of 70% ethanol was added and centrifuged at 10000 rpm for 10 min. The pellet was dried and dissolved in 20 l sterile distilled water. The samples were stored at -20° C.

Quantification of Isolated DNA

The quantity of the isolated DNA was checked in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). From the stock 1 l DNA was mixed with 9- l sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

PCR Amplification

PCR amplification was done in 20 l of reaction volume containing 1x PCR buffer (Kappa, SA); $MgCl_2$- 3 mM; dNTP mix- 0.25 mM; *Taq* DNA polymerase- 0.05 U; primer- 1 pmol and template DNA- 50 ng. Sterile nuclease free water was used as negative control.

Agarose gel analysis of RAPD-PCR

Agarose powder of 2 gram was measured and added to a 250 ml conical flask. To the flask 100 ml TAE (1X) buffer was added and mixed properly. The agarose was melted in a microwave or hot water bath until the solution became clear. The solution was allowed to cool to about 50-55°C with swirling of the flask occasionally to cool evenly. 1-2 μl of Ethidium Bromide solution was added to the melted agarose gel and mixed well. Ends of the casting tray were sealed and combs were placed. Molten Agarose solution was poured into the casting tray without forming air bubbles and was let to cool until it became solid. The combs were carefully pulled out; the tape removed and the gel was placed in the electrophoresis chamber. Sufficient amount of 1X TAE Buffer was added so that there was about 2-3 mm of buffer over the gel. 6 μll of 6x Sample Loading Buffer was added to each 25 μll of DNA sample. Each sample/ DNA ladder was carefully pipetted into separate wells in the gel. Electrode wires were connected to the power supply, making sure the positive (red) and negative (black) are correctly connected. Power supply was turned on to about 100 volts (should not exceed 5 volts/ cm between electrodes). After sufficient run power packwas turn off and the gel was removed by using gloves. The DNA in the gel was visualized with U.V. light, photographed and documented using UV trans-illuminator.

Clove Oil extraction and analysis

Twenty gram of clove sample was powdered separately to extract oil by steam distillation. The powder was added to 150ml of sterile distilled water in a 500 ml round bottom flask for distillation. The distillate was collected in a 125 mlE. flask. The distillate was extracted in ether. Continuous heating was provided from a heating mantle at a steady rate of 1 drop/2-3 seconds. The water level was maintained and distilled until no oily layer was apparent. At least

200 ml of distillate was collected which is a water-clove oil mixture. To separate clove oil from water, the distillate was transferred to a separation funnel and 10 ml of saturated NaCl solution was added. Condenser and receiving flask was rinsed with 5 mL of ether and transferred to the separation funnel. The mixture was allowed to separate after which the ether was separated out. The same was repeated twice to get more purified clove oil and it was stored in airtight glass vials. Thus extracted oil was analysed for Eugenol content by gas chromatography.

RESULTS AND DISCUSSIONS

The intrinsic genetic diversity in present study on *S. aromaticum* accessions was apparent from the analysis of their RAPD profiles and from dendrogram generates where all the accessions had been unambiguously separated from each other. RAPD studies have been widely used for population genetic studies in both wild (Dikshit et al. 2007, Yao et al. 2008) and cultivated plants(Nagaoka and Ogihara 1997; Sikdar et al. 2010). Many unique species-specific alleles were amplified by RAPD markers(Hardik, et al. 2015). The six clove samples collected from different locations were analyzed for their genetic similarity using RAPD. Genomic DNA from six clove leaf samples wereused to generate RAPD patterns with 10 RAPD markers which resulted in different banding pattern ranging from 100 – 3000bpas shown in Fig. 1 - 6. To assess the degree of homology between shared bands, a different set of decamer primers was used on a minimal species set. The primers were OPAA01 (5'AGACGG CTCC3'), OPAA02(5' GAGACCAGAC 3'), OPAA03(5' TTAGCGCCCC3'), OPAA04(5' AGGACTGCTG3'), OPAA05(5' GGCTTTAGCC3'), OPAA06(5' GTGGGTGCCA3'), OPAA07(5' CTACGCTCAC3'), OPAA08(5' TCCGCAGTAG3'), OPAA09(5' AGATGGGCAG3'), OPAA10(5' TGGTCGGGTG3'). These OPAA primers were evaluated as part of a related experiment, and it is assumed that an estimate of homoplasy made with these primers will be applicable to RAPD generated patterns. For construction of a dendrogram with 167 bands scored for presence and absence as 1 and 0, respectively. Using PAST statistical software diversity between the samples were analyzed using paired group algorithm with Euclidean distance matrix.

