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Comparison between the field plants and their micropropagated plants of four *Mentha spp.* using molecular markers and phytochemical analyses

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Abstract

The efficient micropropagation procedures among four *Mentha* species (*M. longifolia*, *M. spicata*, *M. viridis*, and *M. piperita*) were established. In addition, the shoots were produced in 3.0 mg/l BA. *M. longifolia* had the highest percentage of regeneration (70%), whereas *M. piperita* recorded that the lowest value (47%). The field plants and the micropropagated plants were subjected to phytochemical analyses. The gas chromatography and spectrophotometer results indicated that the active ingredients were changed in the micropropagated plants. Nevertheless, the results of molecular markers showed that the thirteen SRAP primer combinations had 80 scorable bands; of them, 58 were polymorphic (72%). Nine ISSR markers showed a total of 60 scorable bands; of them, 44 were polymorphic (73%). Twelve out of the 102 polymorphic SRAP and ISSR markers were found to be genotype unique specific markers. This study aimed to find an effective micropropagation procedure for four *Mentha* species, investigate phytochemical analyses, and assess the genetic variations in the field plants and the regenerated plants.

Keywords: *Mentha, micropropagation, phytochemical, SRAP, ISSR, phylogeny.*

1. Introduction

Mentha is the most important genus in the Labiatae family. It is composed of 25–30 species, which are found in temperate Australia, Eurasia, and South Africa and are widely distributed in semi temperate to tropical agroclimates **(Kamkar *et al*., 2010)**.

The essential oil (EO), obtained from the distillation of the aerial parts of the plant, contains a large variety of aroma chemicals in varying amounts, such as menthol, menthone, menthofuran, isomenthone, carvone, linalyl acetate, linalool, and piperitenone oxide used in pharmaceuticals, food, flavor, cosmetics, beverages, and allied industries **(Zheljazkov *et al*., 2010)**.

Antioxidants and polyphenols have several biological activities, including anti-inflammatory **(Joseph *et al.*, 2016)** and anticancer effects **(Fatiha *et al*., 2015)**.

Micropropagation is a tried-and-true technology for producing true-to-type medicinal plants on a large scale, with constant quality and quantity of plant-derived medicines. *In vitro* propagation methods also allow researchers to link secondary metabolite synthesis to a variety of factors such as nutritional and hormonal composition **(Siwach and Gill, 2014).**

A molecular marker is a type of genetic marker that detects genetic polymorphism in the DNA based on the differences in nucleotide sequence between individuals' genetic materials **(EL-Bakatoushi and Ahmed, 2018)**. The dominant markers based on PCR, such as inter simple sequence repeats (ISSRs) and sequence-related amplified polymorphism (SRAP), have been widely used in studies of genetic diversity **(Sun *et al*., 2018 and Gaafara *et al.*, 2017).**

The inter simple sequence repeat (ISSR) marker based on the amplification of DNA segment using a single primer containing a microsatellite "core" sequence anchored at the 5' or 3' end by a set of 2–4 purine or pyrimidine residues. This marker provided high reproducibility by detecting a high level of polymorphism in a relatively simple process. As a result, it has been widely used to determine genetic diversity and identify cultivars **(Sabboura *et al.*, 2016).**

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2. Materials and Methods

2.1. Materials

Four *Mentha* spp., namely, *M. longifolia*, *M. spicata*, *M. viridis*, and *M.piperita*, were used in this study. They were kindly provided by the Experimental Plants Station, Faculty of Pharmacy, Cairo University, Egypt.

2.2. Methods

2.2.1. Tissue culture

Leaf sterilization

The plants of four *Mentha* species were cultured in four blocks. Each block contained ten rows, was irrigated twice a week, and was fertilized once a month. For sterilization, the leaves were dipped in 70% ethano1 for one minute, then immersed in a 0.1% (w/v) solution of $HgC1_2$ for 1 minute, and rinsed three times in sterile distilled water. On sterilized Whatman filter paper, the leaves were blot-dried **(Sharma *et al*., 2019)**.

Shoots induction

The leaves were transferred to shoot induction medium **(Murashige and Skoog, 1962)**, supplemented with 6-Benzylaminopurine (BA) at different levels (0.0, 1.0, 2.0, 3.0, and 4.0 mg l^{-1}). The cultures were incubated under fluorescent daylight tubes at 25°C and subjected to 3,000 lux for a 16-/8-hour light/dark period. The frequency of shoot formations, the shoot number per explant, the shoot length, and leaf number per shoot were calculated.

Root induction and acclimatization

The developed shoots (8.0 cm in height) were transferred to jars containing MS medium supplemented with 1.5 mg/l IBA (Indole Butyric Acid) for root induction. After 2 weeks, the root number per explants and the root length were calculated.

The plantlets showing a well-developed root system were transferred to peat moss in 15 cm plastic pots and kept in the controlled greenhouse under a mist system **(Arumugam *et al*., 2020)**.

