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ORIGINAL PAPER

Micropropagation of centennial tertiary relict trees of *Liquidambar orientalis* Miller through meristematic nodules produced by cultures of primordial shoots

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Abstract
Key message **This paper provides an important micropropagation method that might be used for conservation and commercial production of tertiary relict tree *Liquidambar orientalis*.**
Abstract The *Liquidambar orientalis* Miller is an important tree as a tertiary relict endemic species in terms of plant biodiversity, and has economic value due to the balsam it produces. In the present study, an efficient micropropagation method was developed for the *L. orientalis.* Initially, primordial shoot explants isolated from axillary buds were cultured on Murashige and Skoog and Woody Plant Medium (WPM) containing different plant growth regulators (PGRs). The highest number of shoots per explant was obtained on WPM supplemented with 1.0 mg/L 6-benzylaminopurine (BAP) and 1.0 mg/L indole-3-butyric acid (IBA) (shoot proliferation medium: SPM). These shoots were then subcultured continuously on SPM for a period of 18 months. Meristematic nodule clusters were formed at the base of the cultured shoots in SPM, where multiple shoots developed from them. The number of shoots per explant was increased approximately 2.8-fold by applying various strategies such as different explant type (single shoots and shoot clusters) and culture vessels (Glass Tube, 210-cc Glass culture jar, 400-cc Glass culture jar, and Vitro Vent®). Shoot clusters cultured in ventilated Vitro Vent® vessels on SPM gave best result for shoot propagation. In vitro shoots rooted best on WPM containing 30 g/L sucrose, 120 mg/L ethylenediamine di-2-hydroxyphenyl acetate ferric (Fe-EDDHA) and 4.0 mg/L IBA. All plantlets were successfully acclimatized in a glasshouse and then plants were transferred to the field. This methodology has been adapted by a commercial producer.

Keywords Oriental sweetgum tree · Culture vessel · Fe-EDDHA · In vitro rooting · Ventilation · Conservation · Plant acclimatization

Abbreviations

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
IAA	Indoleacetic acid
MS	Murashige and Skoog medium
WPM	Woody Plant Medium
PGR(s)	Plant growth regulator(s)
SPM	Shoot proliferation medium
Fe-EDDHA	Ethylenediamine di-2-hydroxyphenyl acetate ferric

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GT	Glass tube
210-GCJ	210-cc Glass culture jar
400-GCJ	400-cc Glass culture jar

Introduction

The deciduous tree genus *Liquidambar*, belonging to the Hamamelidaceae family, is spread over a wide geographical area, ranging from North America to East Asia (Hoey and Parks 1991; Öztürk et al. 2008). A total of five species are taxonomically categorized under the genus *Liquidambar*; *Liquidambar orientalis* Miller (western Asia), *L. styraciflua* (disjunct between eastern North America and from central Mexico to Belize), *L. macrophylla* (from Mexico), *L. formosana* (eastern Asia) and *L. acalycina* (eastern Asia) (Ickert-Bond et al. 2005; Özdilek et al. 2010). *L. orientalis* Miller is a relict endemic species, which has managed to survive ever since the tertiary period, and spreads over a narrow range in southwestern Turkey (Hoey and Parks 1991; Özturk et al. 2004). Commonly known as the *Oriental Sweetgum* (English), the tree is also frequently called the *Gunluk Ağacı* (Turkish) meaning frankincense or myrrh tree owing to its fragrance, or the *Sigla Ağacı* (Turkish) owing to the balsam seeping from the tree trunk when injured (Öztürk et al. 2008).

Dense forests of monoecious *L. orientalis* trees have survived as a relict heritage since the tertiary period and form an important genetic heritage, not just for Turkey but for the world at large (Öztürk et al. 2008; Küçükala et al. 2010). The natural production of balsam by the species, which is a rare characteristic among forest trees, also renders *L. orientalis* economically important (Göçmen-Taşkın et al. 2007; Çengel et al. 2010). The balsam is obtained from the trunk of the *L. orientalis* and contains cinnamic acid, styracin, styrol, storesinol and styrogenin (Küçükala et al. 2010).

