Protoplasma (2017) 254:1045–1061
DOI 10.1007/s00709-016-1011-x

ORIGINAL ARTICLE

Genetic elaborations of glandular and non-glandular trichomes in *Mentha arvensis* genotypes: assessing genotypic and phenotypic correlations along with gene expressions

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Received: 10 October 2015 / Accepted: 29 July 2016 / Published online: 11 August 2016

Abstract *Mentha arvensis* (corn mint) is well known for the production of menthol, a widely used commodity in pharma and flavoring industries and provides natural fragrances and products. Glandular trichomes are specialized hairs found on the aerial surface of vascular plants species producing specific secondary metabolite chemistry. Correlations were established among trichomes, oil yield, and major secondary metabolites. Nine improved, elite cultivars representing different *M. arvensis* genotypes were used for analysis. Phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) were estimated; results indicated the presence of considerable amount of genetic variability, thereby emphasizing wide scope of selection. Positive and significant associations were found among glandular trichomes, oil yield, essential oil constituents, and leaf morphology itself, whereas morphological parameters of leaf show positive and negative correlations to average number of trichome and essential oil constituents. Average number of glandular, non-glandular trichomes, their ratios, menthol content, and trichome number showed a good heritability. Trichomes were studied microscopically in leaf parts in all varieties for analyzing their distribution pattern. The trichome number variations showed significant correlation throughout the genotypes with essential oil yield and monoterpenoid constituents. Differential changes were analyzed for Glutathione S-transferases, Glutathione reductase, Malondialdehyde, phenolics, and chlorophyll content. Gene expressions were analyzed for biosynthesis genes and selected transcription factors TRANSPARENT TESTA GLABRA 1 (TTG1), ENOLASE 1, GLABRA 3, GTL 1, NUCLEAR TRANSCRIPTION FACTOR Y SUBUNIT B-6, WRKY transcription factor 22, putative WRKY 33, WRKY 17, WRKY 1, and WRKY 65-like for harnessing their relation with trichome development in *M. arvensis* genotypes.

Keywords Correlation · Glandular secretory trichomes (GST) · Phenotypic coefficient of variation (PCV) · Genotypic coefficient of variation (GCV) · Non-secretory trichomes (NST) · WRKY · GL3

Handling Editor: Hanns H. Kassemeyer

Electronic supplementary material The online version of this article (doi:10.1007/s00709-016-1011-x) contains supplementary material, which is available to authorized users.

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Introduction

Mentha L. species are one of the most important medicinal and aromatic plant species widely distributed and cultivated in the different regions of the world. These are sources of

economically important essential oils that are widely used in food, flavor, cosmetics, and pharmaceutical purposes. *Mentha* is one of the important genera of aromatic perennial herbs belonging to the Labiatae (Lamiaceae) family and distributed mostly in temperate and subtemperate regions of the world (Yaseen et al. 2000; Annicchiarico 2002; Lal 2007). Genus *Mentha* consist of different species that show considerable chemical multiplicity in the essential oil composition and are considered industrial crops as they produce series of monoterpenes which are reasonably and commercially valuable (Aksita et al. 2013). Menthol mint (*Mentha arvensis* L.) is a genus of about 25 species and is widely used in the food, flavorings, pharmaceutical, and cosmetic industries. Genetic improvement(s) in *Mentha* species leads to wider adaptation, higher herbage, and essential oil yield as well as improved quality of oil which will permit economical production of mint related commodities (Khanuja et al. 2000). In addition to being a popular flavoring for food, confectionery, and cigarettes, natural menthol has a cooling, soothing effect on the skin and mucous membranes of the human body, making it a useful ingredient in pharmaceuticals and cosmetics (Rawashdeh 2011). These are sources of essential oils that are widely used in food, flavor, cosmetic, and for pharmaceutical purposes. India is a major producer with 15,000–20,000 t of annual production. Until about 15 years ago, the bulk of the world's corm mint came from Brazil and China. China and India subsequently overtook Brazil and, more recently, India has led the world in the production of this plant and its products (Lal 2013). The site of monoterpenes biosynthesis in mint has been specifically localized to the secretory cells of the glandular trichomes (Gershenzon et al. 1989; McCaskill et al. 1992) located upon the aerial surfaces (Amelunxen 1965 and Fahn 1979). The storage compartment of glandular trichomes usually is located on the tip of the hair and is part of the glandular cell, or cells, which are metabolically active. Eight enzymatic reactions take place which lead to formation of menthol from precursor molecule isopentenyl pyrophosphate. Geranyl diphosphate is first molecule produce in the series by the action of prenyltransferase. The cyclization of geranyl diphosphate leads to formation of limonene by action of limonene synthase, a multiproduct plant monoterpene, which is the first committed step toward the menthol biosynthesis (Croteau et al. 2005). Limonene is further hydroxylated to transisopipertinol by action of limonene hydroxylase (Kjonaas et al. 1985), which is an intermediate compound and converted to isopiperitenone by help of isopiperitenone dehydrogenase (Kjonaas et al. 1985). Isomerization leads to formation of pulegone (Croteau et al. 2005). Pulegone plays a central role in monoterpene metabolism as it serves as a precursor of menthofuran, menthone, isomenthone, and through reduction of the saturated ketones, menthol, neomenthol, isomenthol, and neoisomenthol by pulegone reductase (Croteau et al. 2005). Menthone is finally converted to menthol in the presence of menthone reductase (Croteau et al. 2005).

Trichomes as well as their secretive substance can be extracted relatively easily, and this has permitted a detailed study of their metabolites, as well as the genes and proteins responsible for them. This knowledge now assists classical breeding programs, as well as targeted genetic engineering, aimed to optimize trichome density and working to facilitate customization of essential oil production or to tune biocide activity to enhance crop protection (Glas et al. 2012). The number and size of trichome are playing a very major role in essential oil biosynthesis. (McCaskill and Croteau 1995). The essential oil quality is evaluated by the composition and percentage of their active constituent. We have done our studies with nine different genotype of *M. arvensis* to find out those varieties which had high as well very low number of trichome on dorsal and ventral side. These findings are discussed in relation to changes in glandular trichome and essential oil content. So we had done the trichome patterning and density analysis by the help of stereomicroscope and scanning microscope. Morphological study at different parameters as plant height, number of leaf, leaf breath, petiole length, and menthol content in essential oil plays a major role in genotype selection. The number and size of trichome are doing a very major role in essential oil biosynthesis. The objectives of the present study were to evaluate the essential oil yield with trichome number with other morphological parameters of nine commercial cultivars namely Kosi, Kushal, Saksham, Kalka, Himalaya, Sambhav, Damroo, Shivalik, and Gomti of menthol mint (*M. arvensis* L. by CSIR-CIMAP, Lucknow (India)), used for commercial cultivation in different years in India and to determine their correlation with different parameter as well as trichome density and patterning. In genetic assessment of correlation between traits, it is necessary to be able to examine inheritance in a group that also has a variety of morphological features (McLellan 2005). Peltate and capitate are two types of glandular trichomes, located on both the upper and lower leaf surfaces (Werker 2000). *M. arvensis* genotypes have both types. Only the peltate glandular trichomes accumulate monoterpenes. These trichomes contain secretory cells that are responsible for oil synthesis. The density of glandular trichomes on leaves in the genus *Origanum* has been positively correlated with plant essential oil content (Bosabalidis 2002). The purpose of this analysis is to examine and to establish correlations specifically among glandular secretory trichomes (GST) and non-secretory trichomes (NST) with leaf morphological traits, essential oil yields, and secondary metabolite chemistry in diverse populations of the *M. arvensis*, as well as to analyze whether traits correlated in naturally occurring populations are genetically independent of each other. Enzymatic analysis was done to examine and validate the translational activity by superoxide dismutase (SOD), glutathione S-transferases, glutathione reductase (GR),

malondialdehyde (MDA), phenolics, and chlorophyll content.

Material and method

Plant materials

The nine varieties of menthol mint (*M. arvensis* L.), namely, Kosi, Kushal, Saksham, Kalka, Himalaya, Sambhav, Damroo, Shivalik, and Gomti released by CSIR—Central Institute of Medicinal and Aromatic Plants, UP, India for commercial cultivation were evaluated at the experimental research farm of the institute, and their suckers were transplanted in a randomized complete block design with three replications (Table 1). In RBD experiment, each block contains three rows at a distance of 75 cm each. The plants received normal intercultural operations, irrigation, and fertilizer applications (120 kg N_2, 80 kg P_2 O_5, and 60 g K_2O per hectare). Minimum and maximum night and day temperatures ranged in between 3–11 and 12–16 °C, respectively, during planting of suckers and from 22–30 to 33–41 °C, respectively, during harvesting. Average rainfall during the growing season was 5–7 mm according to weather data obtain from Metrological Laboratory of CSIR-CIMAP, Lucknow, UP, India. After 100–110 days of transplantation, data were recorded for various agronomical and considered parameters.

