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***Original Research Paper***

# Comparison of leaf volatile aroma constituents and phenolic acid profiles of the seedling originated polyembryonic mango (*Mangifera indica* L.) genotypes

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## ABSTRACT

**In mango, leaf and fruit volatile aroma profiles are variety specific which can be used as fingerprint of a variety. Such biochemical markers can also discriminate the nucellar and zygotic seedlings in polyembryonic mango varieties. In order to validate the applicability of volatile as well as phenolic acid profiles as biomarkers, the open pollinated seedlings of three polyembryonic varieties of mango were compared with their mother trees. Leaf volatile and phenol acid profiling were done using Gas Chromatography/Mass Spectrometry (GCMS) and Liquid Chromatography/Mass Spectrometry (LCMS) methods respectively. The sesquiterpene hydrocarbons were the most abundant in all the genotypes studied. Monoterpenoids were the major compounds in cultivars Vellaikolumban and Olour, while the sesquiterpenoids were the major compounds in *cv*. Turpentine. While terpinolene was the major monoterpenoid compound in Vellaikolumban and limonene in *cv*. Olour, the sesquiterpene á-gurjunene was the major compound in *cv*. Turpentine. Volatile profiling showed clear differences between the varieties but was similar within a variety. Among the 15 phenolic acids quantified in the leaves, P-coumaric acid, gallic acid, and ferulic acids were predominant whereas, vanillic acid, syringic acid, gentisic acid, benzoic acid, and sinapic acids were low in quantity. Phenolic acid profile did not show significant diversity among the varieties and therefore cannot be used for identification of varieties. The volatile profiling can be used for the identification and differentiation of polyembryonic mango genotypes.**

**Keywords:** GCMS, LCMS, mango, nucellar seedling, polyembryony

## INTRODUCTION

India has a large diversity of mangoes, with more than 1000 varieties (Salvi and Gunjate, 1988) that are grouped based on the number of embryos in the seed into monoembryonic and polyembryonic types (Mukherjee 1997). Most of the commercially grown varieties in India are monoembryonic while the polyembryonic varieties are used as rootstock since their apomictic seedlings arising from nucellus are known to be true to type. Each cultivar is distinguished by a unique combination of characters such as plant architecture, fruit size, color, taste, and flavor. Correct identification of varieties as well as discrimination of zygotic and nucellar seedlings is very important for crop improvement as well as for clonal rootstocks, even though morphological and molecular assessments have greatly aided in cultivar identification (Naik and Gangolly 1950, Ravishankar *et al*., 2000, Karihaloo *et al.,* 2003, Pandit *et al*., 2007). To complement this work, more reliable variety specific biochemical markers are a desirable attribute. There is a reliable variability in the volatile profile in mango cultivars (Andrade *et al.,* 2000). More than 270 aroma volatile compounds have been reported in various mango cultivars, including monoterpenes, sesquiterpenes, esters, aldehydes, ketones, alcohols, acids, aliphatic hydrocarbons (Shibamoto and Tang, 1990). Each of these volatile substances has its own distinct odour, and the combinations, quantities, and ratios of these molecules impart unique fragrance traits (Araguez and Valpuesta 2013). Mango leaves are a rich source of phenolic compounds such as xanthone-C-glycosides, gallotannins, benzophenones, flavonol glycosides, 5-alkyl- and 5-alkenylresorcinols and many other miscellaneous phenols (Barreto *et al.,* 2008) such as

kaempferol, quercetin, catechin, rhamnetin, gallic acid, benzoic acid, ellagic acid, tannins, flavonols, benzophenone, and their derivatives (Mwaurah *et al.,* 2020, Dorta *et al.,* 2014). In this study an attempt has been made to study the variability in leaf volatile and phenolic acid profiles of polyembryonic mango genotypes to identify their suitability as biochemical marker to identify the polyembryonic seedlings.

