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IN VITRO CLONAL MICROPROPAGATION OF IZMIR OREGANO (*Origanum onites* L. cv. "Ceylan-2002")

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ABSTRACT

In this study, which was carried out to develop an *in vitro* clonal micropropagation procedure in elite cultivar of *Origanum onites* L. cv Ceylan-2002, node explants were subjected to pre-washing and different sterilization methods. The highest level of sterilization efficacy was achieved in sterilization trials using diverse durations and commercial bleach doses, with a 100% success rate. Mean regenerated shoots number per explant (0.13), mean shoot length (0.12 cm), and mean leaf number (0.87) were achieved in MS medium+2 mg l^{-1} BAP+0.3 gl^{-1} activated carbon after four weeks of culture. The highest propagation coefficient (3.40) was obtained in the 4th subculture. In the rooting experiments the highest mean stem thickness value (2.02 mm) and the highest mean shoot length (10.02 cm) were obtained in media containing 1.0 g l^{-1} activated carbon. The rooted plantlets (100%) were acclimatized with a survival success rate of 16.25%. Essential oil analysis of well-developed rooted plantlets was performed using GC-MSD. The highest essential oil content (2.00%) was determined in regenerated shoots from MS medium+2 mg l^{-1} IBA+1 g l^{-1} activated carbon. The highest thymol ratio (65.33%) was found in regenerated shoots from ½ MS media supplemented with 0.5 mg l^{-1} IBA+1 g l^{-1} activated carbon. The highest carvacrol ratio (6.82%) identified in regenerated shoots was obtained from MS media, including 2.0 mg l^{-1} IBA+1 g l^{-1} activated carbon.

Keywords: *Origanum onites* L., Ceylan-2002, *in vitro*, clonal micropropagation, essential oil analysis.

INTRODUCTION

Oregano (*Origanum* spp.) is a herbaceous plant belonging to the Lamiaceae family, and it includes many aromatic and medicinal plants. Türkiye, Chile, Peru, Mexico, Greece, Israel, Albania, Indonesia, and Egypt are the most important producers of the oregano plant, which has 40 species worldwide and 35 species in Turkey (Goleniowski et al., 2003; Atilabey et al., 2015; Tunca and Yesilyurt, 2017). The most exported oregano species are *Origanum onites* L., *Origanum vulgare* subsp. *hirtum*, *Origanum minutiflorum*, *Origanum majorana,* and *Origanum syriacum* var. *bevanii* (Bozdemir, 2019).

Oregano is used as a spice and is obtained from *Origanum* species. Although carvacrol and thymol are the predominant secondary metabolites in Origanum species, concentration of these compounds varies considerably between species (Oluk and Cakir, 2009; Bayram et al., 2010; Sevindik et al., 2017). These compounds are effective against cancer cells through some cell collapse, as well as protecting the cell membrane. *O. onites* L. contains rosmarinic acid, γ-terpinene, γ-cimene, α-terpinene and α-pinene compounds with carvacrol and thymol (Atar and Colgecen, 2019). Carvacrol is a natural essential oil with high importance in aromatic compounds. It has been demonstrated to possess a range of beneficial properties, including antioxidant, antimicrobial, anticancer, antidiabetic, cardioprotective, antiobesity, and hepatoprotective effects, as well as antiaging properties (El-Gengaihi et al., 2006; Silva et al., 2012; Memar et al., 2017; Imran et al., 2022). Thymol is known for its antioxidant, antispasmodic, antimicrobial, and anti-inflammatory effects (Memar et al., 2017). The amount of carvacrol and thymol compounds is an important factor in determining the price of oregano trade (Ozyazici and Kevseroglu, 2019).

Although oregano is a perennial herb, its commercial life is about seven years. Traditional production methods include seeds and cuttings (Sokat, 2021). However, in this method, the desired quality and standard production may not be achieved due to foreign fertilization (Bahtiyarca Bagdat, 2006; Colak Esetlili and Cakici, 2010). The collection of oregano plants from the natural flora leads to the extinction of many plant species, the loss of natural gene resources of these plants, and the collection of non-purpose material. Given the numerous challenges

associated with the conventional production of oregano, the development of alternative biotechnological methods is necessary for the large-scale cultivation of oregano species and the attainment of standardized products with consistent quality. (Gungor and Bayraktar, 2005; Bahtiyarca Bagdat, 2006; Tokul, 2015; Sonmez, 2019).

Plant tissue culture is the *in vitro* aseptic culture of cells, tissues, organs, and their components in solid or liquid media under defined physically and chemically controlled conditions (Thorpe, 2006; George and Manuel, 2013). Micropropagation provides rapid production of high-quality, disease-free plant materials bearing the characteristics of the species in a limited area in a short time. Plants can reproduce anywhere under controlled environmental conditions throughout the year, regardless of season and weather (George and Manuel, 2013). Techniques applied for clonal propagation of plants are generally time-consuming and labor-intensive (Datta et al., 2017).

Within the framework of this study, experiments were carried out to ensure the surface sterilization of the oregano cultivar 'Ceylan-2002', belonging to *Origanum onites L.*, and to investigate the effect of the presence of activated carbon (AC) in the medium content on the darkening after sterilization. It was tried to determine the effects of plant growth regulators (PGRs) in different types (BAP, IBA, and NAA) and doses on shoot multiplication in sterilized explants. Experiments were set up in MS and ½ MS media containing different IBA doses for rooting from shoots. The acclimatization of rooted plantlets was attempted. In addition, chemical analyses were made in micropropagated plants to determine the content of essential oils.

MATERIALS AND METHODS

Materials

In this study, node explants of 'Ceylan-2002' cultivar belonging to *Origanum onites* L. species registered by Ege University Faculty of Agriculture, Field Crops Department in Izmir were used as starting material.

Methods

Surface sterilization

After the shoots were collected from the field and separated from their leaves, the node parts were cut and prepared as explants. The explants were pre-washed for five min in water with detergent and then under running water for 10 min. In all trials, in the first step, explants were shaken in 70% EtOH for 1 min. Afterward, the explants were kept in commercial bleach (containing 5% NaOCl) at different doses (15, 20 and 30%) for different times (15-20 min). They were rinsed three times with sterile distilled water. Six different methods were tested for the surface sterilization of node explants (Table 1). After sterilization procedures, node explants were cultured in MS (Murashige and Skoog, 1965) media containing (AK0,3) and without (AK0) 0.3 g l^{-1} of AC. Culture vessels were maintained in a photoperiod of 16 hours light/8 hours dark, at 24±2°C and 3500 lux light intensity.