Analysis of glandular and non-glandular trichomes

For trichome analysis, six leaves from apical region of all varieties of *M. arvensis* grown in experimental field of approximately 100 to 110 days age were collected in replicates. Trichomes were counted on both dorsal and ventral surfaces of leaves for each variety and for each sample, three microscopic fields were analyzed on different phyllotaxical arrangement. Trichomes were observed under stereomicroscope (LEICA KL 200 LED, Schott, Germany) adjusted at 6.25-mm^2 grids at its eyepiece lens at ×40 magnification. Both the types of trichomes, glandular secretory (GST) and non-glandular (NST), were counted on abaxial and adaxial surfaces of leaves for all varieties *M. arvensis*. Leaves were analyzed at different parts, i.e., tip, middle, base, total leaf area, which were also measured with a leaf scanner. Grids covering 6.26 mm^2 areas were fixed at the eye piece lens therefore only those trichomes were counted which were present inside the grid. Trichome were studied in two different life stage of plant i.e. two different developmental stages as early stage of plant after 35 days of transplanting and second mature stage after 110 days of transplanting.

Analysis of surface of leaves by scanning electron microscopy

Leaf samples were cleaned and fixed with 2.5 % glutaraldehyde for 2–4 h and washed thrice using 0.1 m phosphate buffer. The fixed samples were dehydrated using 30, 50, 70, 80, 90, 95, and 100% dehydrated acetone. The dehydrated leaf samples were individually mounted on double-sided carbon tape, which was attached to metal stubs. These stubs were coated with gold-palladium in a sputter coater (JFC 1600; JEOL, Tokyo, Japan) at 20 mA and viewed in a scanning electron microscope (JSM 6490 LV; JEOL, Tokyo, Japan) at different working distances and accelerating voltages. Each specimen was studied extensively and photographed at various magnifications. The leaf morphology of the leaf was assessed based on trichome size, numbers size, and distance between two consecutive trichome. Size of trichome and distance between two trichome were assessed by the help of ruler

Table 1 A detailed description of *M. arvensis* varieties used in genetic analysis

Sr. no.	Menthol mint varieties	Origin/development	Morphological characteristics	References
1	Shivalik	Introduction from China	Long, broad leaves, thick suckers	Kumar et al. (2000)
2	Himalaya	Hybrid of Gomti and Kalka	Dark green, broad leaves erect, thick suckers	Kumar et al. (2000)
3	Damroo	Selection in open pollinated seed progenies of Shivalik variety	Dark green, leathery broad leaves, erect, thick suckers, thick stem	Singh (2005)
4	Sambhav	Single somaclonal variant of cultivar 'Himalaya'	Light green leaves, thin suckers	US Patent PP14538
5	Gomti	Seedling variant of Shivalik	Dark green, broad leaves erect, thick sucker	Kumar et al. (1994)
6	Kushal	Somaclonal selection	Dark green, broad leaves erect, thick suckers, tolerate high moisture in soil	Anonymous (2003)
7	Saksham	Clonal variant of Himalaya	Dark green, broad leaves erect, thick suckers	Khanuja et al. 2001
8	Kosi	Half-sib progeny selection	Light green leaves, bushy, thin suckers	Kumar et al. (1998)
9	Kalka	Cross-hybridization	Light green leaves, thin suckers, susceptible to leaf spot disease	Lal (2013)

and unit was converted according to parameters provided in images obtained.

Morphological parameters analysis

Different morphological parameters of *M. arvensis* genotypes were studied in the field. Experiments were replicated three times. Characters studied were (a) plant height, (b) number of leaves on main stem, (c) number of branches, (d) leaf length, (e) leaf breadth, (f) petiole size, and (g) internode numbers. The morphometrical parameters were recorded for nine genotypes to study for variation of under field conditions of same aged group plants. Observation was tabulated, and graphs were plotted for each morphological parameter for comparative analysis of all genotypes. Five random plants per plot per replication were the selected genotypes for various parameters under study.

Oil extraction and secondary metabolite analysis

Freshly harvested leaf samples of all varieties were hydro distilled in a Clevenger's apparatus for extraction of essential oil. The oil samples were subjected to gas chromatography (GC) analysis (Nucon gas chromatograph model 5765 equipped with FID) using fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness) and stationary phase BP-20 (coated with a carbowax 20M). Hydrogen was used as a carrier gas at the rate of 1.0 ml/min. Injector and detector temperatures were 200 and 230 °C, respectively. The column temperature was programmed from 70 to 230 °C at 4 °C/min, with the initial hold time of 2 min. The injection volume was 0.02 μl. Hydro distillation and GC analysis were done in replicated manner. Essential extraction and constituent were also studied in two different life stages of plant, i.e., two different developmental stages as early stage of plant after 35 days of transplanting and second mature stage 110 days of transplanting.

Statistical analysis of data

The analysis of variance (ANOVA) was performed by using Statistical software 4.0 version, available in the Division of Genetics and Plant Breeding of the CSIR–CIMAP. Statistical analyses were done based on the standard methods as described by Panse and Sukhatme (1989) and Singh and Chaudhary (1985). The pooled values of all the traits were subjected to correlation (Dewey and Lu 1959). Observations were recorded on each cultivar for the most important economic traits. Observations were tabulated, and graphs were plotted for each morphological parameter which compares all plants for different parameters. Five random plants per plot per replication were selected for recording observations on plant height (cm), number of leaves on main stem, number of branches on main stem, leaf length (in cm), leaf breath (in cm), and petiole length (in cm).

RNA isolation and cDNA synthesis

Leaf samples were collected from three different biological replicate, and total RNA isolation was performed with approximately 100 mg of leaf tissues using Spectrum Plant RNA Isolation Kit (Sigma, SA), according to the manufacturer's instructions. Any genomic contamination was removed before complementary DNA (cDNA) synthesis by the treatment with RNase-free DNase I (Sigma, USA). The equipment and glasswares used for RNA isolation were autoclaved and treated with 0.1 % DEPC to make it RNase free. The RNA isolated from leaf tissue collected from three biological replicate. The nucleic acid quality was checked by visual analysis on 1.2 % agarose gel electrophoresis. The purity and concentration of RNA were checked by using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, USA). A260/A280 ratio of each RNA sample was in the range of 1.8–2.0. The first strand of cDNA was synthesized from 3 μg of total RNA with MultiScribe™ Reverse Transcriptase and random primer (ABI, USA) according to manufacturer's instructions. Total RNA samples were denatured at 65 °C for 5 min and then quickly cooled on ice. Reverse transcriptase and other reaction components were added to the samples. These were then incubated for 10 min at 25 °C (primer annealing), followed by 120 min at 37 °C and finally 10 min at 85 °C to inactivate the enzyme. Reverse transcription (RT) negative controls, without the inclusion of the reverse transcriptase enzyme, were performed in parallel to test for the presence of genomic DNA contamination in RNA samples. Rubisco gene of *M. arvensis* was considered as the endogenous gene to check the quality of cDNA on 1.5 % agarose gel.

Primers designed for qRT–PCR

For all genes, qRT–PCR primers were designed using the Primer Express 2.0 software. To ensure maximum specificity and efficiency during PCR amplification, a stringent set of criteria was used for primer designing. These included predicted annealing temperatures (Tm) of 58–60 °C, primer lengths of 18–24 nucleotides, GC contents of 40–60 %, and PCR amplicon length of 100–120 bp. All primers were customized from a commercial supplier Integrated DNA Technology (IDT) India Ltd. Expression analysis was done by ΔΔCT method. Biosynthetic pathway genes were retrieved from NCBI from nucleotide and EST database (Table 2).

Quantitative real-time PCR

qRT–PCR was performed in 384-well plates using the ABI 7900HT RT–PCR detection system. Each reaction mixture

Table 2 Primers used for gene expressions analysis in selected genotypes of *M. arvensis*

Name of genes/transcription factors	Forward primer	Reverse primer
Limonene synthase	CTCGCCTCCGTGTGTCTTG	GGGTTGTAGTTTCCGGATCGT
Limonene hydroxylase	GAATATGCCCCGGTTTGAATT	GGTAAAGAAGTTGTGCCAATGGA
Pulegone reductase	GCAACGGTGCCATTTTGG	TTCCCATGCGAAGAATGAGAT
Deoxy-D-xylulose-5-phosphate synthase	TTGCTCTTGCAGGGCTAACTG	CGAGTTCGGCCCAAAAGAC
Dimethylallyltranstransferas	TCGCTTTTGGCTTACGGTATG	CGCCTGGAGTGTTGTAGTTCAA
Menthofuran synthase	CGACGTCCGCGGTTCTAG	TTTTTAGGGTTCGCGGATTTC
Menthol reductase	TGACATACGTCGAAGACATATCTGAA	CCGACATACACCCCCAAGAG
Transparent testa glabra 1	TACCTCAGATCCTGCTTGTTCCA	CCGATGGTTCCGTAAGAATCTTC
Enolase 1	TAACAAATCATCACCGACGATTTG	CCATTGTATCTATTGAAGATCCATTTGA
Transcription factor GLABRA 3	AAGGCCTACTGCTGCATTATCC	AGAATTACCCTTGGCCGACATT
Trihelix DNA-binding protein (GTL1)	GATGAAGCGAATAGGGTACAATAGAAG	TTGTTGCTTTCTTTCACTTTCTTGA
Nuclear transcription factor Y subunit B-6	GTAGTCGTCGAACCCGAGCTT	AAGAGTGCGTGTCCGAGTACATC
Homeobox protein	AAAAATACCTCGCTTCTGGAATTCT	TCAGCGCCGCCACTTC
Microspore-specific promoter 3	GCAGCACAGCTGAAGATGGTTAT	ACTTTCTTCTTCACGGGACAGTTC
WRKY transcription factor 22	CGAGGGAGTTCCGGTGAGT	GTTGTATGGCGAGGAAGCAAGT
Putative WRKY transcription factor 33	AGTTCGTGAGCCTCGAGTTGTAG	GGATTTCCCTTCACCACTTTCTG
Putative WRKY transcription factor 17	TAATTAGAGTTCCAGCAGTTAGCTCAA	GATGCACGGTATTACCTTGGGTAT
WRKY 1 transcription factor	AACAACAAGCGAGGTGGACATC	CGAGGAAAAGGAGGTTAGAAGATAAA
WRKY transcription factor 65-like	CGAGGACAGTATGAGCACAGACA	CGGCAACTCGCCGAGAT

