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Registration of a pulegone rich variety CIM-Vishisht of *Mentha arvensis*

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ABSTRACT

The new variety of Mentha arvensis named as 'CIM-Vishisht' was developed through half sib progeny selection of the menthol mint cultivar, 'Shivalik'. The new variety has the potential of yielding 60 Kg/ha of essential oil which is rich in pulegone (65 – 68%) and the character is found stable over generations. The plant morphology of this new variety is distinct with medium height, smaller pale green leaves and white flowers, the growth habit is decumbent and has distinct DNA marker profile. This new variety 'CIM-Vishisht' will help opening new avenues for essential oil industry in the country.

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INTRODUCTION

Mints (*Mentha* spp.) belonging to family Lamiaceae, constitute one of the most popular group of essential oil bearing plants that are widely distributed and cultivated in temperate, Mediterranean, and subtropical regions of the world. The existence of different chemotypes, based on qualitative differences within a taxon, is a common feature in most of the *Mentha* species. The mint plants produce a number of commercially valuable essential oils, viz. menthol rich menthol mint oil, carvone or piperitenone oxide rich spearmint oil, menthol and menthone rich peppermint oil, pulegone rich pennyroyal oil, and linalool and linalyl acetate rich bergamot mint oil. These oils find extensive uses in pharmaceutical, cosmetic, food, flavour, beverage, and allied industries.

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Being a valuable compound, pulegone have both positive as well as negative activities. The positive activities described for pulegone are AChE-Inhibitor; antiacetylcholinesterase; antialzheimeran, antibacterial, antihistaminic [20,22], antipyretic, antinociceptive, antibacterial, fumigant, acaricidal [6,17,18,21], cancer-preventive, candidicide, fungicide, encephalopathic, sedative, flavour and perfumery actions etc. In addition, pulegone is mucolytic and is good for congestion of the respiratory system. This is also considered to be an emmenagogue. The negative effects of this compounds are hallucinogenic, nephrotoxic, neurotoxic, hepatotoxic, cerebrotoxic and pulmonotoxic actions. This is toxic to pregnant women but used in folklore medicine as an abortifacient [12]. For the toxicity this is used as herbicide, Insecticide, pesticide and avifuge. The United States food and drug administration (http://www.cfsan.fda.gov/~lrd/fcf182.html) has given a status of "generally recognized as safe" (GRAS) to pulegone [23]. According to the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the

estimated intake of pulegone per capita is 2 µg per day and 0.04 µg/kg bw per day for Europe, and 12 µg per day and 0.03 µg/kg bw per day for the USA [9].

Therefore pulegone can be widely used in aromatherapy, flavouring agents, perfumery, baked goods, candies, ice creams, as a fragrance component in detergents, cosmetics, oral hygiene products, and as an insect repellent [3,4,5,8,11,13,19]. Being a terpene, pulegone has been extensively used to enhance the transdermal permeability of a number of drug molecules, including 5-fluorouracil, propranolol hydrochloride, indomethacin, ketoprofen and tamoxifen [10,15]. Pulgone can be chemically converted into some other important compounds like menthone, carvone or thymol [2] and into high value commercially important menthofuran through biotransformation using the enzyme (+)-menthofuran synthase [1,14]. Considering the large industrial application and lack of chemical diversity for pulegone in menthol mint, it became imperative to develop new pulegone-rich chemotypes for industrial usage. An attempt has been made to develop such a chemotype through half sib progeny selection method for varietal development.

MATERIALS AND METHODS

Breeding history and development of the variety 'CIM –Vishisht

The clone MAC/ BS-11, was developed through half sib progeny selection and was identified with unique aroma during the year 2010. This clone was selected from several seed raised progenies of the menthol mint cultivar, 'Shivalik' during the year 2010 at Research farm of Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP) Lucknow (India) and was multiplied vegetatively for evaluation at CIMAP Research Center, Pantnagar. Evaluation trials were conducted for determining its yield potential with seven other commercial varieties of menthol mint during the years 2014 and 2015. The field trials were laid out in a randomized block design with plot size of 5m x 4m during the year 2014 and with 5m x 5m in 2015 in three replications, each with a row to row distance of 70 cm and the suckers were sown end to end.

Chemical Characterization

GC analysis was carried out on a Varian CP 3800 gas chromatograph equipped with TG-WAX MS (30 m 0.32 mm; 0.25 µm film thickness) fused silica capillary column and flame ionization detector (FID). Hydrogen was used as the carrier gas at 7 psi column head pressure. Oven temperature programming was done from 40 – 80 at 3^0/ C (9), 80 – 120 at 2^0/ C, 120 – 250 at 5^0/ C, 68.33, split 1:40. The injector and detector temperatures were 250 °C and 250 °C, respectively.