Table 1. Surface sterilization trials

Sterilization technique[a]	Commercial bleach doses (%)	Application time (min)
A	15	15
B	15	20
C	20	15
D	20	20
E	30	15
F	30	20

[a]: 70% EtOH in each sterilization technique was applied for 1 min.

Shoot regeneration

After the optimum sterilization protocol was determined, sterilized node explants were cultured in shoot regeneration media (KS) containing different PGRs, doses, and combinations, using MS as a basal medium. MS medium without PGR was used as the control group in the experiment (Table 2).

Table 2. Shoot regeneration treatments[a]

Culture medium- Codes	PGRs (mg l^{-1}/L)		
	BAP	IBA	NAA
KS1	-	-	-
KS2	-	0.5	-
KS3	-	1	-
KS4	-	2	-
KS5	0.5	-	-
KS6	1	-	-
KS7	2	-	-
KS8	0.5	0.5	-
KS9	0.5	1	-
KS10	0.5	2	-
KS11	1	0.5	-
KS12	2	0.5	-
KS13	1	1	-
KS14	1	2	-
KS15	2	1	-
KS16	2	2	-
KS17	-	-	0.1
KS18	2	-	0.1
KS19	-	-	0.2
KS20	2	-	0.2

[a] MS medium was used as the main medium in all experiments. 3 g l^{-1} gelrite was added as gelling agent. In addition, 0.3 g l^{-1} AC was added to all trials.

In vitro rooting and acclimatization

Regenerated shoots were cultured in MS and ½ MS media diversified with 0.3 g l^{-1} and 1 g l^{-1} AC and different IBA doses (0. 0.5, 1,0. 2,0 mg l^{-1}) for rooting experiments (Table 3). Eighty rooted plantlets (five plantlets from each medium) of Ceylan-2002 cultivar obtained from 16 different rooting media *in vitro* conditions were selected and acclimatized. The roots of the oregano shoots, carefully removed from the culture vessels, were cleaned by washing them with water to remove the gelling agent residues. Oregano plantlets were transferred to plastic cups containing peat, the bottom of which was pierced with a needle. After watering plantlets, each cup was covered with a perforated nylon bag. Transplanted plantlets were kept in 16 hours light/8 hours dark photoperiod, 3500 lux light intensity, and 24 ± 2ºC temperature conditions. After the acclimatization process, the bags on the plastic cups were periodically removed to adapt to the outdoor conditions, and the plantlets were aerated and watered a little. On the 18th day, the bags were completely removed, and the plantlets were stored for 12 days in laboratory conditions under a photoperiod of 16 hours light/8 hours dark, a light intensity of 3500 lux, and a temperature of 24±2ºC. After the 30-day acclimatization period, the shoots' survival rates (%) were recorded.

Table 3. *In vitro* rooting media

Culture medium- Codes	Basal medium	AC (g l^{-1})	IBA (mg l^{-1})
KK-1	MS	0.3	0
KK-2			0.5
KK-3			1
KK-4			2
KK-5	½ MS		0
KK-6			0.5
KK-7			1
KK-8			2
KK-9	MS	1	0
KK-10			0.5
KK-11			1
KK-12			2
KK-13	½ MS		0
KK-14			0.5
KK-15			1
KK-16			2

Essential oil analysis

Essential oil components analysis of well-developed rooted plantlets of Ceylan-2002 cultivar obtained from seven different rooting media *in vitro* conditions was carried out with GC-MSD in Ege University Drug Development and Pharmacokinetic Research and Application Center R&D Laboratories (ARGEFAR). The essential oil rate was determined by hydro-distillation using the Clevenger apparatus. A total of 50 g fresh leaves of *in vitro* plantlets were used for essential oil analysis.

Statistical analysis

All *in vitro* treatments were applied in a randomized plot design with three replications. The data obtained from the applications were evaluated using the Minitab 17 (Minitab®, LLC, Pennsylvania, USA, 2015) program.

RESULTS

Surface sterilization

The percentage (%) of sterile explants obtained as a result of applied sterilization methods is shown in Table 4. Observations were made four weeks after the explants were cultured. In addition, the problem of hyperhydricity was observed in the study, and observations were conducted to determine its effect (Table 4). Observations regarding hyperhydricity were made four weeks after explants were cultured. An examination of the sterile explant percentage values revealed that the CV values were generally less than 10%. When examined in this context, it shows that the data is consistent.

Table 4. Percentage of sterile explants obtained from different surface sterilization methods applied to node explants and of explants with hyperhydricity (%)[a]

Surface sterilization technique	Percentage of sterile explant (%) ± SE	CV(%)	Percentage of explants showing hyperhydricity (%) ± SE
A	43.33±3.33 c	13.32	56.67±3.33 a
B	100.00±0.00 a	0.00	26.67±3.33 b
C	80.00±0.00 ab	0.00	10.00±10.00 b
D	100.00±0.00 a	0.00	13.33±3.33 b
E	90.00±5.77 ab	10.00	6.67±3.33 b
F	100.00±0.00 a	0.00	6.67±3.33 b

Source	*F value*	*p-value*	*F value*	*p-value*
Sterilization techique	66.40	0.000	14.57	0.000

[a]Applications were made in 3 replications, and 10 explants were used for each replication. The differences between the mean values shown with different letters in the same column are significant at the P≤0.01 level according to the Tukey multiple comparison test. SE: Standard Error CV: Coefficiency of variation

Micropropagation

The mean number of regenerated shoots from node explants of the Ceylan-2002 cultivar was determined four weeks following the culture. This result is presented in Table 5 (Fig 1). Node explants belonging to 'Ceylan-2002' cultivar obtained from shoot regeneration experiments were subcultured nine times in KS-7 medium. Propagation coefficients calculated over the sum of node explants obtained from subcultures performed at four week intervals are given in Table 6. In the study, the mean leaf number, root number, and root length values per explant were also determined. No root formation was observed in the 1st and 2nd subcultures. The study revealed that as the number of subcultures increased, a small number of multiple shoots occurred in the node explants, particularly in subcultures 6-9 (Table 6, Fig 2). In the experiments where the effects of the number of subcultures were determined, the CV value was calculated in all experiments except root regeneration. When the multiplication coefficient values were examined, all values except the 3rd subculture were <20%, and it was seen that the data were consistent. In the values of the mean number of shoots obtained per node, shoot length obtained per explant, and number of leaves obtained per explant, the CV value was determined as <20% except for the 2nd subculture (Table 6).