consist of 2 μL cDNA, 15 μL SYBR green mix (2×) (TaKaRa), 2 μL (5pmol/μL) of both forward and reverse primers, and 11 μL PCR-grade water equating to a final volume of 30 μL. Reaction mix was dispensed in a 396-well PCR plates in triplicates. The thermal profile of the reaction was an initial denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 10 s. Ct and baseline values were automatically calculated by the Sequence Detection Systems (SDS) software version 2.2.1 (Applied Biosystems). Relative quantification was done for transcript abundance using the ΔΔCT method with default parameters. The relative quantity was calculated as $RQ = 2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001; Sanchita et al. 2015). Rubisco allowed use as endogenous control.

Bioinformatics analysis and sequence retrieval of trichome development related genes and different transcription factors

The gene of desired character was retrieved by a completely different approach. First, the sequence for trichome development and TF were collected from TAIR database specifically for *Arabidopsis thaliana* which were functionally annotated. These sequences were searched for homology by using BLAST N algorithm against SRA (SRX118150 and SRX118149) database of *Mentha* spp. Assembly was done for all obtained reads using CAP3 assembler (Huang and Madan, 1999) . Again, Blast X (Altschul et al. 1990) homology was done to capture the non-redundant nucleotides and protein database to know the presence of putative domain in sequence. Alignment was done against closest sequence and for predicting the function. Furthermore, the primers were synthesized for gene expression analysis (Table 2).

Physiological assays

Antioxidants analysis

Frozen leaf segments (0.5 g) were homogenized into a fine powder with a mortar and pestle in liquid nitrogen and add to buffer contains 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 % polyvinylpyrrolidone (PVP). Sample was centrifuged at 12,000 rpm for 10 min at 4 °C and then the supernatant was used for the following enzyme assays. The amount of soluble proteins was quantified according to the Bradford method with bovine serum albumin as standard. Supernatant was further used for glutathione S-transferases, glutathione reductase (GR) and superoxide dismutase (SOD) estimations as reported earlier (Singh et al. 2015).

Lipid peroxidation analysis

Two hundred milligrams of leaf tissue was homogenized in 1 ml of 0.25 % TBA dissolved in 20 % TCA. This mixture

was centrifuged at 10,000 rpm for 10 min. The supernatant was heated at 90 °C for 30 min. Corrected OD was calculated by taken OD at 532 nm and subtracting the OD taken at 600 nm. The level of lipid peroxidation was measured as micromoles of MDA formed. The total phenolic content of the extract was determined by the Folin–Ciocalteu method. Briefly, 200 L of crude extract (1 mg/mL) was made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 20% (*w*/*v*) sodium carbonate. The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per gram dry weight.

Chlorophyll content analysis

Chlorophyll was extracted by incubating the leaf tissue of controlled and stressed plants in 80 % acetone overnight under dark condition. Chlorophyll content was determined by using the method described by Wellburn (1994). Estimation of anthocyanin content was done by incubating the leaf sample overnight in 1 % acidified methanol. Anthocyanin content was calculated by taking OD at 530 and 657 nm (Porra et al. 1989).

Results

Morphology and phytochemistry of the investigated nine *M. arvensis* genotypes were analyzed for morphological features, light microscopy, quantitative anatomy, scanning electron microscopy, and gas chromatography. All quantitative continuous characters as length of leaf blade, width of leaf blade, plant height, length of petiole were provided as the range measured in the individual populations. Different genotypes of *M. arvensis* show wide range of variations in different morphological and chemical parameter.

Comparative estimation of morphological variance among *M. arvensis* genotypes

Significant morphological variation was found between genotype *M. arvensis* (Table 3). Range wise arrangement of these parameters reflected how genotypes of *M. arvensis* genotypes varied for different of parameters. It is found that a variety of Gomti showed maximum height with higher leaf area, while Kalka has lower plant height. Number of leaves on main stem indicated the herbage yield at the time of maturation. *M. arvensis* varieties Kosi and Himalaya were in highest range for number of leaves, while Kalka showed lowest. *M. arvensis* varieties Himalaya, Kushal, Sambhav, and Kosi showed 15–18 branches at the time of maturity, while Damroo having the least number of branches (7–8), and this is also a key feature for higher herbage at time of maturity. While talking about leaf morphology, Sambhav, Gomti, and Himalaya were in the highest range for leaf length as well as leaf breadth, i.e., leaf area. Himalaya, Saksham, and Sambhav were having longest petiole (Fig. 1).

Comparative estimation of herbage, oil yields, and secondary metabolite constituents

The green herb of all varieties of *M arvensis* was hydro distilled for comparative analysis of essential oil yield (Fig. 2). Herbage was studied in per unit area, and it is found that the maximum herbage was in Himalaya 2.24 kg per unit area, preceded by Gomti and Kosi. Least herbage was found in *M. arvensis* var. Kalka 0.32 kg per unit area of land. Essential oil was measured at the time of distillation, and it is found that variety Kosi was highest in oil yield compared to others, whereas Gomti showed lowest essential oil yield. The chemical

Table 3 A comparative presentation of morphometrical trait for nine elite genotype of *M. arvensis*

Sr no.	Plant height (cm) with SD	No. of leaves on main stem with SD	No. of branches on main stem with SD	Leaf length with SD	Leaf breath (in cm) with SD	Petiole length (in cm) with SD
Saksham	39.8 ± 3.1	16.3 ± 6.3	10.6 ± 2.8	3.7 ± 0.6	2.6 ± 0.2	1.1 ± 0.2
Kosi	47.1 ± 4.0	20.2 ± 2.4	15.5 ± 4.1	4.0 ± 0.5	2.3 ± 0.2	0.9 ± 0.2
Gomti	58.3 ± 2.6	18.3 ± 2.2	10.7 ± 2.1	4.5 ± 5.5	3.3 ± 2.2	0.6 ± 0.1
Himalaya	39.6 ± 15.9	20.9 ± 6.9	15.1 ± 1.6	9.4 ± 4.1	3.0 ± 0.4	1.4 ± 0.5
Damroo	44.8 ± 8.6	21.5 ± 4.3	7.4 ± 2.6	4.3 ± 0.3	3.0 ± 0.3	0.7 ± 0.1
Kushal	38.7 ± 2.1	19.0 ± 2.4	15.3 ± 4.5	4.0 ± 0.3	2.8 ± 0.3	0.9 ± 0.1
Sambhav	53.1 ± 8.2	17.9 ± 3.3	18.4 ± 2.6	4.4 ± 1.4	2.6 ± 0.3	1.0 ± 0.2
Kalka	29.7 ± 6.7	13.8 ± 1.3	10.7 ± 3.6	3.0 ± 0.9	1.9 ± 0.4	0.8 ± 0.3
Shivalik	50.4 ± 0	22.4 ± 2.9	13.7 ± 3.4	4.2 ± 0.5	2.7 ± 0.3	0.6 ± 0.1

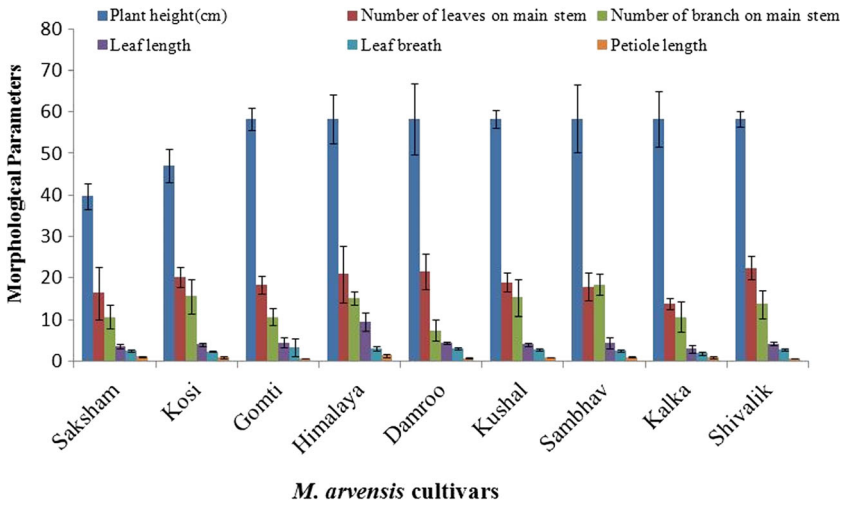

Fig. 1 Comparative analysis of all genotypes of *M. arvensis* analyzed for various morphological parameters

composition of essential oil obtained was analyzed by gas chromatography. The GC separated compounds that were identified for each *M.* arvensis variety, and economically important major secondary metabolites were identified as menthol (72–80 %), menthone (1.5–11.0%), menthyl acetate (0.5–5.3 %), isomenthone (2.1–3.9 %), limonene (1.2–3.3 %) and neomenthol (1.9–2.5 %). All the varieties were rich in menthol and found in a range of 73.7–85.8 %. The menthone content varies between 4 and 13.0 %, with the highest in Gomti (11.0 %) followed by Damroo (11.2 %), while Kalka contains a relatively low amount (2 %) as depicted in Fig. 2. Looking toward metabolite composition and dynamics of essential oil, it was found that during different developmental stage, there were huge changes in essential oil content. The content of essential oil during development of *M. arvensis*, i.e., early stage (35 days) after transplanting and mature stage (110 days after transplanting) and increase of essential oil content and simultaneous increase in menthol content in genotypes of *M. arvensis* (Fig. 10).