The physico-chemical data like specific gravity, optical rotation and refractive index were measured using standard procedures with instruments KEM model DA – 500, Horiba model SEPA – 300 and ATAGO model – 7000 α, respectively.

Molecular characterization

The DNA analysis was carried out to assess genetic variations and relatedness of new clone MAC/BS-11 compared to other released varieties *viz*. Shivalik, Gomti, Himalaya, Kosi, Saksham, Sambhav, Kushal, Damroo, CIM-Kranti and Saryu. RAPD and ISSR analysis were used for molecular identification as well as for phylogenetic analysis. Random decamer primers of MAP series, OPM primers (procured from Operon Inc.) and 15 ISSR primers were used for the DNA analysis to detect the variations among released varieties of *Mentha arvensis* and newly developed chemotype MAC/ BS-11. RAPD and ISSR fragments were scored as present (1) or absent (0) for the estimation of the similarity among all the analyzed samples. The matrices of similarity were calculated as per Nie (1978) method [16] and a phylogenetic tree was constructed by clustering according to the Unweighted Pair-Group Method with Arithmetic averages (UPGMA). Polymorphism percentages were estimated by dividing the number of polymorphic bands over the total number of bands.

RESULTS AND DISCUSSION

Morphology, yielding potential and oil quality evaluation

The plant showed distinct morphology and the growth habit as shown in **Figure 1.** The morphology of this new chemotype is distinct with medium height, smaller pale green leaves, white flowers and a decumbent growth habit (**Table 1).** Gas chromatographic fingerprint of the essential oil (**Figure 2**) reveals higher pulegone content in the new variety. The oil quality parameters over three generations during 2013 to 2015 are given in the **table 2** indicating that the oil contains pulegone in the range of 65 to 68% and the character is found stable over generations. The physico-chemical characteristics studied for the different commercial varieties of menthol mint and this new pulegone rich variety shows its' uniqueness over other varieties with respect to its optical rotation (**Table 3**). The new clone MAC/BS-11 (CIM-Vishisht) evaluated for oil yielding potential in subsequent generations under pilot scale performance trials during summer crops in the year 2014 and 2015 at CRC Pantnagar was found to be in the range of 58 kg to 60 kg per hectare (**Tables 4 and 5)**.

Molecular characterization

MAP 1 to 20 primers provided a total of 135 fragments, out of which only 67 were monomorphic while the rest were polymorphic (57.2%). The chemotype MAC/BS-11 showed 91.8% of polymorphism in comparison to other varieties. Maximum number of bands were generated by the primer MAP 14 and a minimum by primer MAP 12. The clone MAC/BS-11 showed distinct RAPD profile with MAP 19 (**Figure 3**). The characteristic DNA profile of chemotype MAC/BS-11 developed by using MAP series primers (**Figure 4**). OPM series primers were further used to evaluate the distinctiveness of new chemotype MAC/BS-11 with Gomti, Shivalik and CIM-Kranti (**Figure 5**). In ISSR analysis, 16 ISSR primers generated only 136

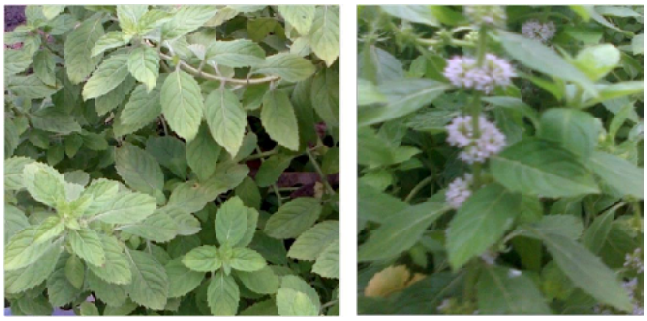

Figure 1: Morphology of the variety 'CIM –Vishisht

Table 1: Morphological characters of *Mentha arvensis* variety Kosi and Clone MAC/ BS-11

Characteristics	Variety Kosi	Clone MAC/BS-11
Habit	Erect	Decumbent
Plant Height	85cm	65cm
	Herbaceous	Woody
Stems	Purplish-Red	Green
	Ciliate above	Wooly above
	Puberulent	Pilose
Internodes/main stem	32	21
Internode Length (cm)	3.8	2.8
	Ovate-Elliptic	Ovate
	Acute	Acute
	Serrate	Serrate
Leaves	Cuneate	Cuneate-Oblique
	Somewhat thick and rough	Thin and smooth
	Recurved longitudinally	Straight longitudinally
	Dark green	Pale green
Leaf–length (cm)	3.9 – 5.0	3.1 – 4.2
Leaf–width (cm)	2.2 – 2.6	1.7 – 2.4
Inflorescence	Verticillaster Cyme	Verticillaster Cyme
Days to flowering	120 -130	130 - 140
Flower colour	Purplish white	White-pale pinkish

Table 2: Essential oil quality parameters of new chemotype MAC/ BS- 11.