Table 5. Some micropropagation parameters determined in MS media containing different types and doses of PGRs [a]

Media	Mean regenerated shoots number per explant±SE	Mean shoot length (cm)±SE	Mean number of leaves ±SE
KS-1	0.10±0.06	0.03±0.02	0.50±0.32
KS-2	0.03±0.03	0.02±0.02	0.30±0.30
KS-3	0.07±0.07	0.02±0.02	0.27±0.27
KS-4	0.10±0.06	0.05±0.03	0.73±0.55
KS-5	0.00±0.00	0.00±0.00	0.00±0.00
KS-6	0.07±0.07	0.02±0.02	0.37±0.37
KS-7	0.13±0.07	0.12±0.09	0.87±0.44
KS-8	0.00±0.00	0.00±0.00	0.00±0.00
KS-9	0.03±0.03	0.02±0.02	0.13±0.13
KS-10	0.00±0.00	0.00±0.00	0.00±0.00
KS-11	0.00±0.00	0.00±0.00	0.00±0.00
KS-12	0.00±0.00	0.00±0.00	0.00±0.00
KS-13	0.00±0.00	0.00±0.00	0.00±0.00
KS-14	0.00±0.00	0.00±0.00	0.00±0.00
KS-15	0.00±0.00	0.00±0.00	0.00±0.00
KS-16	0.00±0.00	0.00±0.00	0.00±0.00
KS-17	0.03±0.03	0.02±0.02	0.13±0.13
KS-18	0.00±0.00	0.00±0.00	0.00±0.00
KS-19	0.00±0.00	0.00±0.00	0.00±0.00
KS-20	0.00±0.00	0.00±0.00	0.00±0.00

[a]Applications were made in 3 replications, and 10 explants were used for each replication. SE: Standard Error

Table 6. Micropropagation parameters obtained in the experiments carried out to determine the effect of the subculture numbers [a]

Subculture	Multiplication coefficient±SE	CV(%)	Mean number of shoots obtained per node±SE	CV(%)	Mean shoot length obtained per explant (cm) ± SE	CV(%)	Mean number of leaves obtained per explant ±SE	CV(%)	Mean number of roots obtained per explant±SE	Mean root length obtained per explant (cm)±SE
1rd	1.08±0.08 a	13.32	0.23±0.03 cd	19.89	2.05±0.11 b	9.31	11.15±1.17 ab	18.14	0.00±0.00 c	0.00±0.00
2nd	1. 88±0.21 cd	19.35	0.25±0.06 cd	38.96	1.87±0.35 b	32.79	10.88±2.01 ab	32.05	0.00±0.00 c	0.00±0.00
3rd	2.33±0.38 bc	28.50	0.18±0.01 d	11.11	3.82±0.81 a	36.77	13.85±0.37 a	4.65	0.17±0.09 abc	0.16±0.08
4th	3.40±0.27 a	13.69	0.35±0.02 abc	10.30	1.79±0.07 b	6.87	9.49±1.33 abc	24.21	0.15±0.05 abc	0.21±0.07
5th	2.10±0.08 bcd	6.68	0.35±0.02 abc	8.33	1.87±0.21 b	19.27	7.94±0.14 bc	3.07	0.72±0.33 ab	1.87±0.97
6th	2.23±0.06 bc	4.95	0.34±0.01 bc	4.45	2.51±0.04 ab	2.42	7.89±0.17 bc	3.62	0.81±0.21 a	1.99±0.74
7th	2.73±0.09 abc	6.27	0.43±0.03 ab	11.84	1.95±0.09 b	7.96	6.65±0.29 bc	7.48	0.09±0.02 bc	0.21±0.08
8th	2.96±0.25 ab	14.38	0.47±0.01 a	4.40	1.84±0.12 b	11.14	6.24±0.10 c	2.79	0.14±0.06 abc	0.35±0.18
9th	3.00±0.23 ab	13.11	0.43±0.02 ab	7.53	2.06±0.05 b	3.97	6.72±0.22 bc	5.54	0.29±0.02 abc	0.66±0.11
Source	***F value***	***p-value***	***F value***	***p-value***	***F value***	***p-value***	***F value***	***p-value***	***F value***	***p-value***
Subculture	10.91	0.000	15.11	0.000	4.32	0.005	7,89	0.000	4.82	0.000

[a]Applications were made in 3 replications, and 10 explants were used for each replication. According to the Tukey multiple comparison test, the differences between the mean values shown with different letters in the same column are significant at the P≤0.01 level. SE: Standard Error CV:Coefficient of variation.

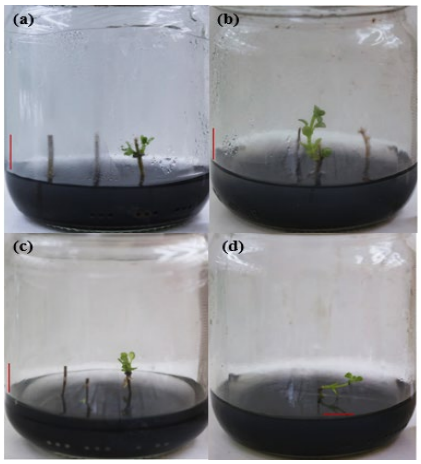

Figure 1. Shoots of Ceylan-2002 cultivar developed in shoot propagation media; a) KS-3, b) KS-4, c) KS-6, d) KS-7. (bar: 1 cm)

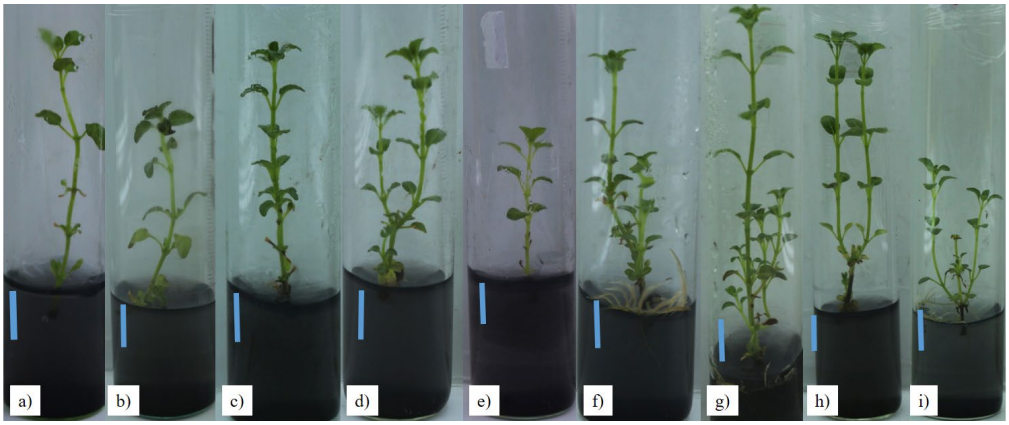

Figure 2. Shoots obtained from the node explants of oregano plants with subcultures in the KS-7 medium; a-i) shoots belonging to subculture numbers (1-9) (bar 1 cm).