The evolution of varieties in distinct agro-climatic zones demonstrates significant levels of variation in response to the selection pressure in the zones (Singh et al, 1998). It is therefore, not surprising to find significant levels of polymorphism among 6 genotypes of clove in RAPD. There are several reports confirming that RAPD technique has been applied to assess molecular polymorphism in *Morchella* sp. (Irfan et al. 2017), pea (Simioniuc et al. 2002). The success of our study in polymorphism was due to the use of number of randomly selected prescreened highly informative primers. Geographically isolated populations accumulates genetic differences as they adapt to different environments. Genetic variation among clove genotypes based on RAPD analysis could be useful to select the best genotype for cultivation. The dendrogram constructed by this method is shown in Fig.7. It revealed that the samples S1 and S6 are genetically more similar, whereas S2 is far distinct from all other samples. S3 shared a close relation with S1 and S6. Samples S4, S5 were separated out but are within the same node.

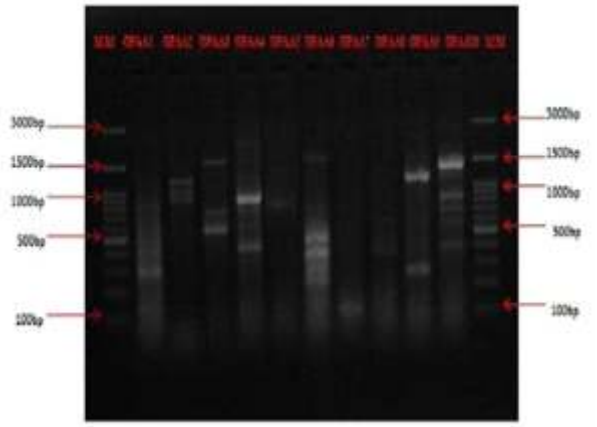

Fig.1. Banding patterns of sample 1 using 10 RAPD primers

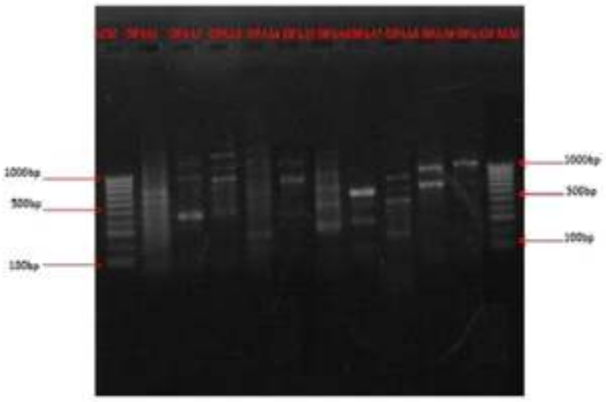

Fig.2. Banding patterns of sample 2 using 10 RAPD primers

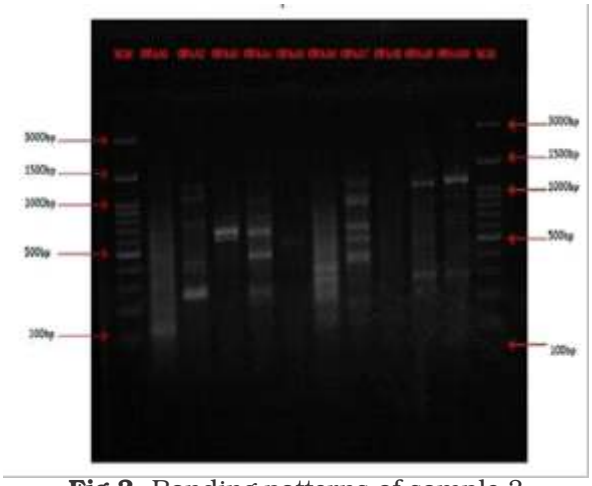

Fig.3. Banding patterns of sample 3 using 10 RAPD primers

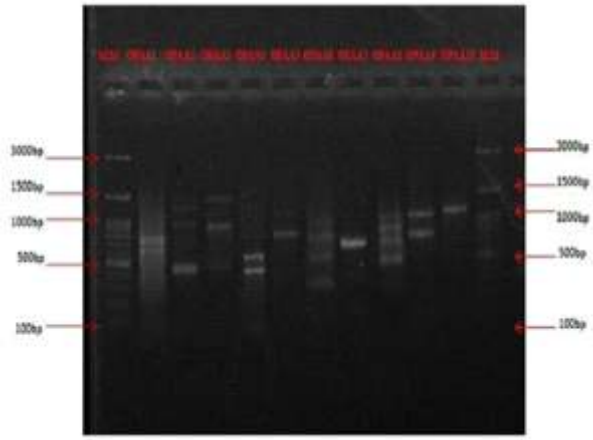

Fig.4. Banding patterns of sample 4 using 10 RAPD primers

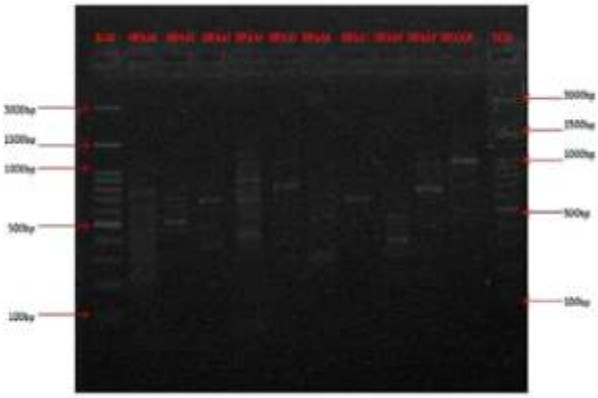

Fig.5. Banding patterns of sample 5 using 10 RAPD primers

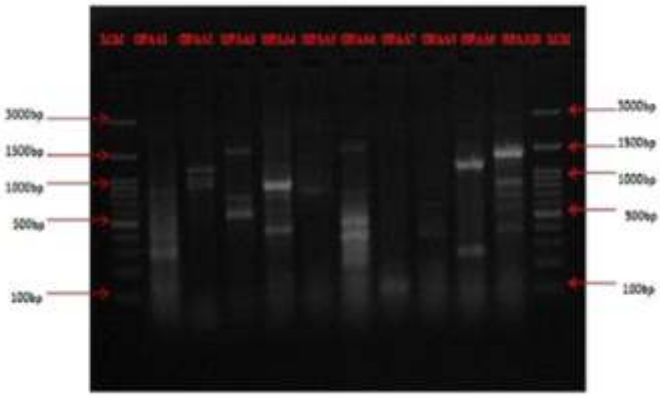

