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Micropropagation and prevention of hyperhydricity in olive (*Olea europaea* L.) cultivar 'Gemlik'

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ABSTRACT

Olive (*Olea europaea* L.) is an economically important crop because of its fruit and oil. Successful olive micropropagation is highly dependence on cultivar, shoot proliferation rate, which is generally low, the rooting of micropropagated olive plantlets is difficult, and the rate of post-transplanting losses is high. In addition, hyperhydricity, a common problem *in vitro* culture was found to be prevalent. The aim of this study was to establish a micropropagation system for the Turkish *O. europaea* L. cv. Gemlik. Initially, five different basal media were tested to determine appropriate medium for establishment of *in vitro* culture and Woody Plant Medium (WPM) was the most efficient. Nodal explants were cultured on WPM containing different plant growth regulators (PGRs) for shoot regeneration. Maximum regeneration frequency and number of shoots per explant were achieved from nodal explants cultured on WPM supplemented with 4.0 mg/L 6-benzyladenine (BA). However, all cultures showed high hyperhydricity and an experimentation was also conducted to resolve the hyperhydricity problem. Hyperhydricity was prevented by changing the gelling agent to Agar-Agar. The shoots regenerated from nodal explants and still attached to initial woody nodal explant were transferred to four different medium formulations each containing 2.0 mg/L zeatin (ZEA) for shoot elongation. Modified Olive Medium (MOM2: OM with three times the concentrations of KNO_3) fortified with 2.0 mg/L ZEA was found to be the best for shoot elongation. The elongated shoots were rooted on Olive medium (OM) containing 160 mg/L Putrescine, 1.5 mg/L naphthaleneacetic acid (NAA), 30 g/L mannitol and solidified with 0.65% (w/v) Agar-Agar. Finally, all plantlets were successfully acclimatized in a climate chamber and the plants were transferred to greenhouse conditions.

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1. Introduction

Olive (*Olea europaea* L.), belonging to the family Oleaceae, is an evergreen Mediterranean plant and originated from Upper Mesopotamia, covering South-eastern Anatolian Region and South Asia Minor (Gökdoğan and Erdoğan, 2018). It is mainly grown to obtain fruit and which can be processed to extract olive oil. Due to its beneficial effects on human health, the economic importance of olive oil has increased and in parallel the area of cultivation of olive has expanded worldwide (Bradaï et al., 2016; Guo et al., 2017). As many as 1250 different cultivars of olive tree have been cultivated globally in 54 countries (Abuzayed et al., 2018). With the total olive cultivation area of 7.379.090 ha., the most important olive producing countries are Spain, Italy, Greece, Tunisia, Turkey, Morocco and Syria (Gökdoğan and Erdoğan, 2018; Guo et al., 2017).

O. europaea is conventionally propagated vegetatively by rooting of leafy stem or softwood cuttings, by grafting pieces of stem (scions) onto seedlings or clonal rootstocks or suckers. Among these techniques, olive is most commonly propagated by rooting of leafy stem cuttings under mist, however, rooting ability varies depending on the season, cultivars and availability of healthy viable material (Fabbri et al., 2009; Lambardi et al., 2013; Mangal et al., 2014). In cultivars hard to root, grafting is the only viable technique for clonal propagation; however, propagation by grafting is more expensive, more complex and requires specialized nurseries and skilled grafters (Fabbri et al., 2009; Lambardi et al., 2013). To overcome these problems, *in vitro* propagation has begun to be applied to olive as an alternative to the production by cuttings or grafting. *In vitro* propagation of the olive cultivars through axillary bud has successfully been used and is now use for commercially production in several Mediterranean countries such as Italy and Spain (Fabbri et al., 2009; Lambardi et al., 2013; Sánchez-Romero, 2018). Due to its recalcitrant nature, oxidation of tissues, and difficulties in getting sterile plant material and establishing shoot cultures, the micropropagation of economically important

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olive varieties is difficult to achieve (Lambardi et al., 2013; Yancheva and Kondakova, 2016). The success of olive micropropagation is highly dependence on cultivar, shoot proliferation rate is generally low, the rooting of micropropagated olive cultivars is difficult, and the rate of post-transplanting losses is high (Grigoriadou et al., 2007; Sánchez-Romero, 2018). Notwithstanding that, olive micropropagation has been achieved for many genotypes such as Meski (Chaari et al., 2002), Arbequina, Picual, Empeltre (García-Férriz et al., 2002), Chondrolia Chalkidikis (Antonopoulou et al., 2006; Grigoriadou et al., 2002), Maurino (Leva et al., 2002), Koroneiki (Roussos and Pontikis, 2002), Leccino, Picholine, Pendolino, Frantoio, Arbequina, Barnea, Hojiblanca (Zuccherelli and Zuccherelli, 2002), Nebbiara (Zacchini and De Agazio, 2004), ZDH4, Lucques, Haouzia, Dahbia, Amellau, Salonenque, Picholine marocaine, Picholine du Languedoc (Sghir et al., 2005), Carolea, Nocellara Etnea (Bati et al., 2006), Aglandau, Tanche (Binet et al., 2007), Galega vulgar (Peixe et al., 2007), Canino, Moraiolo, Rosciola, Piantone di Moiano (Mendoza-de Gyves et al., 2008), Moraiolo (Ali et al., 2009), Oueslati (Chaari-Rkhis et al., 2011), Mission (Rostami and Shahsavar, 2012).

A powerful and indispensable tool, plant tissue culture is used in agriculture and horticulture for mainly breeding, a vegetative "true-to-type" propagation, freeing plants from diseases, the cryopreservation of elite germplasm, and genetic improvement (Rugini et al., 2011; Van den Dries et al., 2013). The use of plant tissue culture on the purpose of vegetative propagation (micropropagation) provides an important alternative to classical plant propagation methods and both it is used to propagate "difficult to propagate" species and can provide relatively economic propagation for "easy to propagate" species (Ilczuk and Jacygrad, 2016). A common morphological, anatomical and physiological disorder occurring during micropropagation of many plants is hyperhydricity, the excessive water uptake by the apoplasts and accumulation of water in plant tissues (Gao et al., 2018; Liu et al., 2017; Van den Dries et al., 2013). The major reasons of hyperhydricity have been reported as the high relative humidity (RH) in the culture vessel (Ivanova and Van Staden, 2010). High RH in the culture vessel can be caused by medium components such as, gelling agents, basal medium and the type and concentration of plant growth regulators (PGRs) (Ivanova and Van Staden, 2008, 2011). A hyperhydricity problem was encountered during this study, and therefore, further studies were carried out to resolve the problem.

Olive micropropagation is highly cultivar dependent and it is necessary to develop different micropropagation procedures for each cultivar. Olive 'Gemlik' is one of the most important Turkish olive cultivars in terms of prevalence and fruit/oil production capacity, which constitutes nearly 11% of all olive plantations in Turkey (Çelikkol Akçay et al., 2014). Olive 'Gemlik' has an important place among the olive cultivars grown in Turkey because of showing no intensive periodicity, having high adaptation capacity and cold/disease resistance, early bearing, fruitfulness, processing ease for fruit consumption and olive oil extraction (Çelikkol Akçay et al., 2014; İsfendiyaroğlu et al., 2018). The most distinctive feature of this cultivar is its deep black fruit color at maturity and it has a high oil content which reaches up to 29% (İsfendiyaroğlu et al., 2018). To date, micropropagation of Olive 'Gemlik' has not been reported. The aim of the present study was to develop and optimize an efficient protocol for the regeneration of nodal shoot explants and micropropagation of the olive Gemlik cultivar and the prevention of hyperhydricity during *in vitro* culture.