2.2.2. Phytochemical analysis

Extraction of essential oil

EOs from the leaves of the field plants and their regenerated plants were extracted using the steam distillation method **(Mazari *et al.*, 2010).**

Compositional analysis by GC

Gas-liquid chromatography (GLC) of *Mentha* EO was carried out using Agilent Technologies 6890 Model Gas Chromatograph equipped with a flame ionization detector (FID) and HP-5 column, Model G1530A, USA, according to **(Rohloff, 1999)** guidelines. The constituents of *Mentha* EO were identified by comparing their relative and absolute retention times with those of authentic standards. The EO composition was reported as a relative percentage of the total peak area.

Determination of total flavonoids, total phenols and total antioxidant

Polyphenols

Total polyphenol content was determined by spectrophotometry, using gallic acid as standard, according to the method described in **(Aliyu *et al*., 2009)**.

Flavonoids

A colorimetric assay was used to measure the total flavonoid content of dried leaves, according to **Benabdallah *et al.* (2016)**.

Total antioxidant activity (AA %)

Total antioxidant was determined according to the method described by **Mensor *et al.* (2001)** and modified by **Aliyu *et al.* (2009)**.

2.2.3. Molecular markers analysis: SRAP and ISSR markers

The field plants and their regenerated plants were analysed at the molecular level using SRAP and ISSR markers.

DNA Extraction

The CTAB method was used to extract the genomic DNA of the field plants and the regenerated plants, according to the method described by **Rogers and Bendich (1985)**. Every plant's leaves were used to extract DNA from each accession. The DNA quality was determined using an agarose gel (1.2 %) electrophoresis and UV light.

SRAP marker

Polymerase chain reaction (PCR) was performed in a total volume of 20 µl containing 10 ng DNA, 200 µM dNTPs, 1 µM of reverse and forward primers, 0.5 units of Red Hot Taq DNA polymerase, and 10X Taq polymerase buffer (ABgeneHousse, UK). SRAP primers (Delta) combinations (Table 1) were screened by PCR. Biometra thermal cycler (2720) was used for DNA amplification as follows: 94°C for 5 minfollowed by 5 cycles at 94°C for 30 sec, 35°C for 1 min, and 72°C for 30 sec; followed by 35 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec; finally, 72°C for 5 min.

ISSR marker

Nine ISSR primers (Table 1) were used in the PCR analysis (Delta). The reaction mixture (20 µl) contained 10 ng DNA, 200 µM dNTPs, 1 µM primer, 0.5 units of Red Hot Taq DNA polymerase (ABgeneHousse, UK), and 10X Taq polymerase (ABgeneHousse, UK). For DNA amplification, a Perkin Elmer Thermal Cycler (2720) was programmed as follows: denaturation step at 94ºC for 4 min; followed by 30 cycles at 94ºC for 30 sec, then at 44ºC for 45 sec, and at 72ºC for 1 min and 30 sec; finally, extension at 72ºC for 10 min.

Table (1): Nucleotide sequences of ISSR and SRAP primers.

ISSR code	Sequence of primer 5 ′3′	SRAP primer	
		Fowared primer (5′-3′)	Reverse primer (5′-3′)
808	(AG)8C	EM1:GACTGCGTACGAATTAAT	ME1:TGAGTCCAAACCGGATA
810	(AG)8T	EM2:GACTGCGTACGAATTTGC	ME2:TGAGTCCAAACCGGAGC
811	(GA)8C	EM3:GACTGCGTACGAATTGAC	ME3:TGAGTCCAAACCGGAAT
817	(CA)8A	EM4:GACTGCGTACGAATTTGA	ME4:TGAGTCCAAACCGGACC
825	(AC)8T	EM5: GACTGCGTACGAATTAAC	ME5:TGAGTCCAAACCGGAAG
834	(AG)8YT	EM6: GACTGCGTACGAATTGCA	
841	(GA)8YC		
UBC824	(TC)8G		
UBC826	(AC)8C		

Band scoring and cluster analysis

The SRAP and the ISSR gel images were scanned using the Gel Doc 2000 Bio-Rad system and analyzed with Quantity One software v.4.0.1 (Bio-Rad Laboratories, Hercules, CA, USA). The bands were sized and then binary-coded by 1 or 0 for their presence or absence in each genotype. The SYSTATver. 7 computer program was employed to calculate the pairwise difference matrices and plot the dendrogram among the field plants and the regenerated plants **(Yang and Quiros, 1993).** Cluster analysis was conducted based on similarity matrices obtained by the unweighted pair group method(UPGMA) using the arithmetic average to estimate the phenogram.

3. Result and Discussion

3.1. Tissue culture

The maximum stimulation of shoots occurred at 3 mg/l BA. Moreover, the shoot induction frequency of *M. longifolia* was 70%, followed by 65% for *M. spicata*, 50% for *M. viridis*, and 47% for *M. piperita* (Figure 1A and B). **Tütüncü (2020)** reported that the maximum shoot regeneration percentage were 82.5% and 80% at MS medium supplemented with 2 mg/L BA +2 mg/L GA3, 2 mg/L BA +1.5 mg/L GA3, respectively. In contrast, According to **(Liu *et al*., 2018),** 1.0 mg L BA supplemented to MS medium was optimal for inducing adventitious shoots.