Dynamics of glandular and non-glandular trichomes: their number and distribution on leaf lamina

Both glandular and non-glandular trichomes were present on leaves of all *M. arvensis* varieties. Glandular trichomes are hair-like structures usually which may serve as a mechanical barrier against various external factors, such as herbivores and pathogens, UV-B radiation, extreme temperatures, and excessive water loss (Werker 2000). Trichomes were also studied microscopically in different parts of leaf in different varieties for their distribution pattern (Fig. 3). The abundance of leaf hairs and their distribution on leaf differed among the varieties of *M. arvensis* studied. Hairs were more abundant on the ventral leaf surface of all varieties of *M. arvensis*. The menthol mint var. Gomti contains maximum number of non-glandular trichomes, while variety Kosi showed minimum number of non-secretory trichomes while analyzing all released varieties. It was observed that glandular trichomes were more abundant on dorsal surface compared to ventral surface of leaves

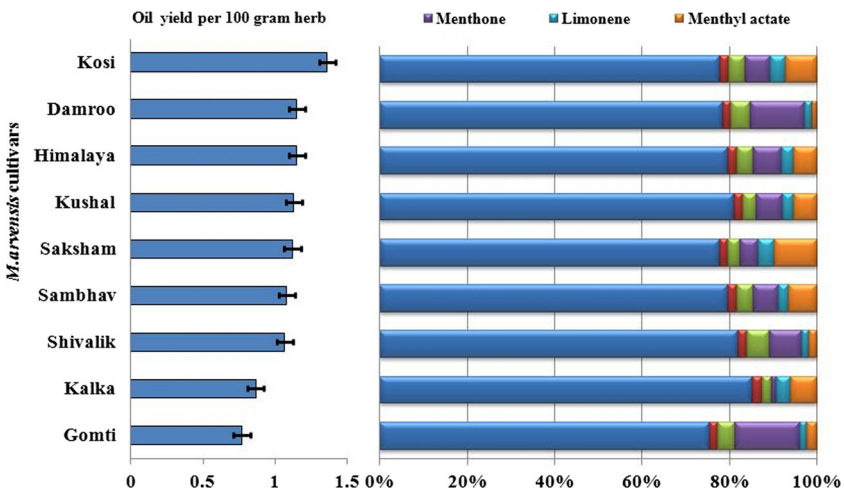

Fig. 2 Essential oil yield in selected genotypes of *M. arvensis* with comparative analysis of major constituents as analyzed by gas chromatography

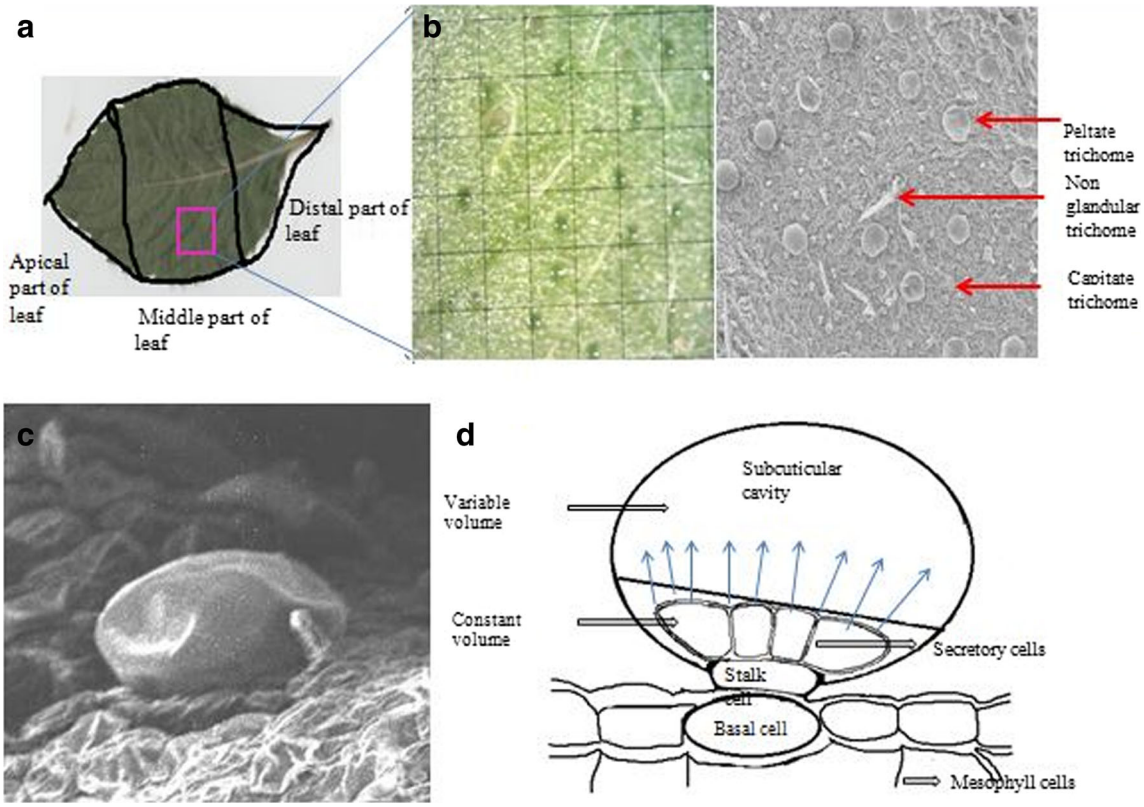

Fig. 3 Representation of trichomes present on leaf surface as visualized by stereomicroscope and scanning electron microscopy. Figure shows the area observed in leaf of *M. arvensis* under microscope. **a**, **b** Scanning electron micrograph of trichome and graphical representation of trichome arrange on leaf surface. **c**, **d** (Estepa et al. 2010, Fahn 1988)

(Fig. 4). A microscopic observation of leaf surface demonstrated that the size and there distribution pattern of glandular trichomes was the same within genotype but differs significantly from other genotypes (Fig. 5). A wide range of variations was observed in size of trichomes and the distance between two trichomes. Glandular trichomes were observed on both surfaces of leaf and the density of glandular trichome was higher on dorsal surface. It was observed that during scanning electron microscopy. *M. arvensis* var. Kalka showed largest trichomes, while variety Shivalik has smallest size of trichomes among all varieties. Analyzing distance between two trichomes provided a critical view of trichome density in each variety, as density is inversely proportionate to distance between two objects and higher distance between two trichomes will decrease the density in per unit area It was found that Kalka genotype goes toward higher side while Himalaya having maximum distance (Fig. 6). The variety Kalka had lower oil yield while variety Kosi showed maximum oil yield. Graph depicted relation between trichome density and oil yield (Supplementary Table 1). It was found that number of trichomes changes during development of plant per unit area. In early stage, each and every genotype possesses low number of trichomes compared to mature stage with the increase in number of trichomes during development. It could be analyzed clearly that in most of the genotypes, the number of glandular trichomes increases compared to non-glandular trichome which is revealed by ratio of GST versus NST (Figs. 4 and 10). A whole trichome contains a stalk cell, attached to the base cell and eight secretory cells, and this system is covered by elevation of subcuticular cavity, which imparts an ovoid or spherical shape. The whole cavity could be divided into two parts: first, it contains eight secretory cells and second is remaining subcuticular cavity. The size of subcuticular cavity is dependent upon secretion of monoterpenoid from secretory cells. There are three stages of trichomes, namely, presecretory, secretory, and postsecretory stages. Cell differentiation takes place in prestage followed by monoterpenoid secretion, which is maximum in secretory stage and it ceased in post secretory stage. Tuner et al. (2000) also described that it takes 33 h long to fill the whole cavity, which can be observable as biggest size of trichome in leaf surface on both sides. The size of glandular trichome depends on monoterpenoid exudates secreted by secretary cells. The more the content of monoterpenoid is, the bigger the size of trichome (Tuner et al. 2000) (Fig. 3).