2. Materials and methods

2.1. Plant material and sterilization

Two-year-old olive plants (*O. europaea* L. cv. Gemlik) obtained from Olive Research Institute (Izmir, Turkey) were maintained in the greenhouse belonging to Bioengineering Department of Ege University and a 3 g/L fungicide solution (3.0 g/L BENOLEX-Active compound: 50% BENOMYL) and 1.5 g/L NPK 20–20–20 fertilizer were applied to these plants as regular spraying every two weeks for three months. New shoots, not lignified, that emerged from these olive plants were used as explant source for all experiments. Single nodal explants (approximately 1.5–2.0 cm and each containing two opposite buds) were prepared from the region between the third and sixth nodes from vigorous growing shoots for initiation culture (Fig. 1a).

The above mentioned nodal explants were prepared and washed under running tap water for 15 min. They were rinsed with 70% ethanol for 10 s and then disinfected in 0.1% (w/v) mercury (II) chloride ($HgCl_2$) (Merck, Darmstadt, Germany) solution containing 0.1% (v/v) Tween 20 (Merck, Darmstadt, Germany) for 3 min. Finally, the nodal explants were washed with sterile distilled water three times.

2.2. Multiplication

2.2.1. Selection of efficient basal medium and plant growth regulators for multiplication

To determine an efficient basal medium for micropropagation of cultivar 'Gemlik', the sterilized nodal explants were cultured in glass tubes (23/24 × 140 mm, Lab Associates b.v., Oudenbosch, The Netherlands) each containing 10 mL of five different basal medium: (1) Olive initial medium (OIM; Rugini, 1984), (2) Modified Driver and Kuniyuki walnut medium (MDKW; Roussos and Pontikis, 2002), (3) Olive medium (OM; Rugini, 1984), (4) Woody Plant Medium (WPM; Lloyd and McCown, 1980), (5) Modified Olive medium (MOM1; OM with twice the concentrations of FeNaEDTA, $MgSO_4$, and $MnSO_4$; Brito and Santos, 2009) (Table A). OIM was supplemented with 2% (w/v) mannitol (Merck, Darmstadt, Germany); MDKW, OM and MOM1 were supplemented with 3% (w/v) mannitol and WPM was supplemented with 3% (w/v) sucrose. The experiments were conducted in three replications with fifteen explants in each replication. Forty-five explants were tested in total per treatment. The data were recorded 30 days after culture initiation.

After determining an efficient basal medium, another experiment was conducted to find appropriate PGRs for multiplication. For this purpose, single nodal explants as specified above were cultured in glass tubes containing 10 mL of WPM supplemented with zeatin (ZEA), 6-benzyladenine (BA), kinetin (KIN) or Gibberellic acid (GA_3) at the concentration of 0.5, 1.0, 2.0 and 4.0 mg/L, 3% (w/v) sucrose. The experiments were conducted in three replications with fifteen explants in each replication. Forty-five explants were tested in total per treatment. The data were recorded 45 days after culture initiation.

All the media were solidified with 0.65% (w/v) Plant agar (Duchefa Biochemie B.V., The Netherlands) (pH 5.8). The stock solutions of ZEA and GA_3 were filter-sterilized through a 0.22 m syringe Millipore filter (Minisart®, Sartorius, Germany), and then added to the autoclaved WPM aseptically at the desired concentrations. The stock solutions of BA and KIN were added to the media at the desired concentrations before autoclaving.

2.2.2. Prevention of hyperhydricity

High hyperhydricity formation in all cultures was seen; therefore different gelling agents were tested to prevent this abnormality. For this purpose, single nodal explants as specified above were cultured in media with Difco™ Agar (Becton, Dickinson and Company, USA; 0.65% (w/v)), Agar-Agar (Merck, Darmstadt, Germany; 0.65% (w/v)), Phytagel (Sigma-Aldrich, Co., USA; 0.3% (w/v)) or Fluka® Analytical (Sigma-Aldrich, Co., USA; 0.65% (w/v)). WPM supplemented with 4.0 mg/L BA was used as multiplication medium for hyperhydricity experiments. The experiments were conducted in three replications with fifteen explants in each replication. Forty-five explants were tested in total per treatment. The data were recorded 30 days after culture initiation.

2.3. Shoot elongation

2.3.1. Experiment 1

The induced shoots still attached to initial woody nodal explant or removed from initial woody nodal explants (approximately 0.5-cm-long,