In vitro rooting

Rooting is required to acclimatize the shoots produced in vitro conditions. In the studies carried out for this purpose, various parameters related to rooting were tried to be determined. (Table 7 and 8). In addition, the root number values produced *in vitro* were also examined in the study. (Table 7 and 8) Another parameter observed in the study was the root length of regenerated shoots (Table 7, 8, and Fig 3). In the observations made as a result of the culture period of four weeks, it was determined that the amount of AC had a positive effect on stem thickening. The highest mean stem thickness value (2.02 mm) and the highest mean shoot length (10.02 cm) were obtained in media containing 1.0 g l^{-1} activated carbon (Fig 4). When the CV values were examined, it was seen that the majority were <10%, but values >20% were also seen. In applications with <20%, the heterogeneity of the study can be observed due to small sample groups. In the context of plant tissue culture studies, it is a common finding that the CV tends to be high, particularly when considering the culture conditions, including light, temperature, pH, and the type of explant utilized, as well as the inherent physiological differences among plants. (Table 7).

Table 7. Effect of MS and ½ MS media containing different doses of IBA and AC on different root properties[a]

	Root regeneration percentage±SE (%)			Root number per explant±SE (unit)			Mean root lenght±SE (cm)		
		CV (%)			CV(%)			CV(%)	
KK-1	93.33±0.03	6.19		7.47±1.30 ab	30.11		1.82±0.27 ab	25.42	
KK-2	73.33±0.15	34.32		7.63±1.11 ab	25.07		1.87±0.08 ab	7.56	
KK-3	90.00±0.00	0.00		8.47±0.94 ab	19.20		2.16±0.10 ab	8.12	
KK-4	83.33±0.12	24.98	87.50 b	6.47±1.39 ab	37.12	7.32 b	1.67±0.37 ab	38.69	1.81 b
KK-5	90.00±0.06	11.11		7.23±1.03 ab	24.74		1.89±0.23 ab	20.77	
KK-6	93.33±0.03	6.19		8.60±0.25 ab	5.07		1.63±0.38 ab	40.06	
KK-7	96.67±0.03	5.97		6.90±0.87 ab	21.88		1.78±0.07 ab	6.74	
KK-8	80.00±0.10	21.65		5.80±1.61 b	48.06		1.65±0.32 ab	33.74	
KK-9	96.67±0.03	5.97		9.5±0.55 ab	10.04		1.53±0.19 b	20.96	
KK-10	100.00±0.00	0.00		10.67±0.33 ab	5.33		1.77±0.17 ab	17.29	
KK-11	96.67±0.03	5.97		9.13±1.01 ab	19.16		1.77±0.03 ab	3.27	
KK-12	96.67±0.03	5.97	97.92 a	12.03±1.62 a	28.92	9.44 a	1.92±0.08 ab	7.53	2.05 a
KK-13	96.67±0.03	5.97		7.83±0.38 ab	8.50		2.33±0.23 ab	17.32	
KK-14	96.67±0.03	5.97		9.23±1.02 ab	19.08		2.72±0.07 a	4.25	
KK-15	100.00±0.00	0.00		7.03±1.21 ab	29.84		2.42±0.21 ab	14.97	
KK-16	100.00±0.00	0.00		10.10±1.69 ab	28.92		1.98±0.31 ab	11.64	

Source	***F value***	***p-value***	***F value***	***p-value***	***F value***	***p-value***
Medium (M)	0.94	0.339	2.25	0.143	4.98	0.033
AC	12.02	0.002	11.32	0.002	5.30	0.028
IBA	0.84	0.482	0.88	0.461	0.95	0.429
BMxAK	0.48	0.493	0.69	0.414	12.77	0.001
BMxIBA	0.58	0.630	0.43	0.730	0.80	0.502
AKxIBA	1.10	0.365	1.83	0.161	0.78	0.512
BMxAKxIBA	1.25	0.308	0.34	0.795	1.34	0.279

[a]Applications were made in 3 replications, and 10 explants were used for each replication. The differences between the mean values shown with different letters in the same column are significant at the $p \leq 0.01$ level according to the Tukey multiple comparison test. SE: Standard Error CV: Coefficiency of variation