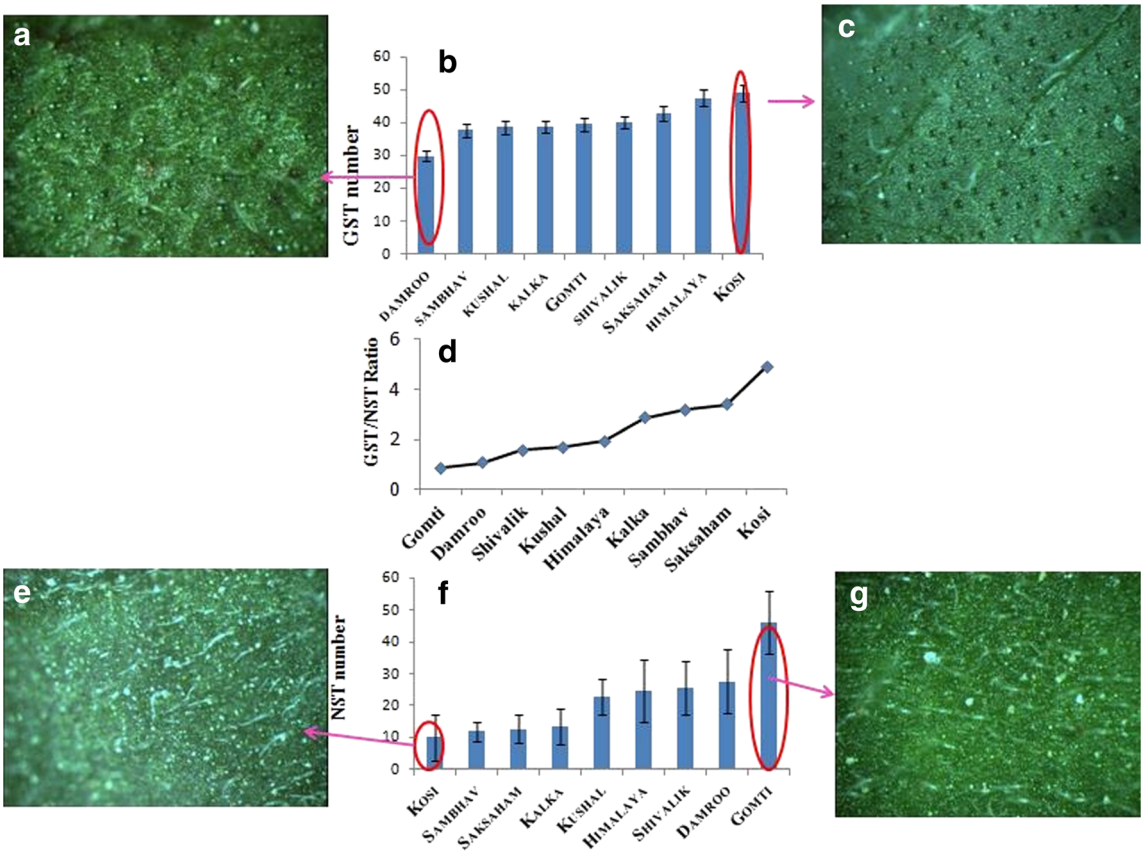

Fig. 4 Glandular and non-glandular trichome density of mature plant on dorsal surface of leaves (**a**, **c**, **e**, **g**). Glandular trichomes appeared as bright green spots. Graphical representation of ratios of glandular trichome and non-glandular trichomes present in different genotypes (**b**, **d**, **f**)

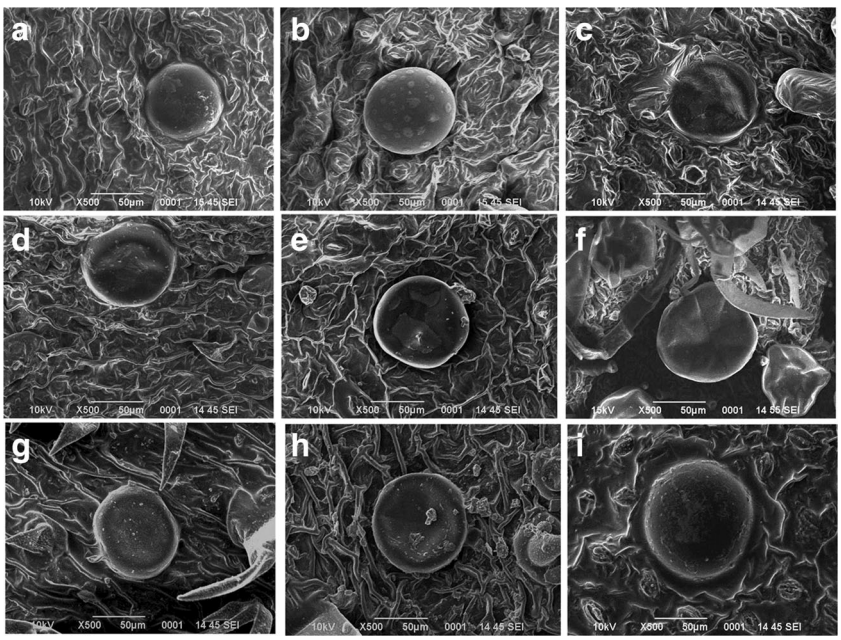

Fig. 5 Scanning electron micrograph of trichomes present in different genotypes of *M. arvensis*. **a** *Saksham*, **b** *Kosi*, **c** *Shivalik*, **d** *Gomti*, **e** *Himalaya*, **f** *Sambhav*, **g** *Kushal*, **h** *Damroo*, **i** *Kalka*

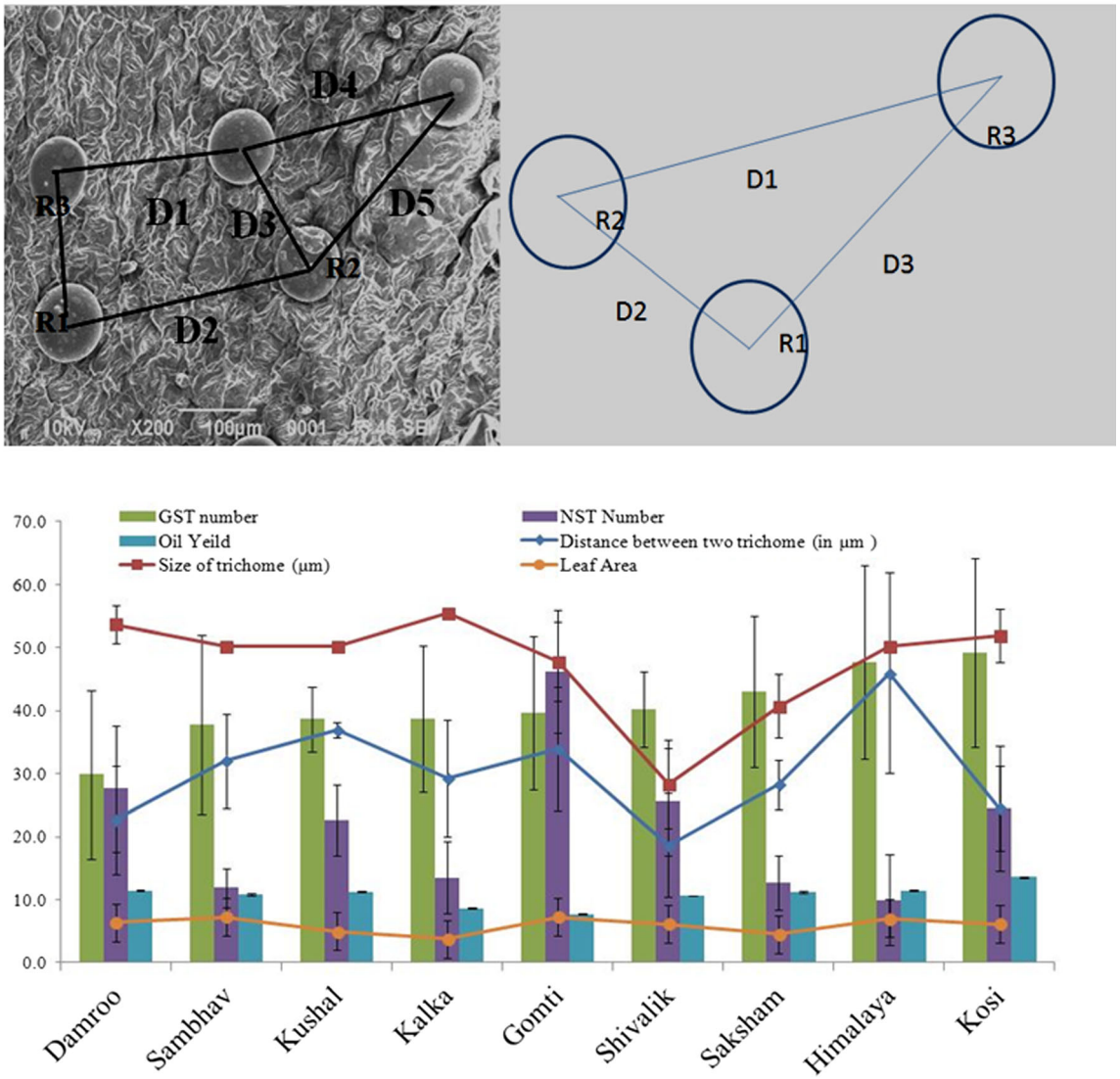

Fig. 6 Representative diagram for estimation of trichome area and distance in between consecutive trichomes. Graph showing correlation between trichome densities to oil yield and a comparative analysis of trichome dynamics with detailed comparison of distances between two consecutive trichomes in different genotypes of *M. arvensis*

