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# Chemical and genetic stability of methyl chavicol-rich Indian basil (*Ocimum basilicum* var. CIM-Saumya) micropropagated *in vitro*

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ABSTRACT

Indian basil *i.e. Ocimum basilicum* L., an important essential oil yielding plant, is used in high-grade perfumes, flavoring, dental and oral products. A methyl chavicol-rich, high essential oil yielding variety of Indian basil CIM-Saumya, released by the Central Institute of Medicinal and Aromatic Plants, India, has immense industrial potential due to the occurrence of two important aromatic components, *i.e.* methyl chavicol (62.5–77.6%) and linalool (14.4–34.1%). The *Ocimum* being highly cross-pollinated and seed propagated, produce heterogeneous progeny thereby restricting its commercial application. In the present investigation, an attempt has been made to produce a large number of genetically and chemically uniform plants of this improved variety CIM-Saumya for the first time using nodal explants. Multiple shoot cultures developed on Murashige and Skoog's medium (MS) supplemented with BA ($N^6$-benzyladenine, 2.67 μM) and these shoots were further transferred to half strength MS medium supplemented with NAA (α-naphthalene acetic acid, 0.27 μM) for rhizogenesis. These plantlets were maintained under *in vitro* conditions for more than three years. The established plantlets were successfully shifted in a glasshouse and finally in the field. Flow cytometry, inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers, were employed to evaluate genetic similarity amongst micro-clones and the mother plant. In all, 28 primers (18 RAPD and 10 ISSR) generated 178 distinct, monomorphic and reproducible bands. Head space gas chromatography showed the similarity in the qualitative profile of 11 major volatile constituents. These results clearly demonstrated that the developed protocol can be efficiently utilized to generate genetically and chemically uniform population of an industrially important variety CIM-Saumya.



## 1. Introduction

Indian basil (*Ocimum basilicum* L.: Family Lamiaceae), an annual culinary herb native to Central Asia and North-West India, is widely distributed in tropical and warm temperate regions of the world. It is extensively cultivated in Egypt, France, Greece, Hungary, Indonesia, Morocco and the United States of America (Lal et al., 2004). The herb is well-known to produce essential oils, monoterpenes, sesquiterpenes and phenylpropanoids such as rosmarinic acid, linalool, methyl chavicol, eugenol and limonene. All these metabolites contribute for the insecticidal, nematicidal, antimicrobial and antifungal activities that make this herb a potential candidate for the cosmetic, pharma and food industries (Wei and Shibamoto, 2010).

The *Ocimum* in general exhibit regular occurrence of interspecific hybridization within the genus that generates a large population of non-identical plants (Heywood, 1978). Thus, maintaining the genetic and chemical uniformity in *Ocimum* becomes very challenging. The heterogeneity amongst the plants of the same species also contributes towards inconsistent yield of phyto-constituents causing the batch to batch variation, thus, affecting the quality of many *Ocimum* containing pharmaceutical preparations. To overcome these limitations, micropropagation can play an important role in terms of production of true-to-type, high yielding plant material (Mohanty et al., 2008; Paul et al., 2010; Lata et al., 2011; Saha et al., 2014; Prasad et al., 2015). Many *in vitro* studies have been conducted in *Ocimum* species using nodal explants for micropropagation *viz. Ocimum kilimandscharicum* (Saha et al., 2010), *Ocimum gratissimum* (Gopi et al., 2006), *Ocimum sanctum* (Singh and Sehgal, 1999) and Sweet basil-*O. basilicum* (Sahoo et al., 1997; Siddique and Anis, 2008; Daniel et al., 2010; Asghari et al., 2012). No such work has so far been reported in the industrially important Indian basil variety CIM-Saumya. Molecular

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markers, flow cytometry and GC profiling have been widely used as valuable tools for establishing genetic as well as chemical stability of the micropropagated progeny in various plant systems (Paul et al., 2010; Bhatia et al., 2011; Ghimire et al., 2012; Vujovic et al., 2012; Faisal et al., 2014; Saha et al., 2014; Agarwal et al., 2015; Largia et al., 2015; Prasad et al., 2015).

This particular variety of Indian basil CIM-Saumya, possesses many beneficial characters like early maturation, short duration, dwarf nature and high essential oil yield. It is commercially more valuable because of the presence of two important aromatic components, namely methyl chavicol (62.5–77.6%) and linalool (14.4–34.1%) in higher amounts throughout the year (Lal et al., 2004). In contrast, other *Ocimum* varieties produce either methyl chavicol or linalool at one time. In addition, this variety also contains a group of essential oils and other bioactive terpenes such as β-caryophyllene, (*E*)-β-ocimene, α-*trans*-bergamotene and germacrene D. Although, the accumulation of dual metabolites in CIM-Saumya variety makes it more valuable for the production of various herbal formulations, but high degree of genetic and biochemical variability (because of cross-pollination behavior) restricts its commercial applications.

The present study was undertaken to overcome these limitations by developing an efficient micropropagation protocol of this important variety of Indian basil CIM-Saumya. The genetic and chemical stability of the regenerated progeny, multiplied and maintained for more than three years, has been evaluated using flow cytometry, molecular markers (RAPD and ISSR) and HS-GC analysis. To the best of our knowledge this is the first report wherein genetic and chemical uniformity of micropropagated progeny of CIM-Saumya variety has been successfully established for its efficient commercial utilization.