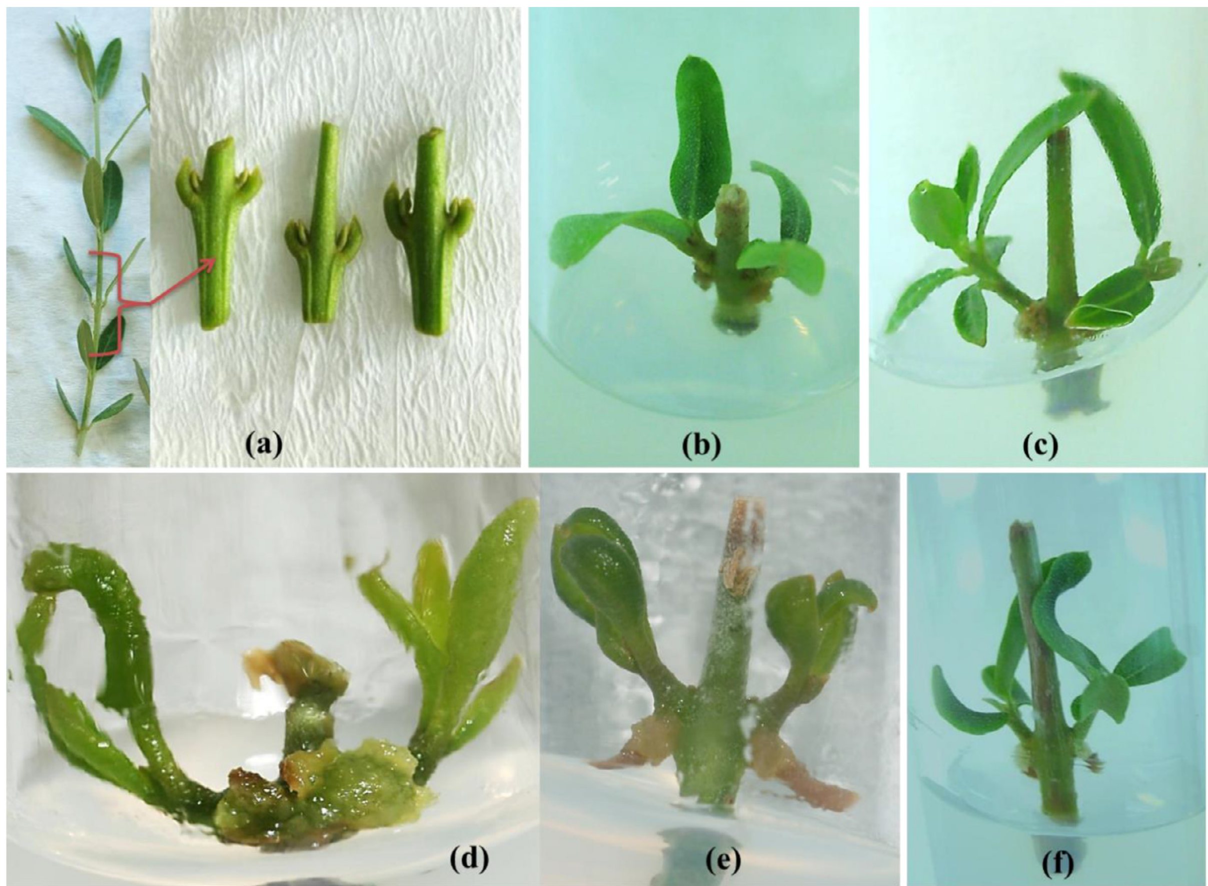

Fig. 1. *In vitro* shoot regeneration of *Olea europaea* L. cv. 'Gemlik': (a) Single nodal explants; (b) shoot regeneration from nodal explants cultured on PGRs free WPM after 30 days from the culture initiation; (c) shoot regeneration from nodal explants cultured on WPM supplemented with 4.0 mg/L BA at 45 days after the culture initiation; (d-e) hyperhydricity in regenerated shoots grown in PGRs supplemented media; (f) shoot regeneration from nodal explants cultured on Agar-Agar gelled medium after 30 days of culture from the beginning. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4 well developed leaves) were subcultured in glass tubes containing 10 mL of: (i) WPM supplemented with ZEA, BA or KIN at the concentration of 1.0 or 2.0 mg/L and 3% (w/v) sucrose, (ii) OM supplemented with ZEA, BA or KIN at the concentration of 1.0 or 2.0 mg/L and 3% (w/v) mannitol, (iii) MDKW supplemented with ZEA, BA or KIN at the concentration of 1.0 or 2.0 mg/L and 3% (w/v) mannitol. All media were solidified with 0.65% (w/v) Agar-Agar. The experiment was conducted in three replicates with ten explants in each replication. Thirty explants were tested in total per treatment. The data were recorded 40 days after culture initiation.

2.3.2. Experiment 2

None of the media above in experiment 1 gave satisfactory results; therefore, additional media were tested. The induced shoots still attached to initial woody nodal explant were cultured in glass tubes containing 10 mL of (i) OM; (ii) MOM1 (modified OM; OM with twice the concentrations of FeNaEDTA, $MgSO_4$, and $MnSO_4$; (iii) MOM2 (modified OM; OM with three times the concentrations of KNO_3); (iv) MOM3 (modified OM; OM with five times the concentrations of KNO_3). All media were supplemented with 2.0 mg/L ZEA, 3% (w/v) mannitol and 0.65% (w/v) Agar-Agar. The experiment was conducted with three replicates with ten explants in each replication. Thirty explants were tested in total per treatment. The data were recorded 40 days after culture initiation.

2.4. In vitro *rooting*

For root formation, shoots raised *in vitro* (approximately 1.0–2.0-cm-long, 3–6 well developed leaves) were transferred to the glass tubes containing 10 mL of OM supplemented with 160 mg/L Putrescine (Sigma-Aldrich), 1.5 mg/L naphthaleneacetic acid (NAA), 3% (w/v) mannitol and solidified with 0.65% (w/v) Agar-Agar.

2.5. *Acclimatization*

Plantlets, 1.0–2.0-cm long with well-developed roots, were removed from the glass tubes, and the roots were washed with water to remove agar medium residues. The plantlets were transferred to 5 cm diameter pots containing a 1:3 vermiculite:peat mixture. They were covered with perforated transparent bags to prevent moisture loss and kept in a chamber at approximately 25 °C, 70% humidity, 50 mol $m^{-2}s^{-1}$ irradiance (cool white fluorescent light) and 16-h photoperiod. The potted plants were ventilated for 20–25 min by removing the bags from the plants once a day for a period of 2 weeks. The acclimation bags were removed at the end of the 2-week period. The plants were watered as needed with $^1/_2$ OM liquid medium which contains no mannitol or growth regulators. The plants were then transferred from the climate chamber to the greenhouse conditions 4 weeks after the beginning of acclimatization. Completely acclimatized plants were then transferred to the larger pots.

2.6. *Media and culture conditions*

The pH of all the media was adjusted to 5.8 with 1 N HCl or 1 N NaOH prior to the addition of the gelling agent. They were autoclaved at 121 °C at 1.04 kg cm^{-2} for 15 min. All the cultures were incubated in a growth room at 24 ± 2 °C under cool white fluorescent light (50 mol $m^{-2}s^{-1}$) and with a 16-h photoperiod.

Table A
The basal nutrient medium composition of Olive Initial Medium (OIM), Modified Driver and Kuniyuki Walnut Medium (MDKW), Olive Medium (OM), and Woody Plant Medium (WPM).

Basal medium	OIM	OM	WPM	MDKW
Macronutrient components (mg/L)				
KNO_3	500	1100	–	–
$CaCl_2$	–	–	72.5	112.5
$CaCl_2 \cdot 2H_2O$	40	440	–	–
$Ca(NO_3)_2$	–	–	386	–
$Ca(NO_3)_2 \cdot 2H_2O$	–	–	–	1664.64
$Ca(NO_3)_2 \cdot 4H_2O$	–	600	–	–
KCl	–	500	–	–
KH_2PO_4	50	340	170	265
K_2SO_4	–	–	990	1559
$MgSO_4$	–	–	180.54	361.49
$MgSO_4 \cdot 7H_2O$	250	1500	–	–
NH_4NO_3	100	412	400	1416
Micronutrient components (mg/L)				
$CuSO_4 \cdot 5H_2O$	0.006	0.25	0.25	0.25
$FeSO_4 \cdot 7H_2O$	13.9	–	–	–
Tritriplex(Na_2EDTA)	18.6	–	–	–
FeNaEDTA	–	36.7	36.7	44.63
H_3BO_3	1.55	12.4	6.2	4.8
$MnSO_4.H_2O$	–	–	22.3	33.8
$MnSO_4 \cdot 4H_2O$	5.58	22.3	–	–
$Na_2MoO_4 \cdot 2H_2O$	0.06	0.25	0.25	0.39
$ZnSO_4 \cdot 7H_2O$	2.15	14.3	8.6	17
KI	0.21	0.83	–	–
$CoCl_2 \cdot 6H_2O$	0.006	0.025	–	–
Vitamins (mg/L)				
Nicotinic acid	0.25	5	0.5	1
Pyridoxine-HCl	0.25	0.5	0.5	0.5*
Thiamine-HCl	10	0.5	1	2
Biotin	–	0.05	–	–
Folic acid	–	0.5	–	0.5*
Myo-inositol	50	100	100	100
Amino acids (mg/L)				
Cystine	–	–	–	10*
Glutamine	–	2190	–	1200*
Glycine	1	2	2	2
Carbon sources (mg/L)				
Sucrose	–	–	30,000	–
Mannitol	20,000	30,000	–	30,000

*Adapted from Roussos and Pontikis (2002).
**Adapted from Brito and Santos (2009).