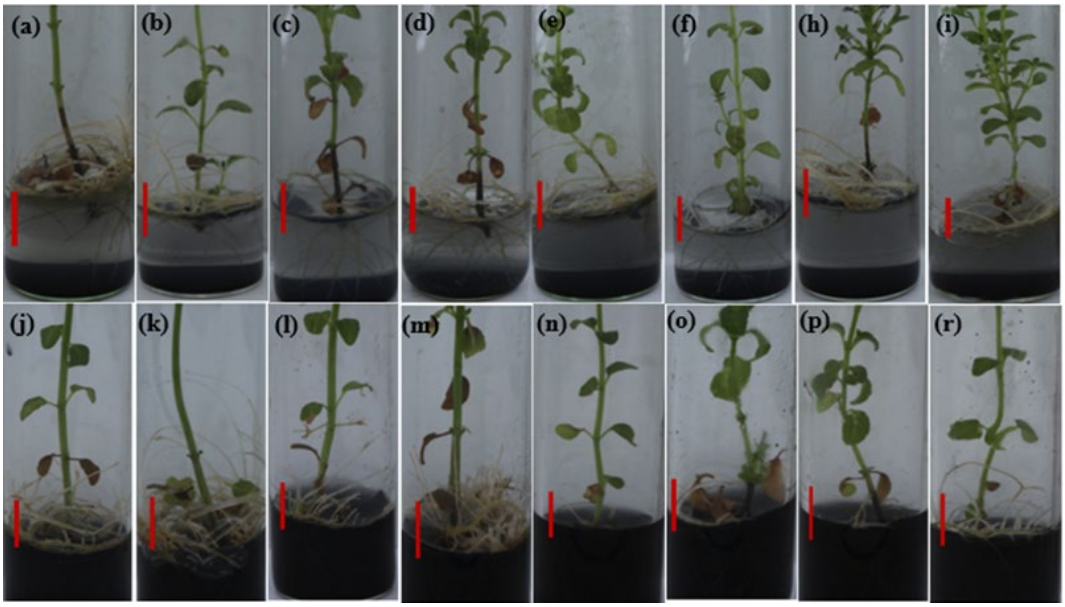

Figure 3. Rooting of regenerated shoots in media containing different doses of IBA and AC; a-r) Root lengths of shoots rooted in KK-1-16 media. (bar 1 cm)

Table 8. Determination of the effect of basic medium and AC on different root properties[a]

Media		Root regeneration percentage (%)		Root number per explant (unit)		Mean root length (cm)	
MS	AK 0.3	85.00 b	91.25	7.51 b	8.92	1.88 b	1.81 b
	AK 1	97.50 a		10.33 a		1.74 b	
½ MS	AK 0.3	90.00 ab	91.25	7.13 b	8.92	1.74 b	1.81 b
	AK 1	98.33 a		8.55 ab		2.36 a	
***p*-value**		0.00		0.00		0.00	

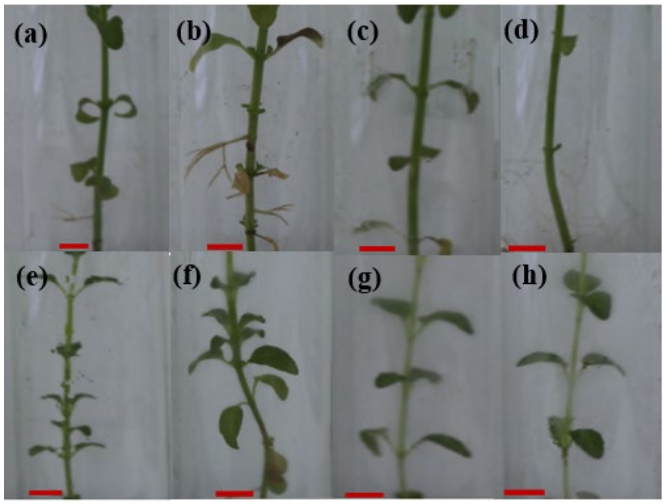

Figure 4. Stem thickening in AC-containing media; a-d) Stem thicknesses in media containing 1 g l^{-1} AC, e-h) Stem thickness in media containing 0.3 g l^{-1} AC (bar 5 mm).

Acclimatization

At the end of the 18th day, the acclimatized plants were kept for 12 days in laboratory conditions with a photoperiod of 16 hours of light/8 hours of darkness, a light intensity of 3500 lux, and a temperature of 24 ± 2°C. The highest survival rate (80%) was recorded from shoots obtained from KK-12 (2 mg l^{-1} IBA + 1g l^{-1} AC) MS medium. The lowest survival rate (0%) was determined in all media containing 0.3 g l^{-1}AC and shoots produced from KK-9 (0 mg l^{-1} IBA + 1 g l^{-1} AC) MS medium. At the end of 30 days, 13 shoots were successfully acclimatized. Acclimatization success was recorded as 16.25% (Fig 5 and 6). In studies carried out for acclimatization, the survival rate of plantlets was 32.50% in media containing 1.0 g l^{-1} AC.

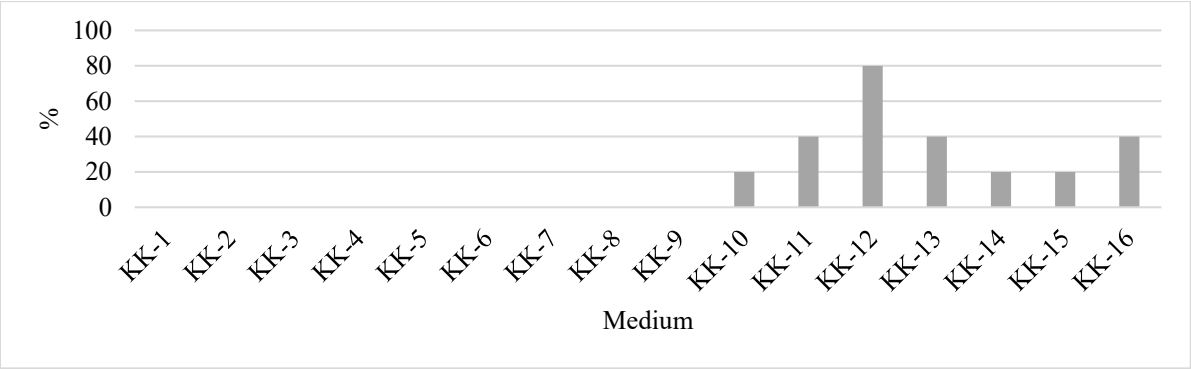

Figure 5. Survival rates after acclimatization

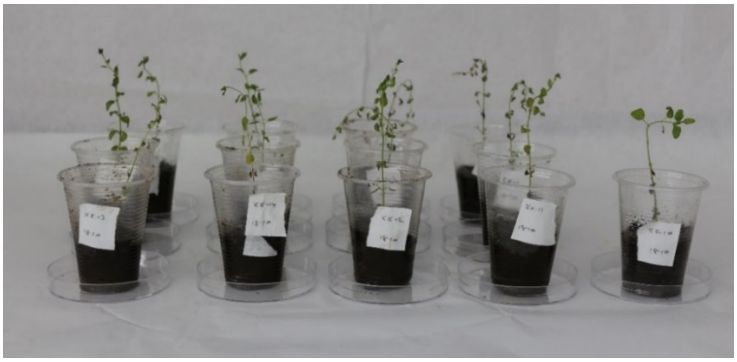

Figure 6. Plants acclimatized after 30 days.

Essential oil analysis

The study also included analyses to determine the essential oil ratios and contents in plants growing *in vitro*. Firstly, the dry matter percentage of the plants was examined prior to the initiation of the analysis. The highest dry matter percentage value (26.30%) was reached in ½ MS medium coded KK-15 containing 1.0 g l^{-1} AC supplemented with 1.0 mg l^{-1} IBA (Table 9).