Correlation studies among various characters studied in *M. arvensis* genotypes

Variations among all varieties were highly significant (**$P < 0.01$, *$P < 0.05$) for all sixteen traits examined (Table 5). The study of analysis of variance, standard errors, and critical difference (CD) revealed highly significant differences among varieties of *M. arvensis*. All sixteen characters studied indicating thereby existence of considerable genetic variability. Phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) were estimated for studied parameters, and results indicated the presence of considerable amount of genetic variability, thereby emphasizing wide scope of selection for the improvement of these characters. The sampling variance was moderate to high for nearly all the traits examined as GCV ranged from 3.22 to 56.89 and PCV from 3.8 to 77.24. A high heritability estimate (HER(BS)) with corresponding high genetic advance (GA) is more reliable for selection in comparison to low GA. The heritability ranges from 33 to 90.3. The GA shows the three range of character as more than <5, >5 to 10 and more than 10. Glandular trichome present in different parts of leaf, trichome average, and menthone contents show a good range of GA, while other show small genetic advance. These traits might be highly amenable to direct selection for their genetic improvement over a short span of time. Besides, heritability in broad sense (HERB) and GA, the associations among characters, also have a good criteria for selection of genotype as well as

Table 4 Estimation of phenotypic and genotypic variance, genotypic and phenotypic coefficient of variation; heritability in broad sense HER(B), genetic advance of nine agronomic traits in *M. arvensis*

Traits studied	Genetic coefficients of variation (GCV)	Phenotypic coefficient of variation (PCV)	Genetic advance	Hertability (broad sense)
GST apical	22.35	28.062	14.79	63
GST lower	19.70	26.93	6.64	53
GST middle	25.63	29.45	16.16	75
Leaf breath	23.34	35.81	0.56	42
Leaf length	23.26	34.76	0.37	44.5
Menthol content	3.22	3.80	3.55	71
No. of branch on main stem	24.61	36.36	2.87	45
Oil yield	14.75	19.02	0.19	60
Petiole length	23.18	33.72	0.19	47
Plant height	17.30	23.31	8.81	55
Trichome average	15.85	17.92	7.20	78
Menthone	56.89	61.16	6.70	86
Menthyl acetate	44.74	77.24	1.40	33
Neomenthone	5.93	8.03	0.12	54.4
Isomenthol	24.07	25.91	1.40	86
Limonene	27.16	39.04	0.65	48
ST/NST ratio	53.85	56.65	2.41	90.3

characters. In the present study, yield and its components were highly heritable with moderate to high level of genetic advance (Table 4).

Positive and significant associations were found among glandular trichomes, oil yield, essential oil constituents, and leaf morphology itself, whereas morphological parameters of leaf show positive and negative correlations to average number of trichome and essential oil constituents. There was a significant correlation between different morphological and yield parameters. Considering the oil yield and trichome numbers, it was found that glandular trichome present on the basal side of leaf shows significant positive genotypic correlation with oil yield (0.799), whereas total number of trichomes showed positive but comparatively less interaction with oil yield. It was also reflected that genotypes having long petiole with large leaf area had more number of trichome present in apical part. Average of trichome number was in positive genotypic correlation with menthone (0.691) and neomenthone (−0.77). Menthone showed positive genotypic correlation with neomenthone (0.863) and negative genotypic correlation with menthyl acetate (0.863) and limonene (0.92). Menthyl acetate showed positive genotypic correlation with neomenthone (0.65), isomenthol (1.49), limonene (1.49), and ST/NST ratio (0.97) (Table 5).

Gene expression analysis in menthol biosynthetic pathway genes

qRT-PCR was done with selected biosynthetic pathway genes deoxy-D-xylulose-5-phosphate synthase (DXP), dimethylallyl transferase (DMAPP), limonene synthase (LS), limonene hydroxylase (LH), pulegone reductase (PR), menthofuran synthase (MS), and menthone reductase (MR) of menthol biogenesis with three genotypes showing good contrasting character in oil yield as well as percentage of major secondary metabolite constituents. It was found that expression of most of the genes was significantly increased in *M. arvensis* var. Kosi compared to Gomti, whereas Saksham was taken as control. It was observed that menthone reductase was drastically high in Kosi which converts menthone to menthol, i.e., high content of menthol in essential oil therefore may be an important factor and responsible for high yields of menthol from Kosi oil (Fig. 7). As evident from microscopy, it was found that among these three genotypes, the size of trichomes of Kosi was higher as evident by higher secretion of monoterpenoids synthesized within the trichomes and which may be due to the higher activity of these genes which are highly expressed in Kosi compared to Gomti and Saksham (Figs. 5 and 7).

Analysis of differential changes for SOD, GST, GR, MDA, phenolics, and chlorophyll content

Enzymatic assays were performed for better understanding of translational activity in *M. arvensis* varieties/cultivars. Superoxide dismutase (SOD), glutathione-S-transferase, malondialdehyde, total phenolic, and chloroform were quantified and compared among three selected genotypes which show high contrasting values of essential oil yield, trichome numbers, and gene expression analysis. It was found that SOD was 15, 32, and 12 units, respectively, in Saksham, Kosi, and Gomti, or GST it was 30, 50, and 31 units, whereas MDA showed 5, 6, and 14 units, respectively. Phenolics content was nearly equal in all of these genotypes with little higher in Saksham. Pigment-like anthocyanin and chlorophyll were lower in Kosi followed by Gomti and Saksham (Fig. 8).

Analysis of transcription factors in relevance to trichome development

Different transcription factors related to trichome development and patterning viz. transparent testa glabra 1, enolase 1, transcription factor GLABRA 3, Trihelix DNA-binding protein (GTL1), nuclear transcription factor Y subunit B-6, homeobox protein, microspore-specific promoter 3, WRKY transcription factor 22, putative WRKY transcription factor

Table 5 GCV and PCV between different morphological and chemical characters (* P < 0.05. And ** P < 0.01.)

	GST lower	GST middle	Leaf breath	Menthol content	Number of branch	Oil yield	Petiole length	Plant height	Trichome average	Menthone	Menthyl acetate	Neomenthone	Isomenthol	Limonene	St/nst ratio
GST apical	0.20	0.00	0.10	−0.30	0.50	0.10	0.78*	0.06	0.08	0.32	0.96**	0.22	0.50	0.93**	0.51
	0.00	0.10	−0.10	−0.30	0.20	0.20	0.40	0.07	0.03	−0.31	0.60	0.0077	−0.40	0.32	0.42
GST lower	–	0.50	−0.50	0.50	0.30	0.79**	0.20	−0.47	0.03	−0.718*	0.52	0.7631*	−0.50	−0.01	0.08
	–	−0.20	−0.10	0.40	0.20	0.23	0.10	−0.23	0.16	−0.443	0.025	0.4526	−0.40	0.27	−0.40
GST middle		–	0.10	−0.30	0.20	−0.27	0.00	0.13	−0.10	0.155	−0.181	−0.236	0.50	0.15	0.40
		–	0.00	−0.20	0.30	−0 .34	0.10	0.077	0.10	0.143	−0.195	−0.198	0.30	0.12	0.31
Leaf breath				−0.92**	−0.24	0.34	−0.40	0.99**	1.01**	1.01**	1.01**	−0.870**	0.50	−0.93**	−0.58
				0.68*	0.03	0.19	−0.10	0.42	0.66**	0.76*	0.76*	−0.506	0.30	−0.35	−0.37
Menthol content				–	−0.12	−0.20	0.00	−0.83**	−0.71	−0.76*	0.163	1.00**	−0.30	0.29	−0.13
				–	−0.25	−0.18	9.76***	−0.60	−0.58	−0.66*	0.053	0.60	−0.30	0.17	−0.08
No. of branch on						0.31	0.50	0.40	−0.18	−0.40	0.663*	0.30	0.10	0.13	0.671*
						0.05	0.33	0.30	−0.05	−0.07	0.02	0.10	0.10	0.10	0.40
Oil yield						–	−0.40	0.50	0.49	0.01	−0.05	0.20	−0.20	−0.16	0.00
						–	−0.46	0.20	0.18	0.02	0.17	0.10	−0.20	0.10	0.10
Petiole length								0.60	−0.25	0.52	1.05**	0.40	−0.50	0.82**	0.40
								0.30	−0.14	0.36	0.262	0.30	−0.3	0.30	0.30
Plant height								–	0.53	0.84**	−0.673*	−0.76*	0.70*	−0.70*	0.10
									0.39	0.49	−0.16	0.41	0.55	−0.46	0.10
Trichome average									–	0.691*	−0.518	−0.77*	0.31	0.61	−0.40
									–	0.58	0.267	−0.44	0.28	0.44	0.40
Menthone											−0.864*	0.86**	0.64	−0.92**	−0.50
											−0.595	−0.59	0.54	−0.49	−0.50
Menthyl acetate											–	0.66*	−1.01**	1.49**	0.97**
												0.13	0.46	0.42	0.50
Neomenthone												–	−0.71*	0.60	0.10
												–	−0.41	0.23	0.018
Isomenthol														−0.77*	−0.26
														−0.62	−0.29
Limonene														–	0.87**
														–	0.57