## MATERIALS AND METHODS

### Plant material

Three weeks old fresh mango leaves (top three) were taken from the OP seedlings of polyembryonic genotypes (Vellaikolamban, Olour, and Turpentine) conserved in the field gene bank of ICAR- IIHR, Bengaluru for HS-SPME and phenol profiling analysis. The volatile flavor constituents were analyzed by headspace-solid phase micro-extraction (HS-SPME) technique using GC–MS/MS and the phenol profiling were done by LC-MS/MS technique.

### Volatile profiling

### Solid phase micro extraction (SPME) of volatiles

The adsorption of analytes from the coated phase of fused silica fibre and partitioning of analytes between the stationary phase of the fibre and the extraction medium as gas constitute solid phase micro extraction. It consists of a 1-2 cm long fused silica fiber, coated with a stationary phase such as poly dimethyl siloxane (PDMS), divinyl benzene (DVB) and carboxen (CAR) or the mixture of all the three and bonded to a stainless-steel plunger and holder. These fibres are to be first conditioned at 250°C for 2-3 hours in the injector port of GC with the continued flow of Helium gas. In our study, ten grams of the fresh leaf was powdered using liquid nitrogen and taken in 100 ml conical flask along with a magnetic stirrer and then previously conditioned SPME fibre (Facundo *et al.,* 2013) was inserted to absorb the head-space volatiles for 2 hours. Fibre was subsequently injected into the GC-MS for the separation and identification of compounds.

### GC-MS analysis

GC-MS analysis was performed on Varian-3800 gas chromatograph coupled with Varian 4000 GC-MS/MS ion trap mass selective detector. The MS column was a fused-silica capillary column of 30 cm x 0.25 mm id, 0.25mm film thickness for the analysis. The injector temperature was set at 250ºC and all injections were split-less mode for 0.2 min, detector temperature was 270°C, and the temperature programs for the column was as follows: 40°C for 2 min at an increment of 3°C/min to 190°C, held for 1 min, then 5°C/min to 220°C and maintaining the constant temperature for 5 min. The mass spectrometer was set in the external electron ionization mode (EI) with the carrier gas helium at 1.5 mL/min; injector temperature at 250°C; trap temperature at 180°C, ion source-heating at 190°C, transfer line temperature at 260°C, EI-mode at 70 eV, with full scan-range 50-350 amu (Atomic mass unit). The total volatile production was calculated by the individual peak areas in the chromatogram, individual compounds identified by comparison of the spectra against the retention index determined using homologous series of n-alkanes (C5 to C32) as standard using two spectral libraries available as Wiley and NIST-2007, and expressed as relative percent area.

### Profiling phenols by LCMS

The phenolic acids for LC-MS/MS analysis was extracted using 80% methanol as previously described by Weidner *et al*. (2000) and Chen *et al*. (2001) with slight modification. 10 g sample was homogenized in methanol (80%), centrifuged and made up to 50 mL. 20 mL extract was taken and evaporated near to dryness under vacuum at 45°C and then diluted to 5 mL with water later extracted thrice with petroleum ether then in 40 mL of ethyl acetate using separating funnel. The aqueous layer was discarded and extract was ethyl acetate evaporated to dryness under vacuum at room temperature. To the dry residue, 4 mL of 2N NaOH was added and allowed to hydrolyze for overnight. Once acidifying to pH 2 using 5 mL 2N HCl, again re-extracted with 50 mL ethyl acetate. Ethyl acetate layer was again re-extracted twice with 25 mL of 0.1N NaHCO3. The ethyl acetate layer which carried the flavonoids was evaporated to complete dryness under vacuum, the residue was dissolved in 2 mL MS grade methanol filtered through 0.2µm nylon filter prior to injection in LCMS MS for flavonoids estimation. The aqueous layer was further acidified to pH 2 with 5 mL 2N HCl and extracted thrice with 25 mL ethyl acetate, the ethyl acetate layer was dried completely in rotary evaporator and the residue was dissolved in 2 mL MS grade methanol filtered through 0.2µm nylon filter prior to injection in LCMS MS for phenolic acid estimation.