Since the beginning of the twentieth century, the total range of *L. orientalis* forests has receded by 79 % from 6312 to 1337 ha. (Göçmen-Taşkın et al. 2007). This rapid reduction is due to many factors; such as excessive damage to the tress for oil extraction, clearance of forested areas for tourism development, housing and agricultural purposes, drainage of wetlands, and inadequate conservation strategies (Küçükala et al. 2010). The protection of *L. orientalis* and its genetic heritage for future generations is of crucial importance and proposals for solutions towards maintaining its sustainability are paramount.

Liquidambar orientalis is traditionally produced by seed which requires 1–3 months of stratification and can sometimes take up to 2 years to germinate. Germination rates are often poor. Seasonally dependent, seed sowing takes place early in the year. It is reported that the individuals which produced balsam represent only 40 % of the population; therefore, commercially vegetative production is preferred. Vegetative propagation via semi-ripe woody cuttings is problematic and collection of donor material from tall trees is very difficult (Parlak 2012). Vegetative propagation by layering takes 12 months. Consequently, in vitro clonal propagation represents an alternative for commercial and conservation purposes of *L. orientalis*.

The current in situ and ex situ methods that are utilized for the preservation of plant genetic sources are reinforced by in vitro techniques (Krishnan et al. 2011). Shoot apical or axillary meristem-based micropropagation is one of the in vitro techniques used in the preservation of plant biodiversity (Reed et al. 2011). Micropropagation allows the rapid and true to type production of valuable and endangered plant species, which may be difficult to produce using traditional methods. The production of an endangered species such as *L. orientalis* can be facilitated using only a small amount of plant material without further harming the species.

In vitro propagation has been reported previously for *L. styraciflua* (Sutter and Barker 1985; Brand and Lineberger 1988, 1991; Kim et al. 1997; Ďurkovič and Lux 2010), *L. formosana* (Xu et al. 2007), hybrid sweetgum (*L. styraciflua* × *L. formosana*) (Vendrame et al. 2001) and *L. orientalis* (Genç 1999; Erdağ and Emek 2005). Genç (1999) obtained indirect regeneration of *L. orientalis*, however; indirect regeneration through a callus phase can cause undesirable somaclonal variation. Erdağ and Emek (2005) achieved direct adventitious shoots from in vitro leaves of *L. orientalis*, obtaining 20 shoots/explant, with an 83 % rooting percentage and 80 % survival rate during acclimatization.

The aims of this study were to improve micropropagation of *L. orientalis* by assessing explant type, culture vessels, sucrose concentrations and the addition of Fe-EDDHA to the culture medium and to adapt the in vitro-obtained plantlets to the external environment. The authors hope this study will act as a roadmap for the protection of this species as it has already been applied for commercial production of balsam-producing trees by a tissue culture company.

Materials and methods

Plant material and sterilization

Plant material was collected in January 2010 from five approximately 100-year-old trees that grew in the *L. orientalis* forests (Köyceğiz district of Muğla) (Fig. 1a).