2.7. Statistical analysis

The experiments were set up in a completely randomized design, and all factors/treatments were replicated three times. For the basal medium selection (Table 1), shoot regeneration (Table 2) and hyperhydricity experiments (Table 3), each replicate comprised of fifteen explants. For the *in vitro* elongation (Table 4,5) experiments, each replicate comprised of ten explants. All data were analyzed using standard ANOVA procedures. The significant differences among the mean values were compared by the Duncan's multiple range test at $P = 0.05$ using SPSS Version 16.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. Multiplication

3.1.1. Selection of efficient basal medium and plant growth regulators for multiplication

To determine a suitable basal medium for micropropagation of *O. europaea,* nodal explants were cultured on five different media. The chemical composition of the culture media tested significantly affected regeneration frequency from nodal explant (Table 1). The highest regeneration frequency (97.78%) was observed on WPM (Fig. 1b) followed by OM (71.11%). The lowest regeneration frequency (6.67%) was obtained on MOM1. Statistically no significant difference in regeneration frequency was found between MDKW (11.11%) and MOM1 media, and therefore they were placed in the same statistical group. The highest shoot number per explant was observed on MOM1, on which an average of 1.71 shoots formed followed by WPM (1.69 shoots per explant) and OM (1.47 shoots per explant) and these three media placed at the same group statistically. Basal medium treatments were observed to have no effect on shoot length. The mean shoot lengths ranged between 1.27 and 1.5 mm. MDKW and MOM1 gave the best response regarding leaf number per explant (3.02 leaves per explant) and leaf length (4.47 mm), respectively. Some abnormalities like hyperhydricity and browning were observed in cultures. The hyperhydricity rate ranged between 84.19% and 95.24% without significant differences among treatments. The highest frequencies of browning (82.22%) were observed on MDKW medium. The shoots regenerated from WPM medium showed no browning.

Based on our results, WPM basal medium which supported shoot regeneration in 97.78% of explants and the highest number of shoots per explants and showed no browning was selected as suitable medium for further studies.

Following determination of an efficient basal medium, to find suitable PGRs for multiplication of Olive 'Gemlik', WPM supplemented with different PGRs was tested (Table 2). Of the four PGRs (ZEA, BA, KIN, and GA_3), ZEA and BA were more effective than KIN and GA_3 in terms of shoot regeneration. The highest regeneration rate (93.33%) was found on the medium supplemented with ZEA or BA at the concentrations of 0.5, 4.0 mg/L, respectively (Fig. 1c). This result was followed by PGRs free control medium (WPM) (84.45%) and WPM containing 1.0 mg/L ZEA (86.67%), 2.0 mg/L BA (84.45%) or 4.0 mg/L KIN (84.45%). They gave statistically the same response regarding regeneration rate. The highest number of shoots (1.87

Table 1
Effect of different basal media on the shoot regeneration and growth of *Olea europaea* L. cv. Gemlik.

Medium Type	Regeneration rate (%) ± SE	Shoot number per explant ± SE	Shoot length (mm) ± SE	Leaf number per explant ± SE	Leaf length (mm) ± SE	Hyperhydration rate (%) ± SE	Browning rate (%) ± SE
OIM	**42.22 ± 2.22 c**	**0.85 ± 0.02 b**	**1.27 ± 0.01**	**2.72 ± 0.22 ab**	**4.07 ± 0.02 ab**	**84.19 ± 0.43**	**33.33 ± 3.85 c**
MDKW	**11.11 ± 2.22 d**	**0.93 ± 0.04 b**	**1.50 ± 0.01**	**3.02 ± 0.03 a**	**4.07 ± 0.02 ab**	**86.03 ± 0.32**	**82.22 ± 2.22 a**
OM	**71.11 ± 4.44 b**	**1.47 ± 0.15 a**	**1.37 ± 0.01**	**2.66 ± 0.28 ab**	**3.93 ± 0.02 ab**	**85.32 ± 4.74**	**15.55 ± 2.22 d**
WPM	**97.78 ± 2.22 a**	**1.69 ± 0.05 a**	**1.33 ± 0.01**	**2.33 ± 0.04 b**	**3.63 ± 0.01 b**	**87.78 ± 2.94**	**0.00 ± 0.00 e**
MOM1	**6.67 ± 0.00 d**	**1.71 ± 0.02 a**	**1.37 ± 0.01**	**2.77 ± 0.08 ab**	**4.47 ± 0.03 a**	**95.24 ± 4.76**	**53.33 ± 0.00 b**

Each value represents the mean ± SE of three replicates. The same *letter* within a *column* denotes statistically equal means with the Duncan's multiple range test at $p \leq 0.05$
OIM olive initial medium (Rugini, 1984), ***MDKW*** modified Driver and Kuniyuki (1984) walnut medium (Roussos and Pontikis, 2002), ***OM*** olive medium (Rugini, 1984), ***WPM*** woody plant medium (Lloyd and McCown, 1980), ***MOM1*** modified olive medium: Doubling the Fe, Mg and Mn concentrations (Brito and Santos, 2009).

Table 2
Effect of different types and concentrations of PGRs on the shoot regeneration and growth of *Olea europaea* L. cv. Gemlik.

Concentrations of plant growth regulators (mg/L)				Regeneration rate (%) ± SE	Shoot number per explant ± SE	Shoot length (mm) ± SE	Leaf number per explant ± SE	Leaf length (mm) ± SE	Hyperhydration rate (%) ± SE
ZEA	BA	KIN	GA_3						
–	–	–	–	84.45 ± 2.22 ab	1.67 ± 0.04 abc	1.27 ± 0.01 b	2.72 ± 0.22 ab	4.07 ± 0.01 ab	84.19 ± 0.43 cd
00.5	–	–	–	93.33 ± 3.85 a	1.78 ± 0.08 ab	1.50 ± 0.01 b	3.02 ± 0.03 a	4.07 ± 0.01 ab	86.03 ± 0.32 cd
01.0	–	–	–	86.67 ± 7.70 ab	1.67 ± 0.15 abc	1.37 ± 0.01 b	2.66 ± 0.28 ab	3.93 ± 0.02 ab	85.32 ± 4.74 cd
02.0	–	–	–	75.56 ± 8.01 bc	1.49 ± 0.18 bcd	1.33 ± 0.01 b	2.33 ± 0.04 bcd	3.63 ± 0.01 ab	87.78 ± 2.94 cd
4.0	–	–	–	71.11 ± 2.22 c	1.36 ± 0.04 cd	1.37 ± 0.01 b	2.77 ± 0.08 ab	4.47 ± 0.03 ab	95.24 ± 4.76 ab
–	00.5	–	–	77.78 ± 2.22 bc	1.36 ± 0.04 cd	1.57 ± 0.03 b	2.60 ± 0.28 abc	5.43 ± 0.04 a	70.59 ± 2.07 e
–	01.0	–	–	82.22 ± 2.22 abc	1.47 ± 0.07 bcd	1.30 ± 0.01 b	2.42 ± 0.08 bc	3.33 ± 0.04 ab	81.74 ± 0.79 d
–	02.0	–	–	84.45 ± 2.22 ab	1.53 ± 0.04 bc	1.87 ± 0.04 ab	2.29 ± 0.04 bcd	3.73 ± 0.05 ab	89.49 ± 0.99 bc
–	4.0	–	–	93.33 ± 0.00 a	1.87 ± 0.00 a	1.37 ± 0.02 b	2.32 ± 0.07 bcd	3.10 ± 0.10 ab	100.00 ± 0.00 a
–	–	00.5	–	28.89 ± 2.22 e	0.51 ± 0.02 f	2.07 ± 0.06 ab	2.07 ± 0.07 cd	4.77 ± 0.13 ab	73.81 ± 1.19 e
–	–	01.0	–	48.89 ± 2.22 d	0.84 ± 0.06 e	2.97 ± 0.01 a	1.86 ± 0.21 d	3.40 ± 0.04 ab	84.05 ± 1.16 cd
–	–	02.0	–	77.78 ± 5.88 bc	1.49 ± 0.15 bcd	2.40 ± 0.02 ab	2.34 ± 0.07 bcd	4.83 ± 0.04 ab	83.55 ± 0.22 cd
–	–	4.0	–	84.45 ± 2.22 ab	1.53 ± 0.04 bc	2.33 ± 0.07 ab	2.28 ± 0.08 bcd	5.13 ± 0.08 ab	94.32 ± 2.84 ab
–	–	–	00.5	46.67 ± 0.00 d	0.73 ± 0.10 ef	1.73 ± 0.04 b	2.26 ± 0.17 bcd	2.83 ± 0.05 b	100.00 ± 0.00 a
–	–	–	01.0	42.22 ± 2.22 d	0.67 ± 0.14 ef	2.27 ± 0.05 ab	2.33 ± 0.25 bcd	3.43 ± 0.05 ab	100.00 ± 0.00 a
–	–	–	02.0	71.11 ± 2.22 c	1.20 ± 0.18 d	1.97 ± 0.04 ab	2.67 ± 0.22 ab	3.63 ± 0.01 ab	89.38 ± 1.72 bc
–	–	–	4.0	42.22 ± 2.22 d	0.58 ± 0.08 ef	2.17 ± 0.04 ab	2.64 ± 0.14 ab	5.37 ± 0.18 a	100.00 ± 0.00 a

Each value represents the mean ± SE of three replicates. The same *letter* within a *column* denotes statistically equal means with the Duncan's multiple range test at $p \leq 0.05$.