### LC and MS-MS conditions

The phenolic acids were resolved on the analytical column BEH-C18 (2.1 x 50 mm, 1.7 µm) from Waters India ltd., protected by a Vanguard BEH C-18 (Waters, USA) with the gradient flow of organic and aqueous phase with the flow rate of 0.3mL/min. The column temperature was maintained at 25°C during analysis and the sample injection volume was 2µL. The eluted phenolic acids and flavonoids from the UPLC column effluent pumped directly without any split into the TQD-MS/MS (Waters, USA) system optimized for the analysis of the phenolic acid.

Statistical analysis (Pearson Correlation) was performed by the web-based portal OPSTAT (Sheoran *et al*., 1998).

## RESULTS AND DISCUSSION

### Volatile profiling

In the three polyembryonic seedling originated plants of three varieties, the leaf volatile profile was generated, using GCMS/MS. The volatiles varied significantly among the genotypes. The most abundant hydrocarbons were monoterpenes and sesquiterpenes in all the three genotypes. In Vellaikolumban and Olour genotypes (Table 1 and 2), the monoterpenoids were maximum while the sesquiterpenoids were minimum but in *cv*. Turpentine (Table 4) sesquiterpenes were maximum. Among the monoterpenoids, the terpinolene was the major volatile compound followed by α-Pinene in the 3 seedling originated plants of cv. Vellaikolumban while sesquiterpenoids β-elemene, γ-cadinene and δ-Cadinene were found to be the minor

**Table 1 : Relative peak area (%) of leaf volatile compounds of genotype Vellaikolumban using SPME based GC-MS analysis and their correlation among plants**

| Volatile compound | $VP_1$ | $VP_2$ | $VP_3$ |
|---|---|---|---|
| α-Pinene | 10.577 | 7.218 | 7.195 |
| Camphene | 1.077 | 0.729 | 0.700 |
| β-Pinene | 3.618 | 2.817 | 3.055 |
| Sabinene | 1.906 | 2.140 | 1.628 |
| 3-Carene | 5.541 | 6.494 | 5.830 |
| α-Terpinene | 2.072 | 2.269 | 1.034 |
| Limonene | 2.416 | 2.359 | 1.974 |
| cis-Ocimene | 1.314 | 1.315 | 1.115 |
| trans-Ocimene | 1.870 | 2.156 | 1.184 |
| Terpinolene | 49.423 | 57.821 | 50.252 |
| α-Copaene | 0.585 | 0.367 | 0.865 |
| (-)-β-Elemene | 0.288 | 0.126 | 0.449 |
| β-Caryophyllene | 3.921 | 3.883 | 6.805 |
| α-Humulene | 2.072 | 1.917 | 4.313 |
| Germacrene D | 3.452 | 0.908 | 3.499 |
| γ-Cadinene | 0.369 | 0.368 | 0.712 |
| δ-Cadinene | 0.801 | 0.612 | 1.890 |
| **Pearson correlation matrix** | | | |
| | $VP_1$ | $VP_2$ | $VP_3$ |
| $VP_1$ | 1 | | |
| $VP_2$ | 0.995** | 1 | |
| $VP_3$ | 0.993** | 0.995** | 1 |

**Table 2 : Relative peak area (%) of leaf volatile compounds of genotype Olour using SPME based GC-MS analysis and their correlation among plants**