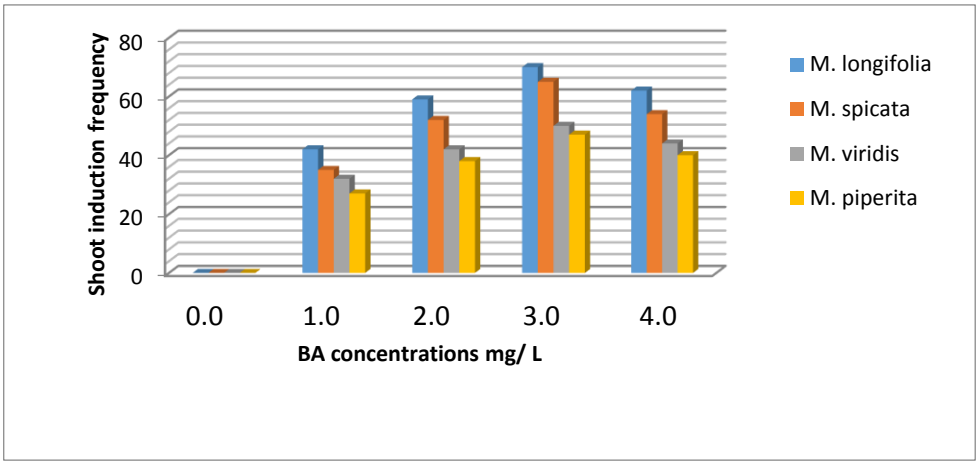

Fig. 1: Shoot induction frequencies in four *Mentha* species under different BA concentrations.

The shoots were transferred to MS medium supplemented with 1.5 mg/l IBA to form the roots (Figure 2C).**Sammaiah *et al.* (2015)** used different concentrations of IAA 0.1 mg/L or IBA 1.5 mg/L for root formation in *Mentha arvensis*. Moreover, **Nedelkova *et al.* (2011)** suggested that the root development was promoted by 1 mg/L IBA.

The plantlets produced *in vitro* were transferred into plastic pots filled with peat moss and kept in the controlled greenhouse under a mist system (Figure 2D). These results were confirmed by previous report **(Arumugam *et al*., 2020)**.

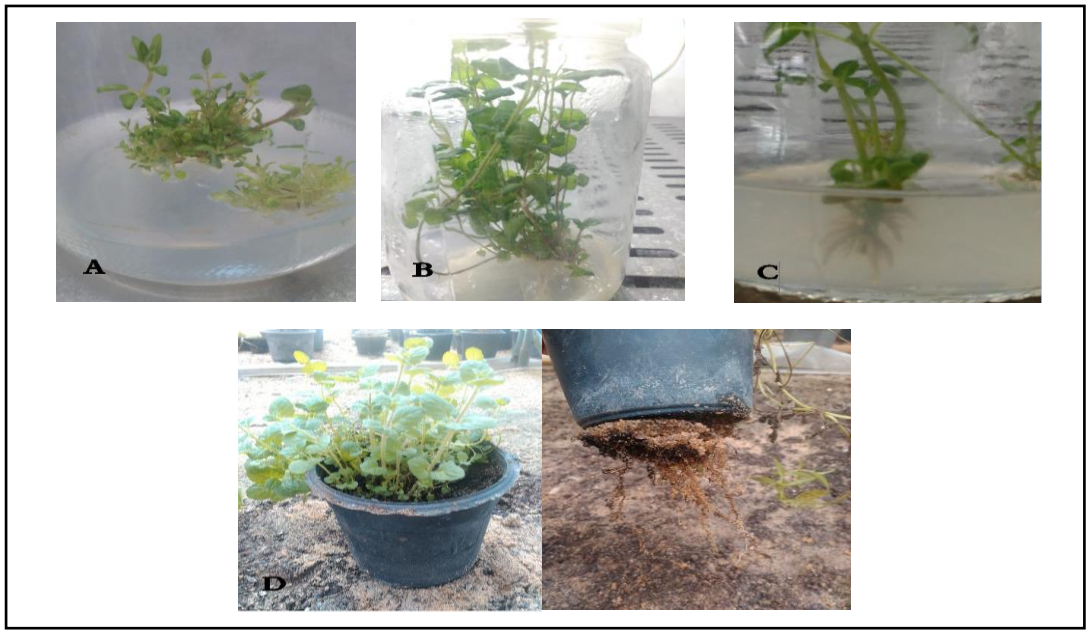

Fig. 2: Micropropagation stages, (A) shoot formation, (B) the whole shoots, (C) root formation and (D) the whole plants in the greenhouse.

M. longifolia gave the highest measurements compared with other species (Table 2).

Table 2.The averageof some measurements of the micropropageted plants.