Table 3
Effect of different types of gelling agent on the shoot regeneration and growth of *Olea europaea* L. cv. Gemlik.

Concentrations and types of Agar% (w/v)	Regeneration rate (%) ± SE	Shoot number per explant (±SE)	Shoot length (mm) (±SE)	Leaf number per explant (±SE)	Leaf length (mm) (±SE)
Plant Agar (0.65)	93.33 ± 0.00 bc	1.87 ± 0.01	1.30 ± 0.02 c	2.17 ± 0.07 b	3.60 ± 0.04 c
Difco™ Agar (0.65)	95.55 ± 2.22 ab	1.83 ± 0.10	3.75 ± 0.15 a	4.25 ± 0.37 a	5.32 ± 0.11 a
Fluka® Analytical (0.65)	88,89 ± 2.22 c	1.80 ± 0.06	3.03 ± 0.18 b	4.14 ± 0.26 a	4.16 ± 0.27 b
Agar-Agar (0.65)	100,00 ± 0.00 a	1.91 ± 0.02	3.24 ± 0.25 ab	4.18 ± 0.11 a	5.49 ± 0.34 a
Phytagel (0.3)	97,78 ± 2.22 ab	1.89 ± 0.02	3.21 ± 0.16 ab	4.18 ± 0.04 a	5.40 ± 0.22 a

Each value represents the mean ± SE of three replicates. The same *letter* within a *column* denotes statistically equal means with the Duncan's multiple range test at $p \leq 0.05$.

Table 4
he response of shoots on different basal medium containing different types and concentrations of PGRs.

Medium	PGR (mg/L)			Shoot response	
	ZEA	BA	KIN	Shoots still attached to initial woody nodal explants	Shoots removed from initial woody nodal explants
WPM	–	–	–	No growth, Light green leaves, Leaf abscission	Weak growth, Substantial leaf necrosis
	1.0	–	–	Weak growth, Leaves with dark green veins	Weak growth, Light green leaves, Leaf abscission
	2.0	–	–	Weak growth, Leaves with dark green veins	Weak growth, Light green leaves, Leaf abscission
	–	1.0	–	Weak growth, Light green leaves, Leaf abscission	Weak growth, Substantial leaf necrosis, Leaf abscission
	–	2.0	–	Weak growth, Light green leaves, Leaf abscission	Weak growth, Substantial leaf necrosis, Leaf abscission
	–	–	1.0	Weak growth, Light green leaves, Leaf abscission	Weak growth, Substantial leaf necrosis, Leaf abscission
	–	–	2.0	Weak growth, Light green leaves, Leaf abscission	Weak growth, Substantial leaf necrosis, Leaf abscission
OM	–	–	–	Weak growth, Light green leaves	Weak growth, Light green leaves, Leaf abscission
	1.0	–	–	Weak growth, Leaves with dark green veins	Weak growth, Leaves with dark green veins
	2.0	–	–	**Well growth, leaves with dark green veins**	Weak growth, Leaves with dark green veins
	–	1.0	–	Weak growth, Light green leaves	Weak growth, Light green leaves, Leaf abscission
	–	2.0	–	Weak growth, Light green leaves	Weak growth, Light green leaves, Leaf abscission
	–	–	1.0	Weak growth, Light green leaves	Weak growth, Light green leaves, Leaf abscission
	–	–	2.0	Weak growth, Light green leaves	Weak growth, Light green leaves, Leaf abscission
MDKW	–	–	–	No growth, Substantial leaf necrosis, Leaf abscission	No growth, Substantial leaf necrosis, Leaf abscission
	1.0	–	–	Weak growth, Leaves with dark green veins	Weak growth, Light green leaves
	2.0	–	–	Weak growth, Leaves with dark green veins	Weak growth, Light green leaves
	–	1.0	–	No growth, Substantial leaf necrosis, Leaf abscission	No growth, Substantial leaf necrosis, Leaf abscission
	–	2.0	–	No growth, Substantial leaf necrosis, Leaf abscission	No growth, Substantial leaf necrosis, Leaf abscission
	–	–	1.0	No growth, Substantial leaf necrosis, Leaf abscission	No growth, Substantial leaf necrosis, Leaf abscission
	–	–	2.0	No growth, Substantial leaf necrosis, Leaf abscission	No growth, Substantial leaf necrosis, Leaf abscission

WPM woody plant medium (Lloyd and McCown, 1980), ***OM*** olive medium (Rugini, 1984), ***MDKW*** modified Driver and Kuniyuki (1984) walnut medium (Roussos and Pontikis, 2002).

shoots per explant) was achieved in cultures established on WPM supplemented with 4.0 mg/L BA. While progressively increasing BA and KIN concentrations in the WPM supported regeneration rate and shoot number, progressively increasing ZEA concentrations in the WPM reduced regeneration rate and shoot number. Shoot length ranged between 1.27 mm (WPM) and 2.97 mm (WPM with 1.0 mg/L KIN), and in general, although KIN and GA_3 led to a reduced number of shoots per explant, they had a positive effect on the shoot length. With 0.5 mg/L ZEA, the highest leaf number (3.02 leaves per explant) was recorded. WPM supplemented with 0.5 mg/L BA or 4.0 mg/L GA_3 gave the best response regarding leaf length as 5.43 mm and 5.37 mm, respectively. PGR-supplemented media caused the

Table 5
Shoot elongation of *Olea europaea* L. cv. Gemlik on different media.

Medium	Shoot length (mm) ± SE	Leaf number per explant ± SE	Leaf length (mm) ± SE	Leaf width (mm) ± SE
OM + 2 mg/L ZEA (Control)	29.84 ± 1.27 a	11.02 ± 0.38 c	8.60 ± 0.21 b	4.20 ± 0.10 b
MOM1+ 2 mg/L ZEA	12.44 ± 0.45 c	5.31 ± 0.19 d	7.38 ± 0.20 c	3.11 ± 0.10 d
MOM2 + 2 mg/L ZEA	31.22 ± 2.18 a	14.00 ± 0.49 a	9.64 ± 0.21 a	5.02 ± 0.13 a
MOM3 + 2 mg/L ZEA	21.29 ± 1.34 b	12.62 ± 0.58 b	8.82 ± 0.24 b	3.67 ± 0.14 c

Each value represents the mean ± SE of three replicates. The same *letter* within a *column* denotes statistically equal means with the Duncan's multiple range test at $p \leq 0.05$
OM olive medium (Rugini, 1984), ***MOM1*** modified olive medium: Doubling the Fe, Mg and Mn concentrations (Brito and Santos, 2009), ***MOM2*** modified olive medium: OM with three times the concentrations of KNO_3, ***MOM3*** modified olive medium: OM with five times the concentrations of KNO_3.

formation of significant hyperhydricity (Fig. 1d). The hyperhydricity rate reached 100% on WPM supplemented with 4.0 mg/L BA or 0.5, 1.0, or 4.0 mg/L GA_3. Hyperhydricity rate ranged between 70.59 and 100%.