In the study, the highest essential oil content (2.00 %) was obtained in the KK-12 medium (MS+1 g l^{-1} AC+2 mg l^{-1} IBA). The essential oil's main components were thymol, carvacrol, P-cymene, β-bisabolene, γ-terpinene, α-thujene, (+)-borneol. When thymol and carvacrol values were compared, it was seen that thymol was synthesized at a higher rate. While the thymol content was in the range of 58.32-65.33 %, the carvacrol ratio was in the range of 3.10-6.82 %. While the highest thymol content (65.33 %) was found in KK-14 medium (½ MS+1 g l^{-1} AC+ 0.5 mg l^{-1} IBA), the highest carvacrol content (6.82 %) was obtained in KK-12 medium (MS+1 g l^{-1} AC+2 mg l^{-1} IBA) (Table 10).

Table 9. Mean wet weight (g), dry weight (g), and percentage of dry matter (g) obtained after 4 weeks of culture.

Medium	Wet weight (g)	Dry weight (g)	Percentage of dry matter (%)
KK-9	1.85	0.24	12.97
KK-10	1.17	0.17	14.69
KK-11	1.50	0.20	13.53
KK-12	1.39	0.20	14.25
KK-14	0.28	0.04	14.11
KK-15	0.23	0.06	26.30
KK-16	0.44	0.05	11.28

Table 10. Essential oil contents obtained in different media after 4 weeks of culture (%).

	Medium						
	KK- 9	KK-10	KK-11	KK-12	KK-14	KK-15	KK-16
Essential oil	1.80	1.70	1.56	2,00	1.12	1.10	1.12
α -thujene	2.75	1.97	2.48	2.68	2.17	2.15	2,05
Camphen	0.38	0.28	0.37	0.36	0.36	0.29	0.23
β -pinene	0.14	0.1	0.13	0.14	0.13	0.12	0.11
Sabinene	0.25	0.17	0.22	0.26	0.15	0.18	0.18
Delta-3-caren	0.1	-	0.1	0.09	-	0.08	0.08
β -myrcene	1.74	1.39	1.68	1.77	0.99	1.14	1.13
α- phellandrene	0.25	0.2	0.25	0.26	0.14	0.17	0.17
α -terpinene	1.44	1.16	1.45	1.49	0.86	0.99	0.98
Limonene	0.37	0.29	0.34	0.35	0.3	0.29	0.3
β - phellandrene	0.27	0.2	0.25	0.27	0.2	0.21	0.21
β -ocymene	0.91	0.79	0.98	0.96	0.51	0.51	0.56
γ -terpinene	3.06	2.7	3.26	3.16	1.8	2.19	2.16
P-cymene	6.8	4.87	6.46	6	7.31	6.5	6.91
α -terpinolene	0.14	0.13	0.15	0.14	0.14	0.11	0.1
1-octen-3-ol	0.41	0.26	0.37	0.35	0.32	0.29	0.3
cis-sabinen hydrate	0.7	0.38	0.58	0.84	0.19	0.51	0.56
Linalool	-	-	-	0.15	-	-	-
trans-sabinen hydrate	0.36	0.36	0.35	0.52	0.16	0.36	0.37
trans- α-bergamotene	-	-	-	-	-	0.15	0.14
trans caryophyllene	1.19	1.44	1.36	1.17	0.16	1.15	1,09
terpinen-4-ol	1.17	1,05	1.19	1.07	0.69	1.15	1.11
trans- β -farnesene	-	-	-	-	-	0.1	0.09
α -terpineol	0.15	0.14	0.14	0.15	0.14	0.12	0.12
(+)-borneol	2.28	2.15	2.22	2.2	1.93	1.54	1.38
Germacren-D	1.45	1.47	1.62	1.56	1.58	1.55	1.47
β- bisabolene	6.33	6.82	6.69	6.38	7.09	7.96	7.29
α- amorphene	0.46	0.51	0.43	0.51	0.45	0.57	0.49
betasesqiphellandrene	0.16	0.18	0.16	0.17	0.02	0.19	0.18
cis- α -bisabolene	0.13	0.15	0.15	0.14	-	0.17	0.14
α -cubebene	-	-	-	0.13	-	-	-
Caryophyllene oxide	-	0.11	-	-	0.18	0.12	0.12
α -copaene	-	-	-	-	-	0.15	0.12
(+) epi-bicylosesquiphellandrene	1.12	1.37	1,08	1.36	1.33	1.47	1.19
thymol	60.60	63.91	60.97	58.32	65.33	63.70	64.92
carvacrol	4.68	5,05	4.35	6.82	3.10	3.37	3.38
unknown	0.22	0.27	0.23	0.23	0.27	0.44	0.36

DISCUSSION

İzmir oregano plant has been demonstrated to possess a variety of beneficial properties and biological activities, including antibacterial, antifungal, antimicrobial, antioxidant, antiviral, insecticidal, antioxidant, anti-inflammatory, and antitumor properties. The extensive range of benefits attributed to this plant necessitates further investigation (Koksal et al., 2010; Alekseeva et al., 2020).

In this study, six different surface sterilization methods were applied in order to determine the appropriate sterilization procedure for the node explants. After the sterilization methods were applied, the node explants were cultured in MS media containing (AK0,3) and without (AK0) 0.3 g of AC. In light of the observations recorded at the conclusion of the four-week period, it was determined that the sterilization treatments B, D, and F, which had demonstrated a 100% success rate, were deemed suitable for incorporation into the sterilization protocol. Although no difference was observed in terms of sterilization success between three different treatments, it was decided to use the B method in order to work at lower bleach doses. In the literature, among the studies carried out for the superficial sterilization of node explants of *Origanum* species, there are different sterilization methods (El Beyrouthy et al., 2013; Sevindik et al., 2017; Turker and Hatipoglu, 2018; Grigoriadou et al., 2019; Pandey et al., 2019). In a study carried out in *Origanum vulgare* subsp. hirtum type, sterilization success was determined to be 93% (Iconomou-Petrovich and Nianiou-Obeidat, 1998). Sterilization success obtained in this study was higher than the result of this literature.