	M. longifolia	*M. spicata*	*M. viridis*	*M. piperita*
Shoot number/explant	10	8	6	5
Shoot length (cm)	11	9	7	6
Root number/explant	2	2	2	2
Root length(cm)	7	6	4	3
Leaf number/shoot	8	6	4	4

3.2. Phytochemical studies

Estimation for the yield of volatile oil (ml/kg dry leaves) in the field plants and their regenerated plants

The results in Table 3 indicated that the volumes of the volatile oil (ml/kg dry leaves) of *M. spicata*, *M. viridis*, and *M. piperita* were decreased in the regenerated plants. On the other hand, the volume of the volatile oil of the regenerated plant of *M. longifolia* was increased from 31.20 to 32.18.

Table (3): Estimation for the yield of volatile oil (ml/kg dry leaves) in the field plants and their regenerated plants.

Species	Volatile oil (ml/kg dry leaves) of field plants	Volatile oil (ml/kg dry leaves) of regenerated plants
M. longifolia	31.20	32.18
M. spicata	30.40	25.32
M. viridis	26.84	22.85
M. piperita	24.79	15.78

Estimation of some active ingredients for the essential oil in the field plants and their regenerated plants by gas chromatogrphy

The active ingredients were changed in the regenerated plants compared with those in the field plants. For example, menthol and menthone of *M. longifolia* were absent in the regenerated plants. In addition, in *M. spicata*, carvone was decreased in the regenerated plant (Table 4).

Bishr and Salama (2018) found 35 components in the EO of three *Mentha* species (in one or more organs); P-menthone (32.24

%) is the most abundant compound in *Mentha piperita* leaves. *Mentha piperita* has seven distinct components, the most significant being 2,4(10)-thujadiene (3.88 %); *Mentha spicata* has 18 distinct components, the most significant being cymene (24.44%); *Mentha pulegum* has 11 distinct components, the most significant being (+)-isomenthol (16.64 %). Using GC-MS to analyze the oils of *Mentha piperita*, *Mentha pulegium*, and *Mentha spicata*, **Ainane *et al*. (2018)** found that menthone (42.97 %) and menthol (27.64%) are the main compounds in *Mentha piperita*; piperitone (31.27%) and piperitenone (22.98%) in *Mentha pulegium*; carvone (33.14%) and trans-carveol (20.06%) in *Mentha spicata*.

Table (4): The differences in the active ingredients content between the field plants and their regenerated plants.

Species		Compound (Area %)				
		α-Pinene	β- Pinene	Menthol	Menthone	Carvone
M. longifolia	Field plants	0.7	1.37	19.83	27.96	41.35
	Regenerated plants	1.23	3.84	-	-	66.79
M. spicata	Field plants	1.33	3.94	1.34	1.75	65.20
	Regenerated plants	0.63	1.68	51.81	22.92	15.07
M. viridis	Field plants	0.79	2.15	21.29	27.19	39.00
	Regenerated plants	1.07	4.23	0.22	1.92	65.86
M. piperita	Field plants	1.27	3.48	1.07	2.22	66.33
	Regenerated plants	3.83	4.13	68.21	56.71	30.85

Estimation of some active ingredients for the essential oil in the field plants and their regenerated plants by spectrophotometer

Total flavonoids

The obtained results in Table 5 indicated that the total flavonoids were decreased in regenerated plants of *M. longifolia*, *M. viridis*, and *M. piperita* compared with those in their field plants. On the other hand, totalflavonoids were increased in the regenerated plants of *M. spicata* compared with those in their field plants.

Benabdallah *et al*. (2016) observed that the uppermost amounts were 31.77 mg RE/g DW, whereas the minimum ones were 9.90 mg RE/g DW.

Table (5): Estimation of total flavonoid content (mg/g DW) in dry leaves of the field plants and their regenerated plants.

Species	Total flavonoids from field plants(mg RE/g DW)	Total flavonoids from regenerated plants (mg RE/g DW)
M. longifolia	8.7	7.6
M. spicata	4.9	7.2
M. viridis	7.5	6.2
M. piperita	18.6	3.2

Total polyphenols

The obtained results in Table 6 showed that the total polyphenols were decreased in regenerated plants of *M. spicata* and *M. viridis* compared with those in their field plants. On the other hand, the total polyphenols were increased in regenerated plants of *M. longifolia* and *M. piperita*. The estimation of total phenolics content was reported in many studies **(Atanassova *et al*., 2011; Benabdallah *et al*., 2016).**

Table (6): Estimation of total polyphenol content (mg/g DW) in dry leaves of the field plants and their regenerated plants.