Year	Oil content (%)	Limonene	Menthone	Iso-menthone	Pulegone	Menthol
7/ 2013	0.75	1.1072	5.6655	1.8397	68.4446	7.8886
8/ 2014	0.80	0.8871	4.4244	0.6965	67.3390	11.0643
4/ 2015	0.80	2.1974	0.9465	0.3334	65.0428	4.5758
7/ 2015	0.80	1.7457	6.1355	0.7377	67.1462	13.3280

Table 3: Physico-chemical characteristics of commercial menthol mint varieties and new chemotype MAC/ BS-11

Variety/ Clone	Refractive index nD^{20}	Specific gravity @ 20^0 C	Optical rotation[α] D^{20}
CIM-Kranti	1.4591	0.9009	(-) 33.3467
Kosi	1.4595	0.9013	(-) 33.3192
CIM-Saryu	1.4595	0.9002	(-) 36.420
MAC/ BS-11	1.4754	0.9221	(+) 3.1380

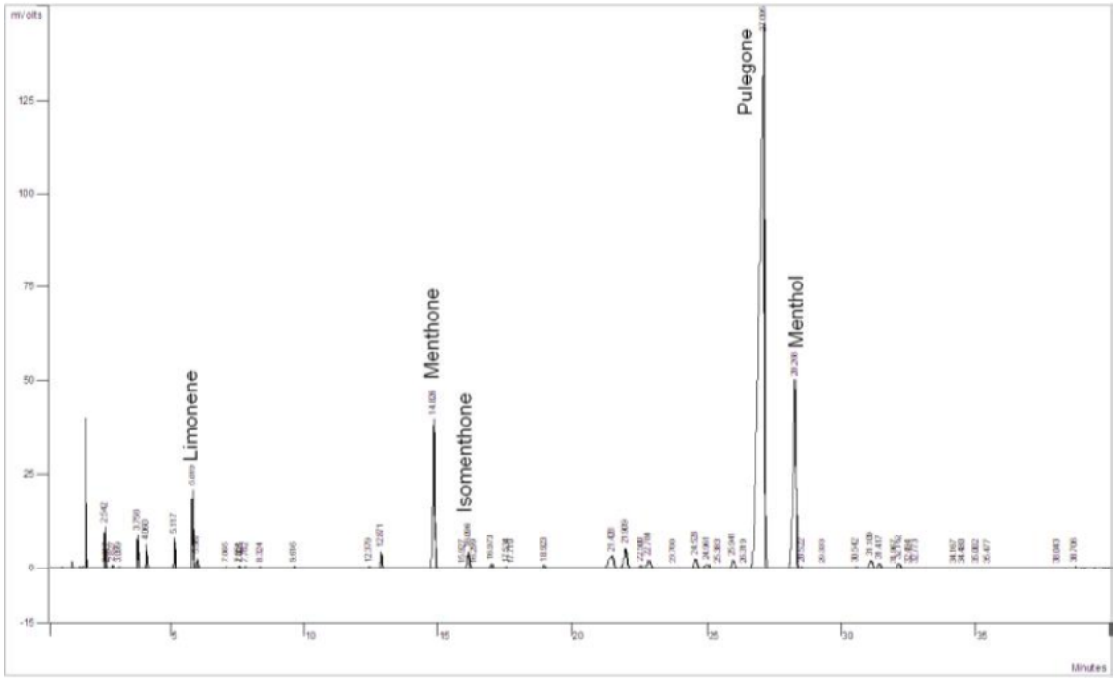

Figure2. Gas chromatographic fingerprint of essential oil

Table 4: Oil yielding potential of menthol mint varieties and the new variety CIM-Vishisht (MAC/ BS-11) during 2014 at Pantnagar