## 2. Materials and methods

### 2.1. Plant material, explant selection, surface sterilization and medium composition

For the establishment of aseptic cultures, nodal segments (2-3 cm in length) were excised from the main stem and branch of one month old plants of CIM-Saumya variety. The nodal segments were treated with Savlon (1–2 min) then washed with alcohol (70%) for 30 s. These explants were then surface-disinfested with 0.1% (w/v) $HgCl_2$ (1–2 min) followed by thorough washing (4–6 times) with sterile distilled water. Modified Murashige and Skoog (1962) contained 0.1 g $l^{-1}$ (w/v) myo-inositol, 30 g $l^{-1}$ sucrose. For shoot and root induction various concentrations/combinations of IAA, NAA and BA, gelled with 8 g $l^{-1}$ agar (Himedia, India), were used for the *in vitro* experiments. All the media combinations were autoclaved under 1.0 kg $cm^{-2}$ $s^{-1}$ pressure at 121 °C for 15–20 min after adjusting the pH to 5.84 ± 0.03 using 1 N NaOH/1 N HCl. The surface-sterilized nodal explants were implanted on modified MS media under aseptic conditions. Nodal segments were used as explants based on our preliminary findings. Cultures were kept in the culture room under cool, fluorescent light (45 μmol $m^{-2}$ $s^{-1}$ photosynthetic flux), 16-h/8-h photoperiod at 27 ± 3 °C and 65–75% relative humidity.

### 2.2. Rooting of regenerated shoots

Healthy regenerated shoots (12–14 cm in length) were excised and transferred onto root induction media, *i.e.* half strength MS medium supplemented with a range of IAA and NAA (0.27, 0.55, 0.83, 1.11 and 0.27, 0.54, 0.80, 1.07 μM) concentrations. Based on the results obtained, another experiment was performed wherein half strength MS media were supplemented with NAA only. Half strength MS media without any plant growth regulator was used as the control. Percent root induction and mean number of roots formed in these media combinations were recorded after six weeks.

### 2.3. Acclimatization of plantlets

Plantlets with well-developed shoots and roots were removed from the culture vessel, gently washed with water to remove adhered agar and transplanted in pots containing sterilized sand and soil (1:2) and kept under diffused light (16/8 h photoperiod) conditions. These plantlets were initially covered with glass jars and watered with half strength MS salt solution whenever needed for initial five days. These pots were maintained in the glasshouse conditions and the hardened plants were shifted to the field.

### 2.4. Flow cytometry

The mother plants as well as micropropagated plants were analyzed for their DNA content that was estimated by flow cytometry. For this, the leaves were collected from the *in vitro* micropropagated and glasshouse-grown individuals. In each sample, 25 mg of young leaf tissue was used for sample preparation to release a sufficient number of nuclei. Nuclear suspensions were prepared using nuclei isolation buffer WPB (woody plant buffer) as per the protocol of Loureiro et al. (2007). Fresh leaf tissue was chopped into 1 ml of the nuclei isolation buffer using a sharp scalpel blade for approx. 90 s to release nuclei. The resulting homogenate was filtered through a 40 μm nylon filter to remove large debris. The nuclear suspensions were treated with 50 μg $ml^{-1}$ RNase to prevent staining of double-stranded RNA. Nuclei were stained with nucleic acid specific stain SYBR Green I (molecular probe, USA) added at a final dilution of 1:10,000 of the commercial solution. Samples were incubated for 15 min before analysis by flow cytometry. Samples were run at a rate of 10 μl $min^{-1}$ on a BD LSR II flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA) equipped with a 488 nm excitation laser and a standard filter setup. The data were acquired and DNA concentration was estimated from all the samples, which were assessed at least three times and included a minimum of 10,000 nuclei per run. The results were acquired using the FACS Diva software (version 6.1.3, Beckman Coulter).

### 2.5. DNA isolation and genetic fidelity assessment

Total genomic DNA was extracted from fresh leaves of nine randomly selected micropropagated plants (C1–C9) along with the mother plant (MP) using the CTAB method (Khanuja et al., 1999). Qualitative and quantitative DNA analysis was done using electrophoresis on 0.8% agarose gel and nanodrop spectrophotometer (ND 1000, Thermo Scientific, USA), respectively. A total of 35 primers (20 RAPD and 15 ISSR-custom synthesized from Sigma Aldrich Chemical Pvt. Ltd., India) were used for genetic fidelity evaluation. RAPD and ISSR primers were amplified in a thermal cycler (Mastercycler, Eppendorf, Germany) using the modified method (Mahar et al., 2011). Amplified PCR products were resolved electrophoretically in 1.2% w/v agarose gel in 1× Tris-Acetic acid-EDTA (TAE) buffer. The amplified fragments were visualized under UV light and photographed using Gel Documentation System (XR-Quantity One, Bio-Rad Laboratories, USA). The sizes of the amplicons were compared with low range DNA ruler 100–3000 bp (Merck Specialities Pvt. Ltd., India). All the reaction each for RAPD and ISSR primers were repeated at least three times and only the distinct and reproducible bands were considered for scoring. For the genetic fidelity studies, data were scored as presence (1) or absence (0) of the band. A pairwise similarity matrix between the mother and micropropagated plants was determined from the band data using Jaccard's similarity coefficient in the Free Tree program (ver. 0.9.1.5) (Pavlicek et al., 1999).

### 2.6. HS-GC-FID/mass spectrometry analysis

The carrier gas ($H_2$) was used at a constant flow rate of 1 ml $min^{-1}$ in the split ratio of 1:25, makeup flow ($N_2$ gas) at 29 ml $min^{-1}$ flow; the