Species	Total polyphenol from field plants (mg GAE /g DW)	Total polyphenol from regenerated plants (mg GAE /g DW)
M. longifolia	20.88	22.54
M. spicata	19.69	19.38
M. viridis	30.98	9.17
M. piperita	32.45	35.61

Total antioxidant

The results showed that the total antioxidant was decreased in regenerated plants of *M. viridis and M. piperita* compared with that in their field plants. On the other hand, the total antioxidant was increased in regenerated plants of *M. longifolia and M. spicata* (Table 7). **Benabdallah *et al.* (2016)** observed that the antioxidant activities of the six *Mentha* species were as follows: 17.00 ± 0.88, 13.33 ± 1.07, 31.66 ± 2.16, 25.66 ± 1.50, 44.66 ± 0.19, and 7.50 ± 0.16 for *M. piperita*, *M. arvensis*, *M. rotundifolia*, *M. pulegium*, *M. villosa* and *M. aquatica*, respectively.

Table (7): Estimation of total antioxidant content in dry leaves of the field plants and their regenerated plants.

Species	Total antioxidant from field plants (DPPH %)	Total antioxidant from regenerated plants (DPPH %)
M. longifolia	25.26	26.78
M. spicata	15.64	17.55
M. viridis	20.98	18.17
M. piperita	19.42	18.85

3.3. Molecular markers analysis

Sequence-Related Amplified Polymorphism (SRAP)

Molecular markers have been known and used in research for many years; they are employed as a tool for studying the polymorphism and genetic relationships among the field plants and their regenerated plants. Therefore, the SRAP banding patterns among the field plants and their regenerated plants using thirteen selected SRAP primers were analyzed. Multiple band profiles were formed by these primers, with a number of amplified DNA fragments ranging in molecular weight from 3 to 9 bands. The primer Me1-Em3 generated the most number of bands (9), whereas primer Me5-Em6 generated three bands. A total of 80 bands were generated, with 58 of them being polymorphic (72%). Me1-Em2 primer showed the highest polymorphism (100%), followed by primer Me1-Em3 (88%). In contrast, the lowest percentage (20%) was produced by Me1-Em4 primers among the tested genotypes (Figure 3 and Table 8).

Namayandeh *et al.* (2017) conducted the SRAP analysis in *Satureja* species. Their results showed that polymorphism was 74.22%. Moreover, SRAP analysis was used by **Zagorcheva *et al.* (2020)** in *Lavandula angustifolia* Mill., **Zhao *et al.* (2020)** in *Paris polyphylla*, and **Yan *et al.* (2019)** in *Mallotus oblongifolius*, showing a polymorphism of 77.2%, 83.24%, and 89.22%, respectively.