Based on our results, WPM containing 4.0 mg/L BA which supported shoot regeneration in 93.33% of explants and had the highest number of shoots per explants was selected as suitable medium composition for further studies.

3.1.2. *Prevention of hyperhydricity*

To resolve the hyperhydricity problem, nodal explants were cultured in WPM supplemented with 4.0 mg/L BA and solidified with different gelling agents (Table 3). High frequencies of shoot regeneration (88.89–100%) occurred on all gelling agent types. The regeneration frequency reached 100% explants on the Agar-Agar (0.65% w/v) containing medium (Fig. 1e). Gelling agent types were observed to have no effect on shoot number per explant. The mean number of shoots per explant ranged between 1.80 and 1.91. The shoot length, leaf number per explant and leaf length showed a significant difference when cultured on WPM solidified with Difco™ Agar, Agar-Agar, Phytagel or Fluka® Analytical compared with Plant Agar. The longest shoot length (3.75 mm) was obtained on WPM solidified with Difco™ Agar (0.65% w/v). The highest leaf number per explant obtained as 4.25, 4.18, 4.18 and 4.14 on WPM solidified with Difco™ Agar, Agar-Agar, Phytagel or Fluka® Analytical, respectively. Leaf length ranged between 3.60 mm and 5.49 mm. The longest leaf length was obtained as 5.49, 5.40 and 5.32 mm from WPM solidified with Agar-Agar, Phytagel and Difco™ Agar, respectively.

Based on our results, WPM containing 4.0 mg/L BA solidified with 0.65% (w/v) Agar-Agar which supported shoot regeneration in 100% of explants, the highest number of shoots per explants, leaf number per explants and leaf length and showed no hyperhydricity was selected as a suitable medium for multiplication.

3.2. *Shoot elongation*

When the shoots regenerated from nodal explants were subcultured onto WPM containing 4.0 mg/L BA solidified with 0.65% (w/v) Agar-Agar, they did not grow further. Therefore, additional experimentation was performed to maintain the growth of shoots which had been obtained from the initial nodal culture. For this purpose, three different basal media (WPM, OM or MDKW) containing different types and concentrations PGRs (ZEA, BA or KIN at the concentration of 1.0 or 2.0 mg/L) and two explant types (shoots still attached to initial woody nodal explant or removed from initial woody nodal explants) were tested (Table 4). After 40 days culture initiation, in the above-mentioned media and in both explant types, no remarkable growth was observed in shoot length, shoot number, leaf number and leaf length. In addition, in most of the media, shoots contain light green leaves, necrotic leaves and leaves with dark green veins and leaf abscission, which made them unsuitable for shoot growth, were observed (Fig. 2a-d). Only in OM supplemented with 2.0 mg/L ZEA, was promising shoot growth observed. However, these shoots produced leaves with dark green veins. For this reason, another experiment was conducted and the effect of four medium formulations on further shoot growth of shoots obtained from initial nodal culture was examined (Table 5). The induced shoots still attached to initial woody nodal explant were used as explant type. Among the four media tested, the highest shoot length (31.22 mm), leaf number per shoot (14.00 leaves), leaf length (9.64 mm) and leaf width (5.02 mm) was observed in MOM2 medium fortified with 2.0 mg/L ZEA (Fig. 3a–d).

Based on our results, modified OM with three times the concentrations of KNO_3 containing 2.0 mg/L ZEA (MOM2) was selected as shoot elongation medium (SEM).

3.3. In vitro *rooting and acclimatization*

To promote *in vitro* rooting, shoots of Olive 'Gemlik' were cultured in OM medium with 160 mg/L Putrescine, 1.5 mg/L NAA, 3% (w/v) mannitol and solidified with 0.65% (w/v) Agar-Agar and 50% of shoots cultured were rooted. Twenty seven *in vitro* plantlets were transferred to a mixture of vermiculite and peat (1:3) for acclimation. Plant acclimation was 100% successful with all plants surviving. The acclimatized plants were later transferred to greenhouse conditions (Figs. 4a and b).

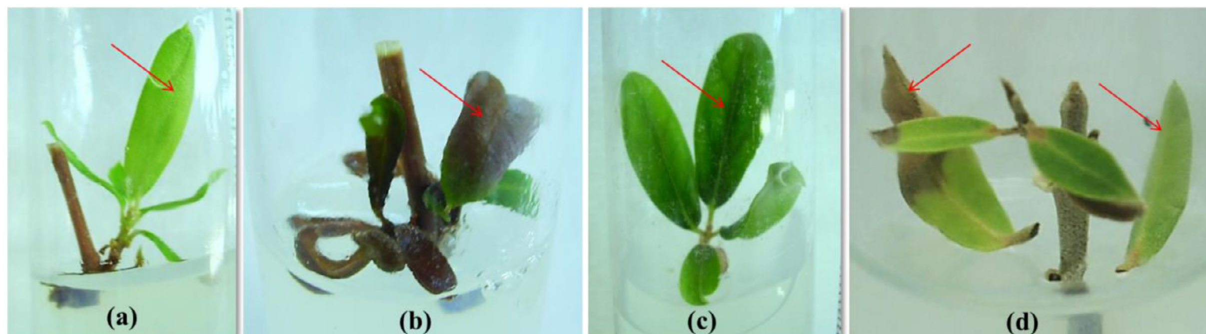

Fig. 2. Some abnormalities observed after the first subculture: (a) Shoots contain light green leaves, (b) necrotic leaves, (c) leaves with dark green veins, (d) leaf abscission. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

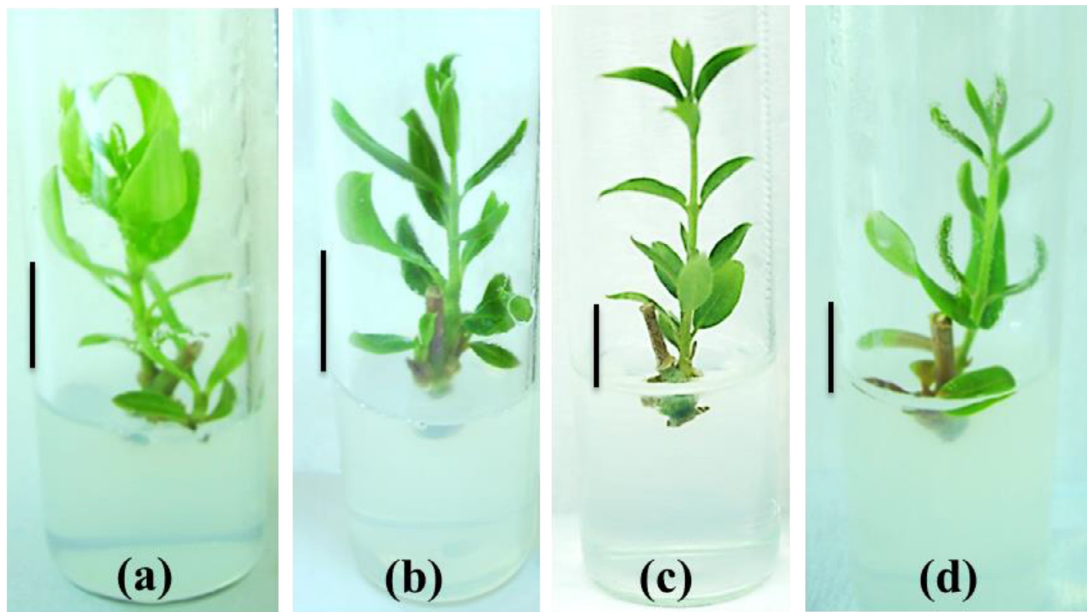

Fig. 3. Shoot elongation in: (a) OM, (b) MOM1, (c) MOM2, and (d) MOM3.