| Volatile compound | $OP_1$ | $OP_2$ | $OP_3$ |
|---|---|---|---|
| trans-2-Hexenal | 0.208 | 0.756 | 0.649 |
| cis-3-Hexen-1-ol | 0.166 | 0.389 | 0.261 |
| α-Thujene | 0.128 | 0.182 | 0.128 |
| α-Pinene | 19.567 | 11.412 | 17.241 |
| Camphene | 0.329 | 0.181 | 0.235 |
| Sabinene | 0.813 | 0.399 | 0.331 |
| β-Pinene | 2.276 | 1.554 | 1.713 |
| trans-Ocimene | 4.101 | 4.111 | 4.447 |
| α-Phellandrene | 5.417 | 5.631 | 5.687 |
| Limonene | 56.958 | 62.001 | 57.140 |
| α-Terpinene | 0.801 | 0.754 | 0.663 |
| Terpinolene | 0.368 | 0.378 | 0.357 |
| Nerol | 0.029 | 0.203 | 0.095 |
| 2-methyl-2-bornene | 0.277 | 0.959 | 0.759 |
| Allo-Ocimene | 0.019 | 0.034 | 0.026 |
| 4-Terpineol | 0.016 | 0.177 | 0.114 |
| Methyl salicylate | 0.495 | 1.229 | 0.570 |
| )-Elemene | 0.199 | 0.261 | 0.368 |
| Germacrene B | 2.733 | 3.478 | 5.044 |
| (-)-α-Cubebene | 0.201 | 0.211 | 0.372 |
| **Pearson correlation matrix** | | | |
| | $OP_1$ | $OP_2$ | $OP_3$ |
| $OP_1$ | 1 | | |
| $OP_2$ | 0.988** | 1 | |
| $OP_3$ | 0.998** | 0.993** | 1 |

volatile compounds. The correlation analysis between the volatile compounds (Table 1) of three plants of Vellaikolumban were found to be significantly and positively correlated to each other (r = 0.993- 0.995). In Olour (Table 2), limonene was the major monoterpenoid followed by α-pinene and allo-ocimene. The correlation matrix (Table 3) indicated that volatiles of all the three plants of cv. Olour were highly correlated to each other (r = 0.988-0.993). In Turpentine (Table 3), sesquiterpenoids were the major group with α-gurjunene being the highest followed by β-sellinene in all the three seedling originated plants. Volatiles of all the 3 plants were highly correlated with each other (Table 4) (r = 0.991-0.998). Genotypes can be identified based on the volatile profile. Monoterpene and sesquiterpene hydrocarbons are the most abundant volatile components in all mango cultivars, accounting for 70–90% of total volatiles. Wetungu *et al*. (2015) studied the chemical profile of six mango varieties and reported that the mango leaves were rich in monoterpenes and sesquiterpenes. The α-pinene, phellandrene, limonene and ocimene were important monoterpene compounds which clearly distinguished the variability among 34 appemidi genotypes and sesquiterpenes composition was observed in genotype Gaddemara (90.39%) followed by Kalwaguda (78.73%). Among sesquiterpenes, α-humulene and caryophyllene were the major

**Table 3 : Relative peak area (%) of leaf volatile compounds of genotype Turpentine using SPME based GC-MS analysis and their correlation among plants**

| Volatile compound | $TP_1$ | $TP_2$ | $TP_3$ |
|---|---|---|---|
| α-Pinene | 2.61 | 3.09 | 2.41 |
| Sabinene | 0.42 | 0.25 | 0.36 |
| α-Phellandrene | 5.62 | 3.09 | 2.36 |
| β-Elemene | 0.48 | 0.57 | 0.52 |
| α-Gurjunene | 40.12 | 37.76 | 38.01 |
| β-Caryophyllene | 14.57 | 16.25 | 15.13 |
| α-Humulene | 5.94 | 7.31 | 6.94 |
| Allo-aromadendrene | 0.33 | 0.48 | 0.41 |
| (+)-9-Aristolene | 3.12 | 3.56 | 4.10 |
| β-Sellinene | 22.56 | 23.53 | 25.69 |
| γ-Gurjunene | 2.69 | 2.94 | 2.58 |
| γ-Cadinene | 1.21 | 1.02 | 1.44 |
| **Pearson correlation matrix** | | | |
| | $TP_1$ | $TP_2$ | $TP_3$ |
| $TP_1$ | 1 | | |
| $TP_2$ | 0.995** | 1 | |
| $TP_3$ | 0.991** | 0.998** | 1 |