Fig.6. Banding patterns of sample 6 using 10 RAPD primers

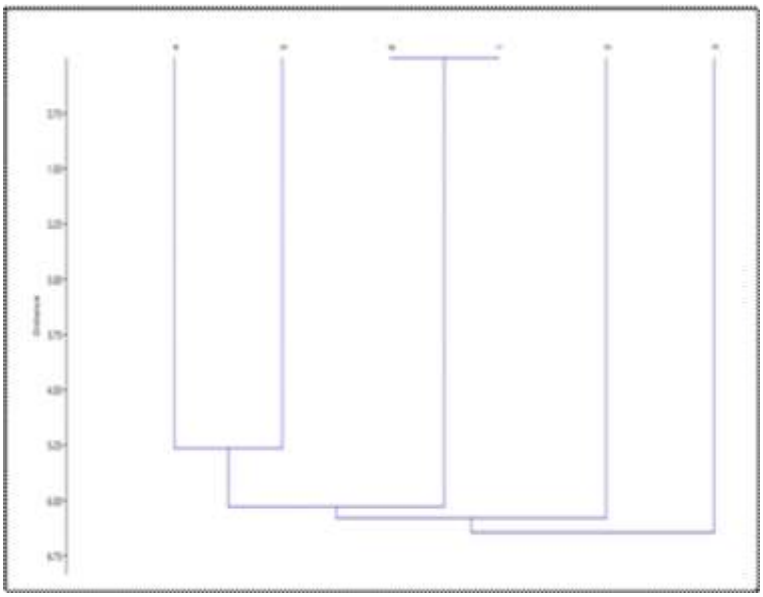

Fig.7. Dendrogram with Euclidean distance

Phytochemical studies of *S.aromaticum*carried out by other workers havereported, several group of compounds including '-sitosterol, nigricin, flavaellagic acid, 2' hydroxyoleanolic acid, 3' hydroxyl-11-oxo-olean-12-en-28-oic acid, etc.(Begum etal, 2014). Clove represents one of the major vegetal sources of phenolic compounds such as flavanoids, hydroxybenzoic acids, hydroxycinamic acids and hydroxyphenylpropens. Eugenol, a phytogenic bioactive component is the most important compound in clove. Eugenol is the main bioactive compound of clove, which is found in concentrations ranging from 9381.70 to 14650.00mg per 100 g of fresh plant material (Neveu et al). Eugenol has been approved to encompass numerous beneficial aspects against a capacious spectrum of life threatening indispositions including oxidative stress, inflammation, hyperglycemia, elevated cholesterol level, neural disorders and cancer(Khalil, 2017). Therefore, in the present study clove oil was extracted and Eugenolpercentage was analysed to compare the genetic diversity with the oil content and quality. Eugenol percentage of the samples S1, S2, S3, S4, S5 and S6 were42.96%, 68.78%, 55.95%, 71.17%, 74.04% and 73.47%, respectively. The Eugenol percentage analyzed from the extracted clove oil were all different for each sample although being genetically similar.