Fig. 4. (a) Rooted shoots and (b) acclimatized plantlets on vermiculite and peat (1:3) after 4 weeks of transplantation.

4. Discussion

We described here the micropropagation of Olive 'Gemlik', one of the most important Turkish olive cultivars with prevalence and high fruit/oil production capacity. Micropropagation of olive can be an important tool to produce large number of selected cultivars, when an efficient micropropagation protocol has been developed.

Unlike the majority of fruit species, at the beginning of the 1990s only a few olive cultivars had been efficiently micropropagated by using explants from zygotic embryos and seedlings; however, initiation of micropropagation using these types of explants is of minor interest when the clonally propagation of selected cultivars or clones is desired (Fabbri et al., 2009). Although shoot organogenesis has been obtained both from zygotic and mature tissues, because of high heterozygosity of olive, mature tissues are preferred instead of zygotic material for genetic stability. In olive studies, somatic embryogenesis has also been successfully developed for some cultivars but with conflicting results. Therefore, it should be considered that unwanted variations may occur before using somatic embryogenesis for propagation true-to-type olive plants (Rugini et al., 2016). In *in vitro* clonal propagation of a selected genotype, shoot tips and axillary buds are preferred as explant source. Plantlets obtained from these explants normally retain the genetic composition of the mother plant and are true-to-type plants (Debnath, 2018; Röck-Okuyucu et al., 2016). It has been reported that the nodal segment, or microcutting, is the preferable starting material for *in vitro* olive culture (Zacchini and De Agazio, 2004). It is therefore important to use nodal explants when clonal micropropagation is the goal and nodal explants were used as the explant source in the present study.

In *in vitro* micropropagation of olive cultivars, the major difficulties are the establishment of sterile cultures and subsequent initially growth of shoots. After collection of tissues from field- or greenhouse-grown plants, rapid oxidation may occur even using high doses of active antioxidants (Rugini et al., 2011). In the present study, when nodal explants excised from two-year-old Olive 'Gemlik' plants were sterilized with 70% ethanol for 10 s and then 0.1% (w/v) $HgCl_2$ solution for 3 min, sterilization-induced oxidation and browning was not observed. This facilitated the establishment of initial cultures for micropropagation in the Olive 'Gemlik'. Due to intense contamination found on olive starting material, $HgCl_2$ has been the usual sterilization method (Peixe et al., 2007; Zacchini and De Agazio, 2004).

In olive, the nutrient demands vary within species and genotypes and the nutrient medium should be determined for each genotype (Bartolini et al., 1990; Brito and Santos, 2009). We conducted

experimentation to find the appropriate basal medium for micropropagation of Olive 'Gemlik'. Although, OM was especially formulated for olive according to data from analysis of main mineral elements found in olive apical shoots and zygotic embryos by Rugini (1984), in our study, WPM showed highest shoot regeneration rate and shoot number per explant and no browning. Therefore, instead of using OM developed for olive, WPM was selected for initial culture of Olive 'Gemlik' micropropagation. OM resulted in the next lowest browning rate after WPM when compared to OIM, MDKW, MOM1 (Table 1). One of the main reasons for the browning of explants *in vitro* studies is the high salt concentration in the medium used. MDKW medium is richer than other media in total concentration micro and macro elements. Therefore, the highest browning rate in this medium might be due to its high salt content (Table 1). OM contains more $MgSO_4$ compared to the other media used. The MOM1 medium is the modified form of the OM medium in which the concentrations of Mg, Fe, and Mn are doubled. These increased concentrations may be the cause of the high browning problem observed in the MOM1 medium.

Forcing the lateral buds on nodal explants for shoot regeneration in olive has been mainly achieved through Zeatin, 6-Benzylaminopurine, Thidiazuron, Metatopolin, Giberellic acid, Dikegulac, Coconut water (Mendoza-de Gyves et al., 2008; Peixe et al., 2007; Rugini et al., 2016). It has been reported that the response of each cultivar to PGRs is different (Santos et al., 2003). In the present study, BA was as effective as ZEA in shoot regeneration from nodal explants of the Olive 'Gemlik'. Also, it has been reported that BA gave good results for olive cultivars such as 'Domat' and 'Memecik' (Seyhan and Özzambak, 1994), 'Kalamon' (Dimassi, 1999), *O. europaea* ssp. *maderensis* (Santos et al., 2003), 'Galega vulgar' (Peixe et al., 2007). In our study, Kinetin at 0.5 mg/L gave the lowest results regarding shoot regeneration rate and shoot number per explant. A similar result was also observed by Peixe et al. (2007) in olive cultivar 'Galega vulgar'. In general, regeneration rate and shoot number per explant with treatments of KIN and GA_3 were lower than those treated with ZEA and BA. A similar result was observed by Dimassi (1999), he reported that GA_3 reduced the rate of shoot proliferation and did not affect shoot length.

Due to strong apical dominance, olive shows a low secondary axillary shoot formation (Mendoza-de Gyves et al., 2008; Micheli et al., 2018). Thus, uni-nodal explants with bilateral buds produce usually just one or, occasionally, two shoots. Consequently, multiplication rate in olive is mainly determined by the number of nodes on the initial explant (Lambardi et al., 2013; Leva et al., 2013; Micheli et al., 2018). In the present study, the node explants exhibited nearly 2 shoots (1.87) with WPM medium containing 4.0 mg/L BA.

In our study, in the efficient basal medium and PGRs selection studies, nodal cultures of Olive 'Gemlik' initiated on all media including PGRs-free media containing 0.65% Plant agar showed severe hyperhydricity ranged between 70.59−100%. This was thought to originate from the type of gelling agent used and different types of gelling agent were tested to prevent hyperhydricity. While Plant Agar promotes hyperhydricity considerably, the other four different gelling agents (Difco™ Agar, Fluka® Analytical, Agar-Agar and Phytagel) used completely prevent hyperhydricity. The relationship between gelling agent types and concentration and hyperhydricity has been shown *in vitro* cultures of some plants such as 'York' and 'Vermont Spur Delicious' apples (*Malus domestica* Borkh.) (Pasqualetto et al., 1988), *Cydonia oblonga* Mill. (Singha et al., 1990), *Prunus avium* (Franck et al., 2004), Malus × domestica (Höhnle and Weber, 2009), *Rosa persica* (Jafarkhani Kermani et. al., 2010), *Aloe polyphylla* (Ivanova and Van Staden, 2011), 'Jonagold' apple (Tabart et al., 2015), *Allium sativum* L. (Liu et al., 2017).