In order to determine the parameters affecting hyperhydricity, the effect of sterilization practices on hyperhydricity was investigated first. As a result of the use of AC, the percentage of hyperhydricity decreased from 50% to 41.67%. In the literature, 5 µM salicylic acid has been used to prevent hyperhydricity in *Thymus daenensis* species (Hassannejad et al., 2012). *In vitro* culturing of *O. vulgare* with *Pseudomonas* spp. prevented hyperhydrism (Gogoi and Borua, 2014; Novak and Bluthner, 2020). In addition, 2.0 g l^{-1} AC was used in *Paeonia lactiflora* Pall. plant to prevent hyperhydricity increased the percentage of non-hyperhydric plants from 4.41% to 34.37% (Wu et al., 2011). Although there is no study on the use of AC to prevent hyperhydricity in plants of Origanum species, the outcomes observed in this study align with the existing literature on the effects of AC on hyperhydricity in diverse plant species.

In order to evaluate the effects of various PGRs and their doses on shoot regeneration, node explants were cultured in MS media at 20 different doses. The results of the observations conducted four weeks after the explants were cultured revealed no significant differences in the impact of these PGRs and their various doses on shoot regeneration. Increasing IBA and BAP doses when used alone led to an increase in the percentage of shoot regeneration. A positive effect on shoot regeneration was not observed with BAP and IBA combinations. The rise in NAA doses alone led to a decrease in the percentage of shoot regeneration. No shoot formation was observed in NAA and BAP combinations. In the literature, the highest shoot regeneration (91.67%) in *Origanum vulgare* L. was obtained in MS medium containing 4.0 µM BAP (Pandey et al., 2019). While the percentage of shoot regeneration was 50% in MS medium containing 0.5 mg l^{-1} BAP in the *Origanum heracleoticum* L. plant, this value was observed as 70% in MS medium containing 1.0 mg l^{-1} BAP (Zayova et al., 2019). In another study, the highest percentage of shoot regeneration (100%) in *Origanum vulgare* L was obtained in MS media supplemented with 1.0 mg l^{-1} kinetin and supplemented with 0.25 mg l^{-1} and 0.75 mg l^{-1} chitosan (Premi et al., 2021). In other research, the highest percentage of shoot regeneration (81.56%) from apical meristem explants was obtained in MS medium with 1.5 mg l^{-1} kinetin after germination of seeds of *Origanum onites* L. (Atar and Colgecen, 2019). 'Ceylan-2002' had a low response rate to *in vitro* culture. The results obtained in this study are not similar to those of other studies due to the use of cultivars belonging to different species and cultural methods.

The node explants obtained from the shoot regeneration experiments were subcultured nine times to evaluate the effect of the number of subcultures on the reproduction coefficient. The data obtained regarding the reproduction coefficient are consistent with those reported in the literature. In a study, the highest reproduction coefficient (5.0) was obtained in an MS medium supplemented with 0.4 mg l^{-1} kinetin +0.1 mg l^{-1} NAA in *Origanum syriacum* L. plant (Arafeh et al., 2003). In another study performed on *Origanum majorana* L. plant, the highest multiplication coefficient (4.4) was observed in MS medium supplemented with 1.0 mg l^{-1} BAP. The reproduction coefficient value obtained in this study shows partial agreement with the value obtained in the literature due to using different species and cultivars.

The mean shoot lengths were observed four weeks after culture to evaluate the effect of various PGRs and their doses on the length of the shoots. After the 3rd subculture, the mean shoot length per explant was relatively reduced. In another study, the highest shoot length (2.36 cm) in *Origanum acutidens* (Hand.-Mazz.) Ietswaart was obtained in MS medium containing 1.8 mg l^{-1} BAP + 0.2 mg l^{-1} NAA (Yildirim, 2013). In another study, the highest shoot length (4.38 cm) in *Origanum vulgare* L. was obtained in MS medium supplemented with 0.75 mg l^{-1} chitosan (Premi et al., 2021). In other research, the highest shoot length (3.25 cm) in *Origanum acutidens* (Hand.-Mazz.) Ietswaart was obtained in MS medium with 1.6 mg l^{-1} BAP (Kizil and Khawar, 2017). As in the reproduction coefficient, the reason for the decrease in shoot length may be due to the inhibitory accumulation of PGRs in the medium with the increase in the number of subcultures (Vujović et al., 2012). This finding of this study is similar to these results.

The mean leaf number of regenerated shoots was observed four weeks after culture, allowing for the evaluation of the effect of different PGRs and their doses on leaf number. In a study, the highest mean (13.63) per explant in the shoot propagation study of *Origanum syriacum* L. was obtained in MS medium with 0.5 mg l^{-1} kinetin (Abdallah et al., 2017). Our study is compatible with this study.

No root formation was observed in the first and 2nd subcultures. After the 6th subculture, the number of roots declined sharply. The increase in the number of subcultures leads to physiological changes in plants. In a study about the *Pinus massoniana* Lamb. plant was subcultured 40 times. In long-term subculturing, a decrease in rooting rate was observed after the 20th subculture (Wang and Yao, 2020). The data we obtained are consistent with these studies. In our study, the highest mean number of shoots per node (0.47) was obtained in the 8th subculture. This study found no data on the effect of the number of subcultures on the number of shoots obtained per node, and it is the first research based on such data. The highest propagation coefficient (3.40) was obtained in the 4th subculture.

The effect of AC doses on root regeneration in MS and ½ MS based media was found to be statistically significant. In a study carried out, the highest root regeneration percentage (96%) in *Origanum sipyleum* L. was obtained in MS medium containing 0.5 mg l^{-1} IBA (Oluk and Cakir, 2009). Another study showed the highest percentage of root regeneration (100%) in *Origanum acutidens* (Hand.-Mazz.) Letswaart plant was found in MS media with 0.2 mg l^{-1} NAA + 0.6, 1.2, 1.8, and 2.4 mg l^{-1} BAP (Yildirim, 2013). In another study, the highest root regeneration percentage (91.80%) in *Origanum vulgare* L. was obtained in an MS medium containing 0.6 mg l^{-1} NAA (Oana et al.,

2008). The data obtained in this study are similar to the literature.

The effect of AC dose on the number of roots obtained per explant was found to be statistically significant in MS and ½ MS-based media. An examination of the impact of the medium on the mean root number obtained per explant revealed that MS-based media were found to be effective. Although the effect of IBA doses on the mean root number per explant was not statistically significant, the highest mean root number per explant (9.04) was obtained in media containing 0.5 mg l^{-1} IBA. As a result, when all these data were examined, it was determined that the most effective medium on the mean root number obtained per explant of the regenerated shoots of Ceylan-2002 cultivar was MS-based medium containing 1.0 g l^{-1} AC.