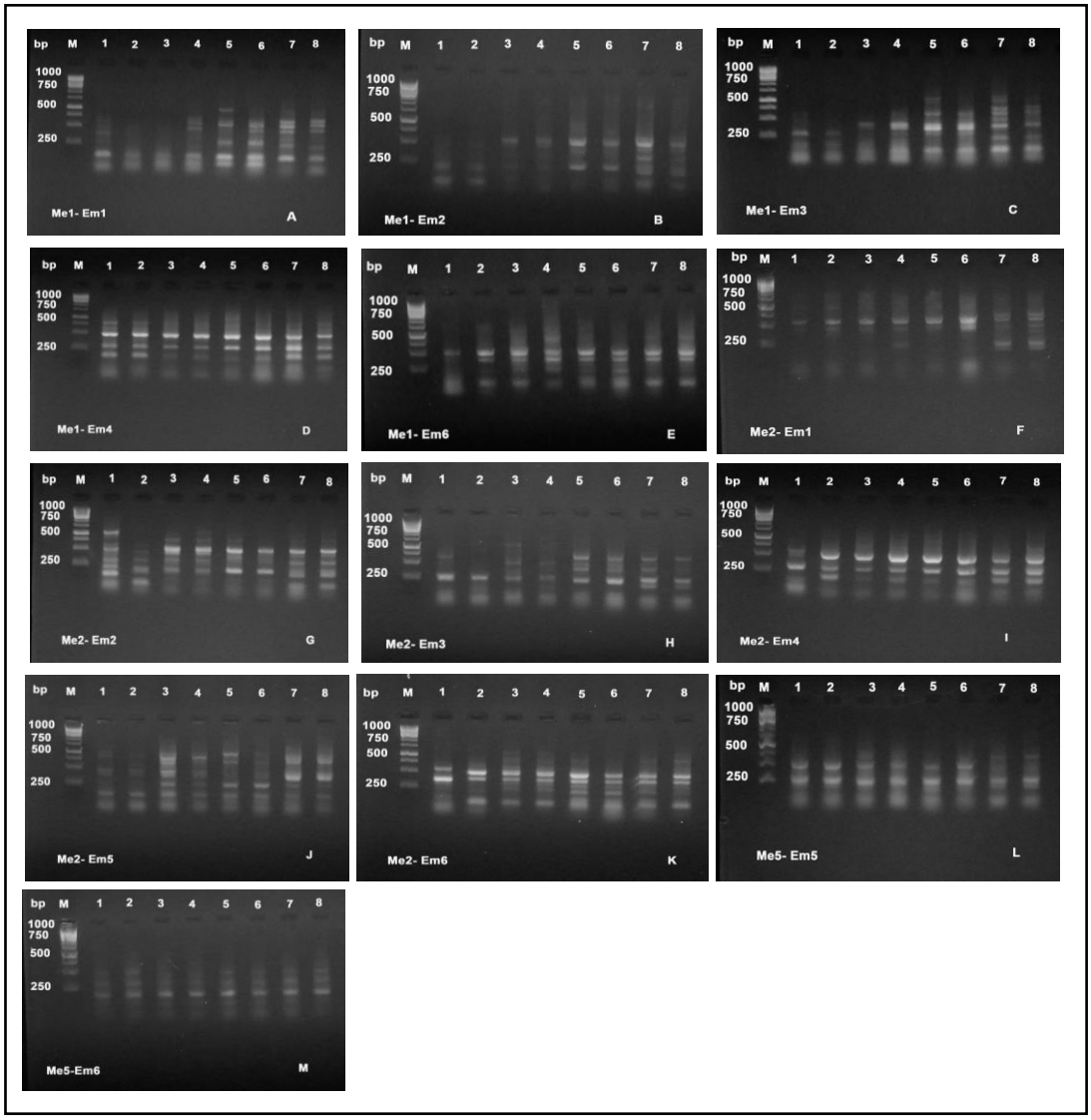

Fig. 3: SRAP banding patterns among the field plants and their regenerated plants using thirteen selected SRAP primers (A, B, C, D, E, F, G, H, I, J, K, L, and M), M: 1 Kb plus DNA ladder; 1, 3, 5, and 7 are the field plants of *M. longifolia, M. spicata, M. viridis, and M. piperita,* respectively; 2, 4, 6 , and 8 are the regenerated plants of *M. longifolia, M. spicata, M. viridis, and M. piperita,* respectively.

Table (8): Polymorphism percentage among the field plants and their regenerated plants by SRAP markers.

Primer name	Generated bands	Polymorphic bands	Monomorphic bands	Polymorphism %
Me1-Em1	7	6	1	85
Me1-Em2	5	5	-	100
Me1-Em3	9	8	1	88
Me1-Em4	5	1	4	20
Me1-Em6	8	7	1	87
Me2-Em1	5	3	2	60
Me2-Em2	6	5	1	83
Me2-Em3	8	6	2	75
Me2-Em4	7	5	2	71
Me2-Em5	4	1	3	25
Me2-Em6	8	6	2	75
Me5-Em5	5	3	2	60
Me5-Em6	3	2	1	66
Total	80	58	22	72

Inter Simple Sequence Repeat (ISSR)

The ISSR banding patterns among the field plants and their regenerated plants using nine selected ISSR primers were obtained. Multiple band profiles were formed by these primers, with a number of amplified DNA fragments ranging in molecular weightfrom 5 to 9. The primer 810 generated the maximum number of bands (9), whereas the primer UBC24 generated the minimum number of bands (5). Furthermore, a total of 60 bands were generated, 44 of which were polymorphic (73%). The primers 834, 810, and UBC26 produced the most polymorphic bands (6 bands), representing 75%, 66%, and 100% polymorphism, respectively. On the other hand, primer 825 produced the least polymorphic bands (3 bands), representing 50% polymorphism (Figure 4 and Table 9). **Hamouda (2019)** studied the genetic polymorphism in *Silybum marianum* L. using ISSR markers. According to their data, the polymorphism was 80%. Moreover, **Abu El-Maaty *et al.* (2019)** employed 9 ISSR primers and 16 SRAP primers to assess the genetic diversity among the three most important *Orobanche* species. The SRAP and ISSR analyses showed that 132 out of 170 and 80 out of 106 markers, respectively, were detected as polymorphic markers (77.6% and 75.4%). Further**, Giachino (2020)** demonstrated that the percentage of polymorphic loci was 51.5% in ISSR profiling in anise (*Pimpinella anisum* L.).