Sl. no.	Variety	Pantnagar ($20m^2$)		
		Oil content (%)	Herb yield (q/ha)	Oil Yield (kg/ha)
1.	**MAS-1**	0.72	117	75.6
2.	**Kalka**	0.83	244	183.2
3.	**Shivalik**	0.82	286	209.9
4.	**Himalaya**	0.72	269	181.2
5.	**Kosi**	0.78	276	194.8
6.	**CIM- Saryu**	0.82	231	169.9
7.	**Kranti**	0.90	272	220.0
8.	**MAC/ BS-11**	0.85	77	58.6
	CD 5%	0.06	13.8	8.4
	CD 1%	0.09	19.3	11.8

Table 5: Oil yielding potential of menthol mint varieties and the new variety CIM-Vishisht (MAC/ BS-11) during 2015 at Pantnagar

Sl. no.	Variety	Pantnagar ($25m^2$)		
		Oil content (%)	Herb yield (q/ha)	Oil Yield (kg/ha)
1.	**MAS-1**	0.68	106	64.7
2.	**Kalka**	0.92	172	141.8
3.	**Shivalik**	0.72	247	159.5
4.	**Himalaya**	0.72	198	133.7
5.	**Kosi**	0.85	201	148.0
6.	**CIM- Saryu**	0/82	191	140.1
7.	**Kranti**	0.82	217	159.7
8.	**MAC/ BS-11**	0.77	88	60.6
		0.07	14	14.8
		0.09	20	20.7

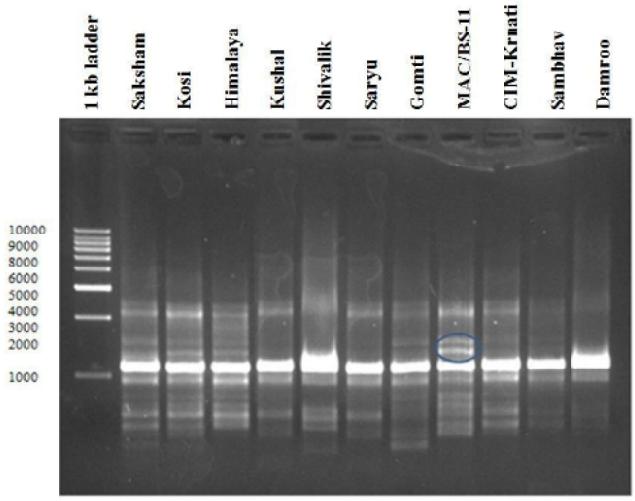

Figure 3: Differentiating fragment of clone MAC/BS-11 among varieties with MAP 19

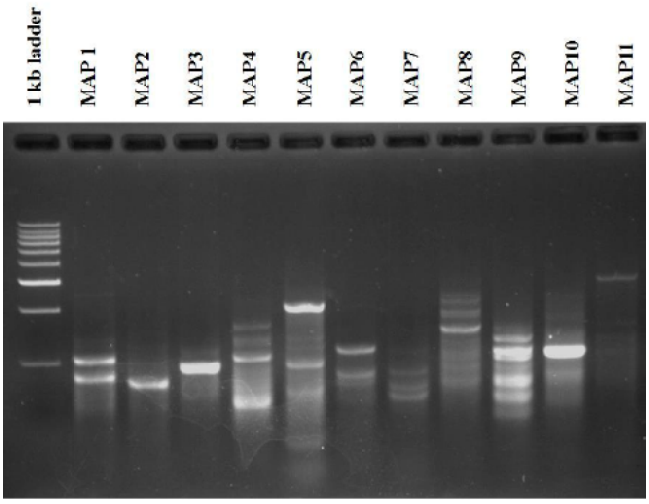

Figure 4: Characteristic profile of novel chemotype of *M. arvensis* clone MAC/BS-11

bands with an average of 8 bands per primer. The 63 bands were found polymorphic with 18 unique loci while the remaining were monomorphic.. Primer 4, 6 and 8 generated maximum number of fragments whereas primer 13 produces only minimum fragments. ISSR primer 11 successfully discriminates the new chemotype MAC/BS-11 from other genotypes (**Figure 6**). In this study, the phylogenetic tree indicated the genetic similarity of MAC/BS-11 to *Mentha arvensis* var Shivalik (64%) (**Figure 7**).