In the literature, the highest mean root length (1.5 cm) obtained in *Origanum syriacum* L. was obtained in an MS medium containing 0.8 mg l^{-1} IAA (Arafeh et al., 2003). In the study we carried out, higher data were obtained. In another study, the highest mean root length (5.5 cm) obtained in the *Origanum sipyleum* L plant was found in MS medium containing 0.5 mg l^{-1} IBA, and the lowest mean root length (1.6 cm) was 1.0 mg l^{-1} IBA (Oluk and Cakir 2009). The results obtained in our study are compatible with Oluk and Cakir (2009), which achieved the highest mean root length in MS medium containing 0.5 mg l^{-1} IBA for *O. sipyleum* L.

This study evaluated the effect of MS and ½ MS-based media with varying doses of AC on stem thickening. It was determined that an increase in AC dose positively affected stem thickness. There is no study in the literature regarding the effect of AC use on stem thickening in oregano plants. However, it was reported that using 10.0 g l^{-1} AC in *Eucalyptus grandis* x *E. urophylla* clones resulted in stem thickening (Jones and Van Staden, 1994). Our results in this study are consistent with Jones and Van Staden (1994) results. In this experiment established to evaluate the effect of MS and ½ MS-based media containing AC at different doses on shoot length, it was determined that the increase in AC doses positively affected shoot length. While the mean shoot length was 4.88 cm in media containing 0.3 g l^{-1} AC, this value increased to 10.02 cm in media containing 1.0 g l^{-1} AC. There is no study in the literature regarding the effect of AC use on shoot length in oregano plants. However, in a study conducted, while the mean shoot length was 0.06 cm in *Thuja occidentalis* L., this value increased to 1.13 cm in the presence of 0.05% (w/v) AC (Nour and Thorpe, 1993). The results we obtained in our study are compatible with the study of Nour and Thorpe (1993).

In studies carried out for acclimatization, the survival rate of plantlets was 0% in media containing 0.3 g l^{-1} AC, while this value increased to 32.50% in media containing 1.0 g l^{-1} AC. In a study carried out by increasing the AC doses from 1.0 g l^{-1} to 5.0 g l^{-1} in Camarosa, Chandler, and Oso Grande strawberry cultivars, the stem diameter values of the plants increased in the acclimatization stage and survival successes in the plantlets rooted in AC media were at the were found as 99% (Adak and Pekmezci, 2011). The findings of the present study are consistent with those of Adak and Pekmezci (2011), who posited that the utilization of AC in various strawberry cultivars enhances the survival rate during acclimatization.

Some of the plants analyzed in terms of the volatile compounds were selected to determine various physiological parameters and to make observations. When wet and dry weight values were examined, higher wet weight values were found in KK-9, 10. 11, and 12 coded MS-based media, while a 5-fold decrease in wet weight values occurred in KK-14, 15, and 16 coded ½ MS-based media. The amount of essential oil obtained during the analysis also showed similar characteristics to this situation.

The highest essential oil content (2.00%) was obtained in MS medium with 2.0 mg l^{-1} IBA; the lowest essential oil content (1.10%) was in ½ MS medium with 1.0 mg l^{-1} IBA in this study. The shoots regenerated from all media had the highest amount of thymol component among the essential oil components. Gurtunca (2011) reported that the essential oil rate of Ceylan-2002 cultivar was determined as 3.43% in 2010 and 4.46% in a study carried out in 2011. In another study, the essential oil ratios of *Origanum onites* L. clones in Bornova and Dikili regions in 2002 and 2003 were between 2.77-4.20% (Avci and Bayram, 2013). In other research, the effect of different water and nitrogen doses on the essential oil composition of Ceylan-2002 cultivar in 2013 was investigated. In the first harvest, it was observed that the amount of thymol varied between 62.45-75.68% and the amount of carvacrol between 5.34-7.56%. In the second harvest, the amount of thymol was between 61.87-69.99%, and the amount of carvacrol was between 5.74-6.83% (Tokul 2015). The data we obtained in our study is compatible with Tokul (2015). Ceylan-2002 cultivar is registered as a thymol-carvacrol type. In this research, the tyhmol ratio was found to be higher than carvacrol. In addition to the composition of mineral and organic compounds and pH value, culture conditions such as temperature, light intensity, and duration are extremely effective factors in the secondary metabolite production of plants grown *in vitro*. In nature, secondary metabolite production is produced by plants in response to environmental stimuli or for defensive purposes. This mechanism can be stimulated by modifying these parameters *in vitro* (Scarpa et al., 2022). In this context in our study, the thymol ratio may have been found to be higher than the carvacrol ratio in oregano plants cultured *in vitro*, where environmental conditions were minimized. Perhaps the thymol ratio may have genetically increased the expression of genes responsible for thymol production by the composition of the medium and the doses of PGRs. In studies on essential oil in *Origanum onites* L. species, carvacrol, thymol, α-thujene, myrcene, camphene, β-pinene, limonene, terpinolene, linalool, α-terpinene, karyopylene oxide, p-cymene, terpinene-4-ol,α-pinene, β-bisabolene, borneol, and α-terpineol were determined as the main components (Tepe et al., 2016). The values obtained as a result of the study were found to be similar to; Kacar

et al. (2006) and Kamatou and Viljoen (2008) for linalool, Kizil et al. (2008) for delta-3-karen, Joshi et al. (2011) for trans-α-bergamoten, Souleles (1991) for trans-β-farnesene, Kizil et al. (2008) and Karık et al. (2021) for β-bisabolene, Aslan-Oz (2017) and Maskovic et al. (2017) for α-cubeben, Karık et al. (2021) for caryopylene oxide and Souleles (1991) for α-copaene. Our study obtained better results than the study carried out by Souleles (1991).

CONCLUSION

Izmir oregano is a plant with high economic value due to its high secondary metabolite content and widespread commercial use. In this study, a micropropagation technique protocol was developed as an alternative method for the production of oregano plants. In the study, the registered local variety Ceylal-2002 was used, and guiding information that can be used to meet the oregano export deficit was presented. Future studies can explore different experiments to reduce hyperhydricity levels. Additionally, elicitation experiments could be performed to enhance secondary metabolite content.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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