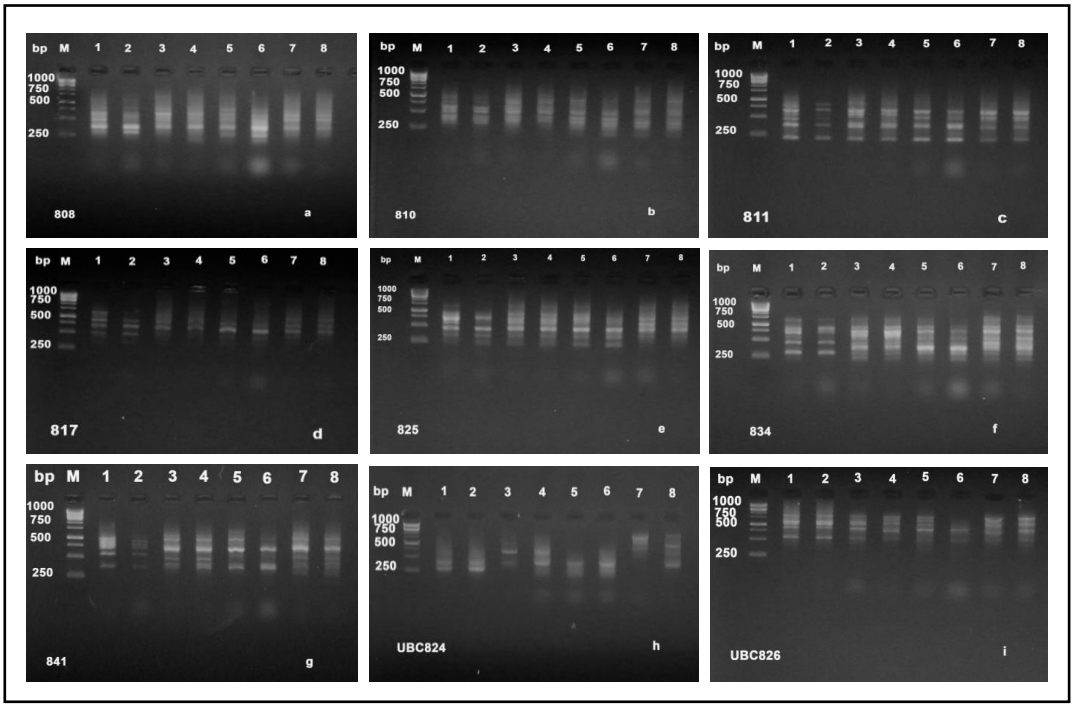

Fig. 4: ISSR banding patterns among the field plants and their regenerated plants using nine selected ISSR primers (a, b, c, d, e, f, g, h, and i); M: 1 Kb plus DNA ladder. 1, 3, 5, and 7 are the field plants of *M. longifolia, M. spicata, M. viridis, and M. piperita,* respectively, whereas 2, 4, 6, and 8 are the regenerated plants of *M. longifolia, M. spicata, M. viridis, and M. piperita,* respectively.

Table (9): The polymorphism percentages among the field plants and their regenerated plants by ISSR markers.

Primer name	Generated bands	Polymorphic bands	Monomorphic bands	Polymorphism %
808	7	5	2	71
810	9	6	3	66
811	7	4	3	57
817	6	4	2	66
825	6	3	3	50
834	8	6	2	75
841	6	5	1	83
UBC24	5	5	-	100
UBC26	6	6	-	100
Total	60	44	16	73

The genetic similarity matrices were produced using the Dice similarity coefficient based on the results of 13 SRAP and 9 ISSR primers for field plants and their regenerated plants (tested plants). The genetic similarity values varied from 0.66 to 0.93 (Table 10). The lowest estimated similarity among the four genotypes and their regenerated plants was 0.66 between *M. spicata* (T) and *M. piperita* (F), followed by 0.67 between *M. longifolia* (F) and *M. viridis* (T) and between *M. spicata* (F) and *M. piperita* (F). However, much higher values were recorded between *M. piperita* (F) and *M. piperita* (T) (0.93) and between *M. spicata* (F) and *M. spicata* (T) (0.91).

Patel *et al.* (2015) showed that genetic similarity coefficients of both RAPD and ISSR markers ranged from 0.21 to 0.90 among *Ocimum* species. On the other hand, **Yousef *et al.* (2015)** indicated that the *Mentha piperita* and *Mentha spicata* var. Moroccan genotypes were found to have the highest similarity values (1.0), whereas the *Mentha viridis* and *Mentha spicata* var. *Longifolia* genotypes had the lowest similarity values (0.21). In addition, **Nazneen *et al.* (2019)** showed that the similarity values ranged from 0.51 to 0.77 of 12 samples of *Tinospora cordifolia* using RAPD and ISSR.

Table (10): Genetic similarity matrices computed according to Dice coefficient from combined data of SRAP and ISSR markers.

Names	*M. longifolia* (F)	*M. longifolia* (T)	*M. spicata* (F)	*M. spicata* (T)	*M. viridis* (F)	*M. viridis* (T)	*M. piperita* (F)	*M. piperita* (T)
M. longifolia (F)	1.00							
M. longifolia (T)	0.88	1.00						
M. spicata (F)	0.72	0.75	1.00					
M. spicata (T)	0.74	0.74	0.91	1.00				
M. viridis (F)	0.72	0.73	0.77	0.79	1.00			
M. viridis (T)	0.67	0.70	0.72	0.73	0.87	1.00		
M. piperita (F)	0.70	0.68	0.67	0.66	0.74	0.73	1.00	
M. piperita (T)	0.72	0.71	0.71	0.71	0.73	0.72	0.93	1.00

The dendrogram of the field plants and their regenerated plants (tested plants) was produced using the results of 13 SRAP and 9 ISSR primers (Figure 5). The dendrogram is grouped into two clusters of species. The first one included *M. piperita* (F) and *M. piperita* (T). The second cluster was divided into two subclusters. The first was subdivided into two branches: the first one had *M. spicata* (F) and the second *M. spicata* (T). The second branch was subdivided into two branches: the first one had *M. viridis* (F) and the second *M. viridis* (T). The second branch was subdivided into two branches: the first one had *M. longifolia* (T) and the second *M. longifolia* (F). The dendrogram results revealed that there are no significant differences between the field plants and their regenerated plants.