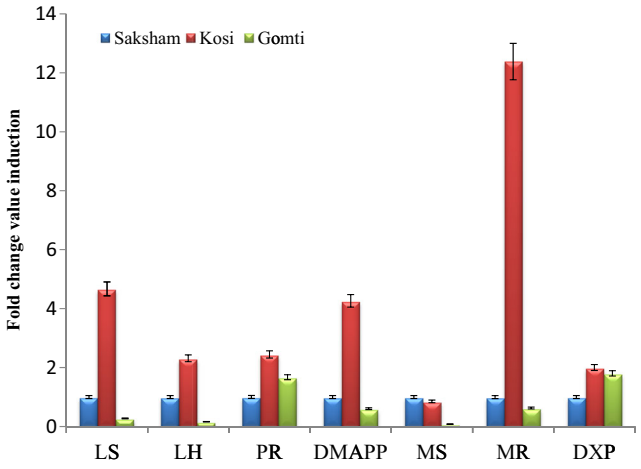

Fig. 7 Gene expression analysis of essential oil biosynthetic genes

33, putative WRKY transcription factor 17, WRKY 1 transcription factor, and WRKY transcription factor 65-like were selected and analyzed for expressions by qRT-PCR in three selected contrasting genotypes on the basis of oil yield, constituents, and trichome number with ratios of GST and NST. It was found that most of the genes were upregulated in *M. arvensis* var. Kosi. The transparent testa glabra1 (TTG1) locus regulates several developmental and biochemical pathways in *Arabidopsis*, including the formation of hairs on leaves, stems, and roots, and the production of seed mucilage and anthocyanin pigments (Walker et al. 1999). Kosi shows threefold upregulation compared to other genotypes studied for TTG1 locus. The recombinant ENO1 protein exhibited enolase activity. ENO1 was localized into plastids and expressed in most heterotrophic tissues including trichomes and non-root hair cells, but not in the mesophyll of leaves. Kosi shows fourfold upregulation of this gene compared to others. Another gene which has an important role in trichome development homeobox protein was almost equally expressed in all genotypes except GL3 which showed a reverse pattern compared to previously studied genes and negatively regulated in Kosi compare to control and shows fourfold inductions

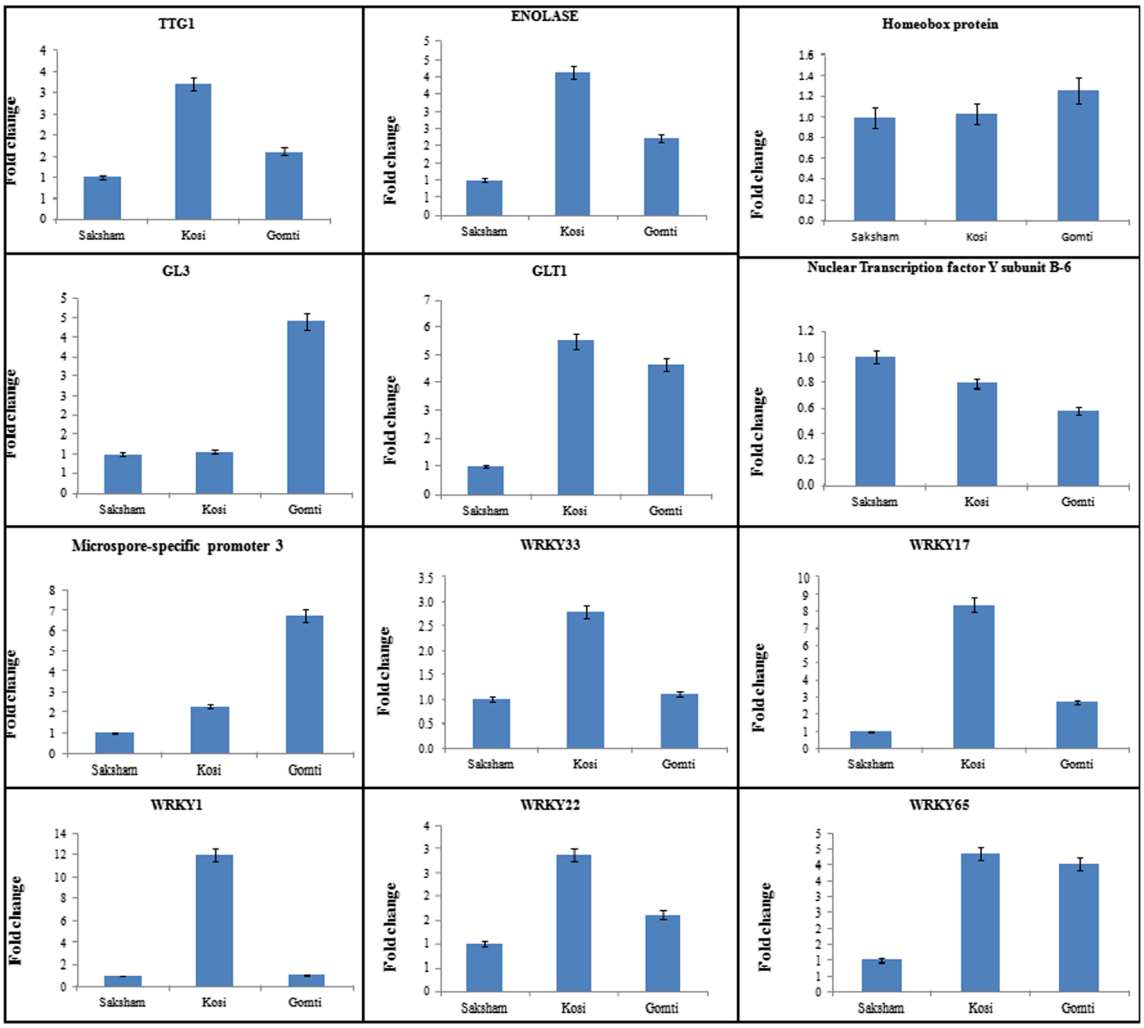

Fig. 8 Gene expression analysis for trichome development related gene and groups of WRKY type transcription factor in selected *M. arvensis* genotypes

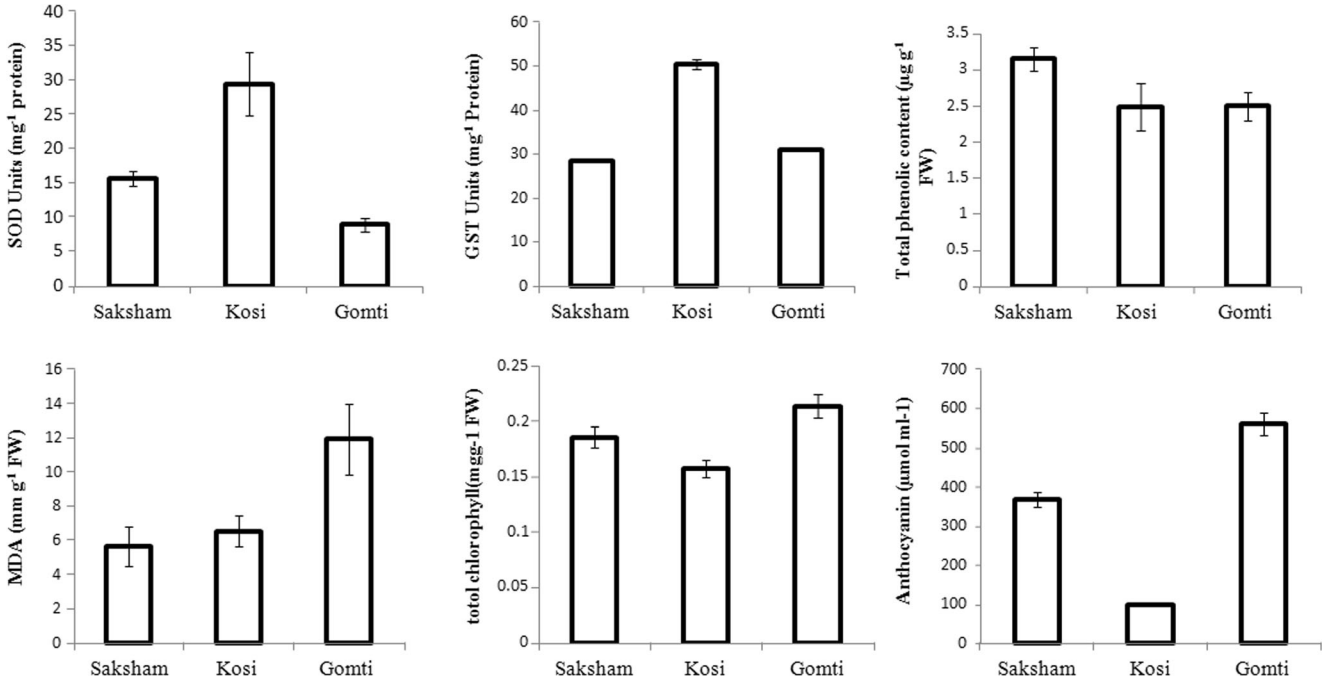

Fig. 9 Comparative analysis of cellular content of superoxide dismutase, SOD content, lipid peroxidation expressed in the terms of malondialdehyde, MDA concentration, and cellular level of glutathione-S-transferase, GST activity with total phenolics, and different pigment content in selected genotypes

in Gomti. Nuclear transcription factor Y subunit B-6 which is also express in trichome and during stress shows downregulation of gene in two genotype compared to Saksham, and microspore-specific promoter 3 gene showed sevenfold up-regulation in Gomti compare to control). Gene expressions were also analyzed with different WRKY transcription