compounds in all the genotypes (Veena, 2018). Ma *et al.* (2018) detected α-pinene and terpinolene in mango varieties and these compounds are considered to be important volatiles. Cultivars Pingguo and Guixiang contained the highest level of α-pinene and limonene respectively. Moreover, limonene was a predominant component in five mango cultivars, including Cuba Delicioso, Super Hadden, Ordoez, Filipino and La Paz (Pino *et al.,* 2005). 3-carene was the dominant volatile in *cv*. Boluoxiang, but limonene was not found. Sesquiterpene hydrocarbons form the second largest group of aroma volatiles in mango (Pandit *et al*., 2009). Significant differences in the composition of total sesquiterpenoids were recorded among genotypes by Donald (2019) and the highest per cent of sesquiterpenoids composition was observed in genotype Rumani (91.48%) followed by H-151 (90.17%), while, the least content was noticed in genotype Dashehari (26.22%). In the case of sesquiterpenoids, caryophyllene, α-gurjunene and α-humulene contributed the maximum to the leaf volatiles in the genotypes studied indicating that the leaf volatile profile can be used as a fingerprint for varietal identification and could be important for clearly distinguishing the variability among mango genotypes (Donald, 2019, Veena, 2018, Gebara *et al.,* 2011, Dzbreveamic *et al.,* 2010, Liu *et al.,* 2013). Dzbreveamic *et al.* (2010) reported that the leaves of *M. indica* was rich in sesquiterpenes (70.3%) and δ-3-carene, α-gurjunene, β-selinene and β-caryophyllene were dominant compounds in mango leaf oil. In conclusion, mango cultivars differ in terms of total volatile concentration, both qualitatively and quantitatively. The volatile profiling of polyembryonic genotype was found to be different between the genotypes, but was strongly correlated with the seedling originated plants within a genotype. The three seedling originated plants of Vellaikolumban, Olour and Turpentine genotypes were also found to be morphologically similar within the group. Hence it is proved that the volatile profiling can be successfully used to identify the seedling originated plants of polyembryonic genotype.

**Phenolic acid profiling**

The phenolic acid profile of mango leaves was determined using liquid chromatography-Mass spectrometry (LC-MS/MS). Fifteen phenolic acids

**Table 4 : Phenolic acid (mg/gm) profiling of genotypes *viz* Vellaikolumban, Olour and Turpentine and their correlation among genotypes**