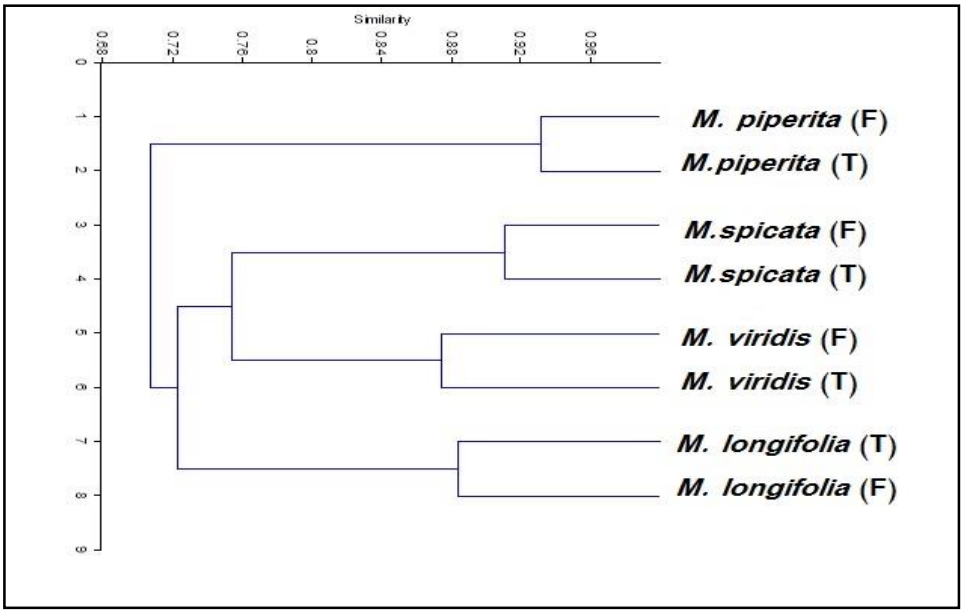

Fig. 5: Dendrogram for the field plants and their regenerated plants (tested plants) revealed by SRAP and ISSR analyses.

The genotype-specific SRAP and ISSR markers of the field plants and their regenerated plants (tested plants) were recorded in Table 11. Seven out of the 60 polymorphic SRAP markers have been identified as genotype-specific markers (11.66%). Therefore, the highest numbers of SRAP-specific markers were recorded for *M. viridis* (F) (3 markers) and *M. longifolia* (F), *M. spicata* (F), *M. piperita* (F), and *M. piperita* (T) (1 marker each), whereas *M. longifolia* (T), *M. spicata* (T), and *M. viridis* (T) did not show any SRAP band scores.

Five out of the 44 polymorphic ISSR markers were found to be genotype-specific (33%). The largest numbers of ISSR-specific markers were recorded for *M. longifolia* T (2 markers) and *M. spicata* (F) and *M. spicata* (T) (1 marker each), while *M. longifolia* (F), *M. viridis* (F), *M. viridis* (T), *M. piperita* (F), and *M. piperita* (T) did not show any scores for ISSR markers.

Table (11): Specific SRAP and ISSR markers in the field plants and their regenerated plants (tested plants).

Species	SRAP		ISSR		Total
	SRAP-specific marker	Total	ISSR-specific marker	Total	
M. longifolia (F)	Me2-Em1	1	-----	---	1
M. longifolia (T)	------	----	817, 808	2	2
M. spicata (F)	Me2-Em4	1	817	1	2
M. spicata (T)	-----	----	24	1	1
M. viridis (F)	Me1-Em4, Me1-Em6, Me1-Em1	3	----	---	3
M. viridis (T)	-----	----	811	1	1
M. piperita (F)	Me1-Em3	1	----	---	1
M. piperita (T)	Me2-Em2	1	----	----	1
Total		7		5	12

4. Conclusions

In this study, an effective and reproducible procedure for direct regeneration of complete plants from leaves was achieved. The most suitable concentration for shoot induction was 3.0 mg/L BA. Furthermore, *M. longifolia* species showed higher values for some measurements (shoot number/explants, shoot length (cm), root number/explants, root length (cm), and leaf number/shoot) compared with other species under the same conditions.

In addition, the variations were evaluated among the field plants and their regenerated plants using two molecular markers (SRAP and ISSR) and phytochemical analyses (total polyphenolic, total flavonoids, total antioxidant, and other active ingredients). The phytochemical data from GC and spectrophotometer revealed variations in the active ingredients of regenerated plants compared with field plants.

The molecular markers SRAP and ISSR analysis showed that the polymorphism percentages were 72% and 73% for SRAP and ISSR, respectively. The field plants and their regenerated plants are characterized by 7 SRAP and 5 ISSR unique markers. These molecular markers can be used for *Mentha* improvement.

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