Clove oil extraction was done using GC-MS technique. Gas chromatography mass spectrometry (GC-MS) has become firmly established as a key technological platform for secondary metabolite profiling in both plant and non-plant species (Kell 2005). The main features of enhanced molecular ion, improved confidence in sample identification, significantly increased range of thermally labile and low volatility samples amenable for analysis, much faster analysis, improved sensitivity particularly for compounds that are hard to analyze are the compelling reasons to use the GC-MS in broad range of areas (ISO/IEC, 2005). GC-MS technique is a very useful tool for quality control, analytical research, impurity profiling and maintenance for human welfare and development (Chauhan 2014). Results of the present study revealed that samples S1 & S6 are genetically more similar but their Eugenol content was 42.96 and73.47 %, respectively. Similarly S4, S5 had71.17 and 74.04 %, respectively and they were genetically similar. It is observed that they have small variation in the oil content. Whereas S2, S3 had68.78 and55.95 % Eugenol content, respectively.

CONCLUSION

From the collected data it is inferred that though the samples shared genetic similarity there observed a considerable difference in the Eugenol content. This information needs to be correlated with the cultivation practices and the soil nutrition along with taxonomical and geographical information based on which commercial cropping could be done to improvise the clove sector of the country for boosting up the economy of the country through development of this species. Also, a specific primer could be designed in the obtained sequence as a SCAR marker for high quality oil yielding variety of *Syzygium aromaticum*.

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