Distinctiveness of new chemo-type MAC/BS-11

The distinctive RAPD profiles for pulegone rich

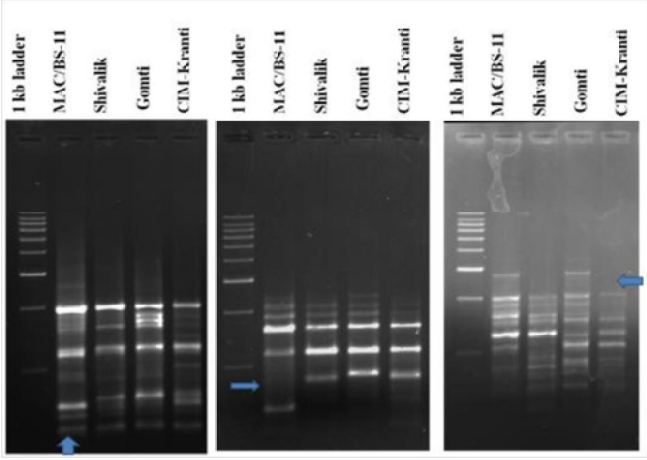

Figure 5: Differtiating RAPD profile of chemtype MAC/BS-11 with Gomti and Shivalik with primer OPM 03,05,07

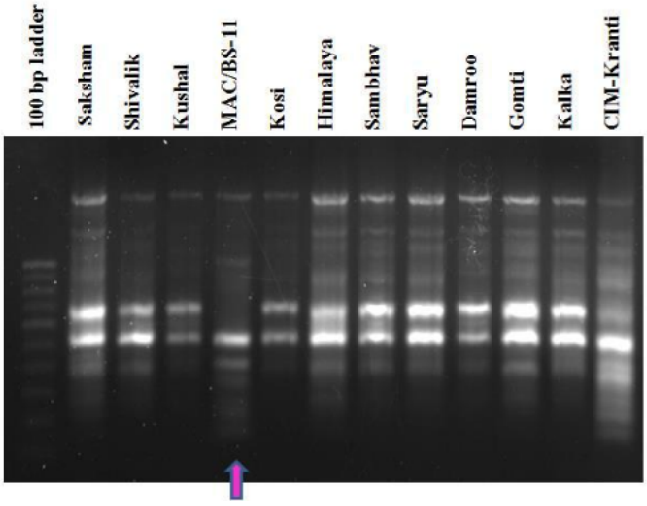

Figure 6: Differentiating fragment of clone MAC/BS-11 among varieties with ISSR-11

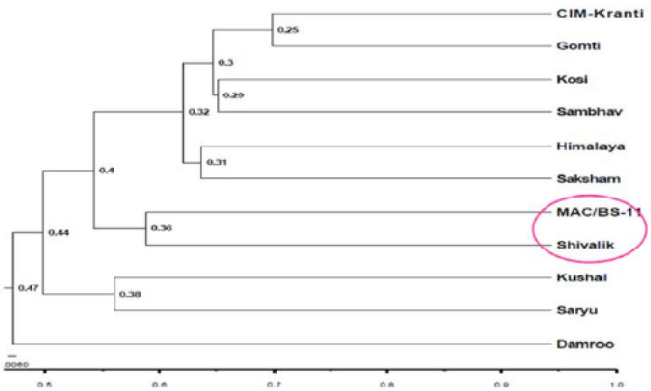

Figure 7: Phylogenetic tree as obtained through combined analysis of RAPD and ISSR

M. arvensis chemotype MAC/BS-11, and for the nine released varieties, demonstrated that the chemotype MAC/BS-11 is novel and distinct among all the varieties. The calculation of genetic distances based on the number of shared fragments shows that the chemotype MAC/BS-11 is much closely related to variety Shivalik with 64% similarity (**Figure 7**). Further, the RAPD analysis of MAC/ BS-11 with Gomti and Shivalik was done with OPM primer series to differentiate the new chemotype with Gomti and Shivalik. The OPM primer 03, 05 and 07 **(Figure 5**) efficiently differentiated the MAC/ BS-11 from Gomti, Shivalik, CIM-Kranti. The RAPD analysis with MAP and OPM primers clearly validated and established the distinctiveness of novel chemotype MAC/BS-11. The ISSR analysis by anchored primers, this new chemotype was quite distinct when compared with other varieties. ISSR primer 11 discriminated new pulegone rich chemotype with other genotypes. These distinctive primers could be further utilized as a molecular probe for MAC/BS-11. This is the only chemotype among all released varieties of *M. arvensis* producing 65 to 68% pulegone consistently in the essential oil.

Statement of distinctness

The proposed variety 'CIM-Vishisht' (MAC/ BS-11) is decumbent in growth and leaves are thin, smooth with pale green colour. Moreover, special feature of the clone is to produce quality mint oil with 65 -68% pulegone. The clone has distinct morphology and RAPD/ ISSR profile.

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