After the initiation culture, the induced shoots still attached to initial woody nodal explant or removed from initial woody nodal explants were subcultured on different basal medium WPM, OM or DKW supplemented with ZEA, BA or KIN at the concentration of 1.0 or 2.0 mg/L for further growth. Results from this study indicate that all media tested, except for shoots attached to initial woody nodal explant, cultured in OM medium containing 2.0 mg/L ZEA, were not suitable for further growth. The shoots cultured did not grow well and showed light green leaves, necrotic leaves and leaves with dark green veins and leaf abscission (Fig. 2a-d). Reduced growth, leaf chlorosis and abscission may be a result of nutrient deficiency during culture and leaf chlorosis may be due to lack of nutrients like Fe, Mg, and Mn required for photosynthesis and chlorophyll synthesis (Brito and Santos, 2009). Doubling of these nutrients in OM prevented chlorosis and abscission and provided green-healthy shoots in *O. maderensis* (Lowe) Rivas Mart. & Del Arco (Brito and Santos, 2009). Therefore, we conducted another experiment for *in vitro* multiplication-elongation. Media tested in our experimental system differ mainly in the concentration of KNO_3, $FeSO_4$, $MgSO_4$, $MnSO_4$ compared to OM. OM containing 2.0 mg/L ZEA was used as control medium. In our study, doubling of Fe, Mg, and Mn in OM medium (MOM1) did not provide the same effect reported by Brito and Santos (2009). MOM1 was less effective than the other media tested in terms of all parameters observed. MOM2 (modified OM; OM with three times the concentrations of KNO_3) supplemented with 2.0 mg/L ZEA, 3% (w/v) sucrose and 0.65% (w/v) Agar-Agar gave best response regarding shoot growth and it was therefore the selected medium for shoot elongation (Fig. 3a-d). MOM3 (modified olive medium: OM with five times the concentrations of KNO_3) showed best leaf number per explant and leaf length after MOM2 but resulted in leaf chlorosis, necrosis and curl (Figs. 5a and b). This adverse effect may be related to toxicity of the KNO_3 dose. Increasing of KNO_3 concentrations in the MOM2 and MOM3 media compared to OM provided only an increase in the leaf number. In MOM2 leaf length increased also.

High K^+concentration results in Mg deficiency in plants and *vice versa* (George and de Klerk, 2008). It can be said that the MOM3 is effective on the number of leaves and leaf growth but at the same time the high concentration of KNO_3 might be toxic or cause Mg deficiency and thus this might result in chlorosis. Nitrogen is essential to plant life and mainly absorbed in the form nitrate (NO_3^-). It is a constituent of both proteins and nucleic acids and occurs in chlorophyll. In most plant cultures, nitrate is an important source of nitrogen. Due to the latent toxicity of the ammonium ion in high concentration, and the need to control the pH of the medium, most tissue culture media contain more nitrate than ammonium ions. Both growth and morphogenesis in tissue cultures are markedly influenced by the availability of nitrogen and the form in which it is presented. Total nitrogen supplied in medium, especially in nitrate form, is effective in stimulating *in vitro* organogenesis and in shoot cultures (George and de Klerk, 2008). In olive plants, the nitrogen form also has significant effect on *in vitro* cultures (Chaari-Rkhis et al., 2011). Rama and Pontikis (1990) modified OM and also increased the concentration of KNO_3 from 1100 mg/L to 1830 mg/L. They achieved successful micropropagation of the olive cv. Kalamon in this modified OM medium.

According to the results of previous olive micropropagation studies, rooting is the critical phase of micropropagation of olive, being influenced by several factors like auxin type, genotype (Haddad et al., 2018), the addition of putrescine and coloring basal medium dark or placing the whole culture in the dark for one week have aided rooting (Rugini et al., 2016). For rooting of olive cultivars, the adding of putrescine to the medium is beneficial (Rugini et al., 2011). Putrescine at 160 mg/L promoted early and effective rooting by increasing total peroxidase activity at the shoot base, which is essential for root induction (Rugini et al., 1997, 2016). In the present study, after rooting of shoots on OM supplemented with 160 mg/L Putrescine, 1.5 mg/L NAA, and 3% (w/v) mannitol, they were transferred to a mixture of vermiculite and peat (1:3) for acclimation. Plant acclimation was 100% successful with all plants surviving. Acclimatized plants were later transferred to greenhouse conditions (Figs. 4a and b).

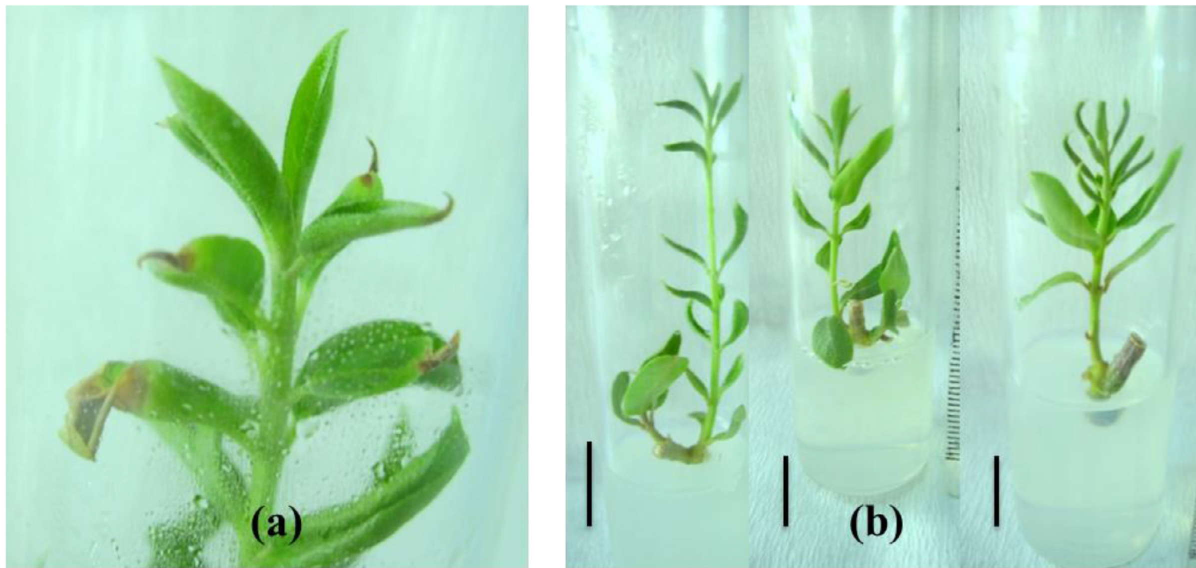

Fig. 5. Shoot elongation in MOM3: (a) Leaf necrosis and (b) curl.

5. Conclusion

Several studies have been carried out to optimize conditions for the micropropagation of Olive 'Gemlik'. Although OM is the most widely used medium for micropropagation of *Olea* cultivars, WPM was found more appropriate basal medium for initiation of micropropagation processes from nodal explants of Olive 'Gemlik'. Best shoot regeneration was observed in WPM containing 4.0 mg/L BA. The hyperhydricity observed in shoots was prevented by changing the type of gelling agent. The shoots regenerated from nodal explants still attached to initial woody nodal explant were elongated in MOM2 (OM with three times the concentrations of KNO_3) fortified with 2.0 mg/L ZEA. Rooting of shoots was carried out with OM containing 160 mg/L Putrescine and 1.5 mg/L NAA and all plantlets were successfully acclimatized. Finally, we believe that this new protocol enables the micropropagation of Olive 'Gemlik' and will facilitate its commercial production further.

Declaration of Competing Interest

Authors declare no conflict of interest.

CRediT authorship contribution statement

Meltem Bayraktar: Writing - review & editing, Data curation, Writing - original draft. **Sadiye Hayta-Smedley:** Writing - review & editing. **Sundus Unal:** Writing - review & editing. **Nurhan Varol:** Writing - review & editing. **Aynur Gurel:** Writing - review & editing, Supervision.

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