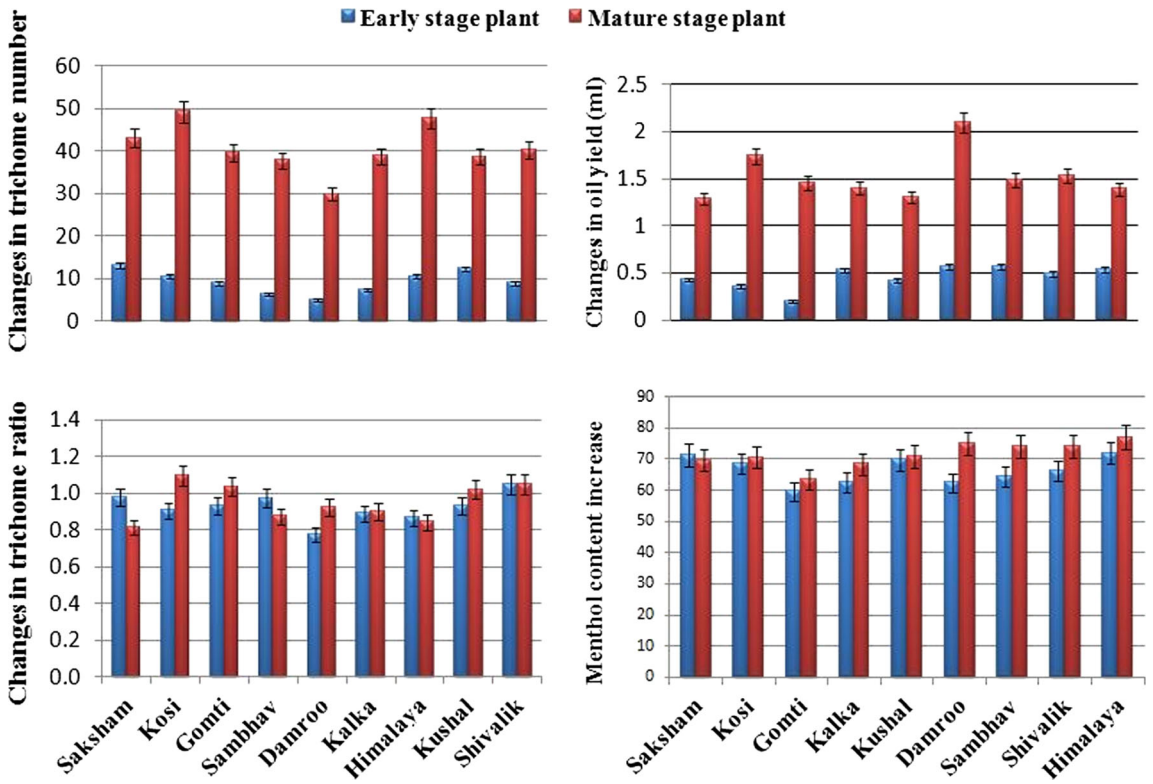

Fig. 10 Dynamics of trichomes and essential oil yield and menthol content in *M. arvensis* genotypes during early and mature stages of development

factors, which were expressed during stress and in trichome, also show upregulation in Kosi compared to other genotypes studied. WRKY 33 shows threefold upregulation. WRKY 1 shows 11-fold, WRKY 17 shows eightfold, and WRKY 22 shows 2.5-fold induction in Kosi compared to other genotypes, which clearly establish the role in trichome development (Figs. 9 and 10).

Discussion

At the time of harvest, when there was no emergence of new branches and leaf on main stem, oil yield was estimated. For high oil yield, a high leaf number and lateral branches are very important than a high leaf area (Loomis 1978) because the trichome density is constant with in a genotype and do not vary with leaf size. After the harvesting, time leaf senescence took place and herbage yield decline. Therefore, the herbage is not only the factor which controls the oil yield. There are several other quantitative continuous characters as leaf morphology, plant height, number of branches on main stem, and trichomes present on dorsal and ventral surfaces of leaves which play important roles in determining the oil yield and its chemical quality which was measured by the percentage of constituents or secondary metabolites. High yielding varieties are dependent upon various characteristics of genotypes and the divergence present in the genetic stocks; we studied genetic variation for nine genotypes of *M. arvensis* for agromorphochemical traits in order to understand genetic variability, genotypic, phenotypic associations, and direct and indirect correlations to environment for various yield components. All these traits were also expressed high heritability (HER(B)) and medium to high genetic advance with positive and significant genetic associations. *M. arvensis* aerial parts showed different types of glandular and non-glandular trichomes similar to those previously described for Lamiaceae. Microscopic studies showed the structure, size, and density variations in studies genotype of *M. arvensis*. *M. arvensis* genotypes demonstrated highly varied constituents of essential oil, as menthol, menthone, neomenthol, etc. and their correlation to various other characteristics studied along with the size, distance, and ratios of glandular and non-glandular trichomes (Fig. 7).

In most of the cases, it was found that GCV is larger than PCV as GCV measures only the extent of genetic variability present for a character; GCV should be considered in combination with heritability and genetic advance while assessing the effect of phenotypic selection (Valarmathi et al. 2004). High heritability does not mean a high genetic advance for a particular quantitative character (Aditya et al. 2011). Though genotypic coefficient of variation measures the amount of variation in character, it is not possible to access the amount of heritable variation based on this estimate. The smaller difference between phenotypic coefficients of variation and genotypic coefficient of variation in certain cases indicated that these characters were less influenced by environment (Reni and Rao 2013). Burton and Vane (1953) have suggested that GCV along with heritability estimates would provide a better idea of amount of genetic gain expected through phenotypic selection. High heritability coupled with high genetic advance observed for herb yield was under additive genetic control and simple selection for these traits would be quite effective. Broad sense heritability estimates are expected to be high because total genetic variance on which these estimates are based is made up of three parts, namely, additive genetic variance, non-additive genetic variance due to dominance, and non-additive genetic variance due to non-allelic gene interactions. There are several studies already done in model plant species for the spacing and distribution pattern of trichomes in which the possible role of gene involves in trichome was discussed. The architecture of peppermint glandular trichomes is a fixed parameter, whereas the program controlling trichome development is flexible (Estepa et al. 2010). During development, trichome density increases per unit leaf area with simultaneous increase in yield parameters (Fig. 10) which clearly depicted that there was a significant enhancement of essential oil yield, due to increase of trichome number and ratios of GST and NST as analyzed during study of trichome dynamics in *Artemisia annua* by Lommen et al. (2006). The role of *GL1*, *TTG*/*R*, and *TRY* was studied in reference to *Arabidopsis* as a model by Schnittger et al. (1999). Hauser et al. (2001) elaborated the possible role of GL1 in trichome distribution in *Arabidopsis thaliana*. Greese et al. (2014) and Failmezger et al. (2013) have generated the mathematical model and tool which help to elaborate the trichome spacing and patterning. In that model, the 3D construction and analysis of relevant aspects of trichome pattern by light microscopy and surface reconstruction and trichome patterning in reference to the understanding of the biological process. There are several reports which show correlation between essential oil content, antioxidants, and lipid peroxidase content. Therefore, it might be an interplay between trichome numbers, essential oil, content, and these enzymatic activities demonstrated by results of this analysis; it suggests that high essential oil containing genotypes have higher values of GST and SOD and low level of MDA; furthermore, it is clearly evident by higher antioxidant and lower lipid peroxidase and may have some crosstalk in higher essential oil content with increase of lipid peroxidase and antioxidant activity. In cultivar Kosi, content of chlorophyll and anthocyanin was less compared to other genotypes and suggested that higher essential oil content leads to depletion of pigments. Our results showed that cultivation only seems to affect the essential oil yield, increasing its content, not affecting the essential oil composition which seems to be more stable and uniform. These are the features that turn this species

and elite varieties and cultivars into interesting products for cultivation and commercialization.

Acknowledgments The authors express their sincere gratitude to the director of CSIR-CIMAP, India for keen interest and providing facilities for the experiments. Anand Mishra was supported by CSIR—Senior Research Fellowship, India. The authors gratefully acknowledge to USIC, B. B. Ambedkar University, India for providing the SEM facility.

Funding This work was supported by the Department of Biotechnology, Bio-CARe award project grant BT/AB/08/01/2008(III) GAP 311 and Council of Scientific and Industrial Research, India (CSIR Network Project BSC0110).

Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

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