| Phenolic acid | $VP_1$ | $VP_2$ | $VP_3$ | $OP_1$ | $OP_2$ | $OP_3$ | $TP_1$ | $TP_2$ | $TP_3$ |
|---|---|---|---|---|---|---|---|---|---|
| Vanillic acid | 0.05 | 0.96 | 4.67 | 0.09 | 2.97 | 4.74 | 7.66 | 7.46 | 9.37 |
| Syringic acid | 0.18 | 0.11 | 0.07 | 0.00 | 0.00 | 0.01 | 0.02 | 0.04 | 0.05 |
| Ferulic acid | 541.31 | 635.65 | 522.05 | 306.61 | 223.91 | 355.38 | 272.26 | 378.66 | 344.17 |
| Caffeic acid | 17.90 | 29.01 | 5.95 | 9.24 | 4.13 | 6.97 | 9.02 | 6.66 | 15.37 |
| Gallic acid | 564.95 | 705.11 | 383.15 | 144.47 | 145.30 | 272.86 | 437.98 | 514.02 | 742.97 |
| p-Coumaric acid | 1096.94 | 1266.06 | 927.67 | 872.10 | 606.84 | 1657.16 | 967.13 | 1088.20 | 1411.17 |
| o-Coumaric acid | 72.08 | 86.21 | 54.47 | 83.80 | 60.77 | 133.59 | 67.77 | 136.14 | 148.89 |
| 2,4-Dihydroxy benzoic acid | 24.44 | 18.81 | 0.68 | 5.28 | 3.21 | 6.60 | 91.88 | 85.89 | 101.45 |
| Gentisic acid | 57.51 | 5.90 | 1.76 | 7.60 | 0.00 | 0.62 | 40.80 | 43.60 | 204.64 |
| Protocatechuic acid | 27.95 | 43.01 | 0.60 | 0.93 | 0.00 | 7.48 | 178.99 | 157.59 | 1.20 |
| p-Hydroxy benzoic acid | 36.30 | 28.96 | 19.79 | 24.03 | 26.99 | 35.94 | 31.80 | 29.34 | 32.63 |
| Salycylic acid | 59.60 | 17.16 | 15.43 | 22.05 | 10.01 | 10.07 | 34.45 | 47.56 | 94.12 |
| Benzoic acid | 4.74 | 1.40 | 9.43 | 3.93 | 3.50 | 1.37 | 3.01 | 0.67 | 0.42 |
| 3-Hydroxy benzoic acid | 49.45 | 35.74 | 24.26 | 30.14 | 34.16 | 48.07 | 40.86 | 40.13 | 39.64 |
| Sinapic acid | 2.51 | 2.01 | 0.52 | 1.80 | 5.26 | 3.65 | 1.92 | 1.92 | 3.81 |
| **Pearson correlation matrix** | | | | | | | | | |
| | $VP_1$ | $VP_2$ | $VP_3$ | $OP_1$ | $OP_2$ | $OP_3$ | $TP_1$ | $TP_2$ | $TP_3$ |
| $VP_1$ | 1 | | | | | | | | |
| $VP_2$ | 0.998** | 1 | | | | | | | |
| $VP_3$ | 0.992** | 0.990** | 1 | | | | | | |
| $OP_1$ | 0.946** | 0.934** | 0.960** | 1 | | | | | |
| $OP_2$ | 0.965** | 0.956** | 0.974** | 0.997** | 1 | | | | |
| $OP_3$ | 0.927** | 0.915** | 0.932** | 0.991** | 0.988** | 1 | | | |
| $TP_1$ | 0.966** | 0.964** | 0.947** | 0.943** | 0.955** | 0.950** | 1 | | |
| $TP_2$ | 0.981** | 0.980** | 0.966** | 0.951** | 0.966** | 0.950** | 0.995** | 1 | |
| $TP_3$ | 0.967** | 0.961** | 0.938** | 0.926** | 0.942** | 0.936** | 0.974** | 0.978** | 1 |

(Table 4) were identified in the leaves of all the 3 genotypes. Among them, P-coumaric acid, gallic acid and ferulic acids were found to be the major phenolic acids. On the other hand, vanillic acid, syringic acid, gentisic acid, benzoic acid and sinapic acids were minor contributors in phenol profiling. P-Coumaric acid was the predominant phenolic acid in all the genotypes followed by gallic acid, ferulic acid in Vellaikolumban and Turpentine but in Olour it was ferulic acid followed by gallic acid. The correlations between the seedlings originated from the same kernel indicated a highly significant correlation (r = 0.915-0.998) (Table 4). Correlations between the genotypes also showed significantly higher values indicating that this parameter is not variety specific. Earlier reports indicate that the proportion and profile of polyphenols in mango vary depending on the variety and also plant part (Ma *et al*., 2011). Ocampo *et al*. (2020) reported variations in the phenolic profiles among mango types. Gallic, vanillic, syringic, and ferulic acids were all

found in the peels of all mango genotypes, while coumaric and chlorogenic acids were not detected. Gallic acid has also been identified as a common phenolic acid present in the mango types Keitt, Sensation, and Gomera 3 (Dorta *et al*., 2014). Our results showed that based on phenolic acid profiling, it is not possible to distinguish the genotypes. On the contrary to these findings, Ocampo *et al*. (2020) reported that the phenolic acid profile could be utilised as a marker/fingerprint in the future to correctly identify types such as the Carabao mango, which is well-known in the Philippines for its flavour.

## CONCLUSION

Volatile aroma and phenolic acid profiling from the mango leaf using GCMS and LCMS/MS techniques indicated that leaf volatile profile is variety specific and can also be used successfully to identify the nucellar seedlings of polyembyonic varieties which are similar to the mother plant. Leaf volatiles are stable which gives unique aroma to a particular genotype. However, the phenolic acid profiling could not differentiate the varieties.

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