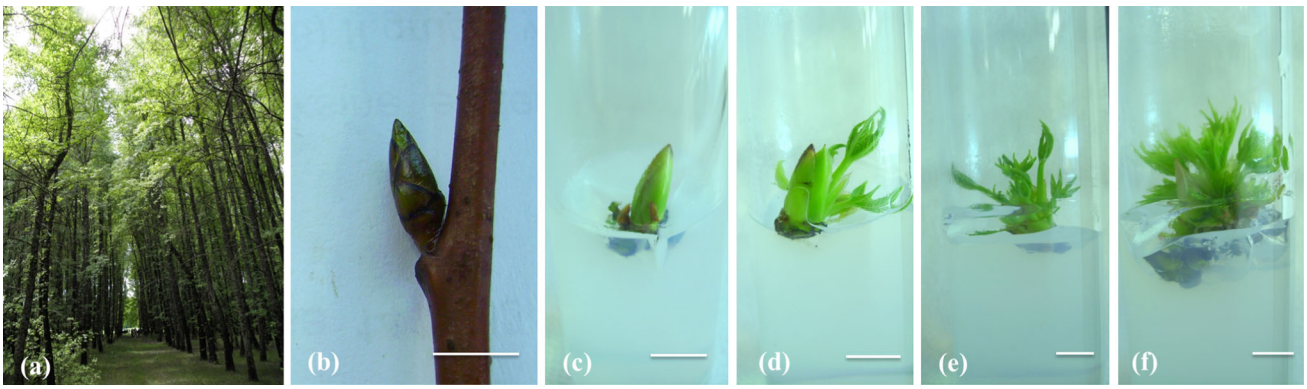

Fig. 1 Multiple shoot formation from an axillary bud explant of *L. orientalis* on WPM containing 1 mg/L BAP and 1.0 mg/L IBA: **a** over 100 years old *L. orientalis* trees, five of which were donors of suckers; **b** an axillary bud from the collected sucker; **c** multiple shoot formation: culture initiation (0 day); after 12 days (**d**); 20 days (**e**) and 30 days (**f**) of the culture (*bars* 0.5 cm)

These trees have been used for balsam production over many years. 20–30-cm-long hardwood cuttings with axillary buds (0.5–0.7 cm) from suckers were used as material (Fig. 1b). The hardwood cuttings were kept in a 3 g/L fungicide solution (1.5 g/L CAPTAN-Active compound: 48.9 % CAPTAN and 1.5 g/L BENOLEX-Active compound: 50 % BENOMYL) for 2 days. Prior to sterilization, the cuttings were cut into pieces (approximately 1 cm and each containing an axillary bud). These explants were washed under running water for 30 min. They were rinsed with water containing commercial liquid anti-bacterial soap (10 drops/1 L water) for 10 min, and afterwards were washed under running water for 15 min. The explants were rinsed with 70 % ethanol for 3 min, and then disinfected in 100 % sodium hypochlorite (Sigma-Aldrich®-available chlorine 10–15 %) for 30 min. The nodal fragments were washed with sterile water three times. The outer bud scales were removed using a scalpel under a stereo microscope (Olympus SZX7, Tokyo, Japan) and discarded. The primordial shoots were then cut from the woody tissue. Subsequently, the explants were soaked in 3 g/L fungicide solution (1.5 g/L CAPTAN and 1.5 g/L BENOLEX) for 30 min and were then transferred to culture media without rinsing.

Establishment of bud culture

Primordial shoots (0.5–0.7 cm in length) were cultured in glass tubes (23/24 × 140 mm, Lab Associates b.v., Oudenbosch, The Netherlands) each containing 10 mL of Woody Plant Medium (WPM; Lloyd and McCown 1980) or Murashige and Skoog medium (MS; Murashige and Skoog 1962) supplemented with various concentrations of different plant growth regulators (PGRs), 3 % (w/v) sucrose and 0.65 % (w/v) agar (Plant agar, Duchefa Biochemie B.V., The Netherlands) (Table 1). Primordial shoots originating from the five donor trees were distributed equally among media treatments. The experiments were conducted in three replications with ten explants in each replication. Thirty explants were tested in total per treatment. The data were recorded 45 days after culture initiation.

Multiplication of shoots

The induced shoots were subcultured in glass tubes containing a 10 mL WPM containing 1.0 mg/L BAP + 1.0 mg/L IBA (shoot proliferation medium, SPM), 3 % (w/v) sucrose and solidified with 0.65 % (w/v) agar. The explants were subcultured monthly for 18 months.

Culture vessel and explant types

An experiment was set up to investigate the effect of different culture vessels and explant types on shoot propagation and growth. Two different explant types [single shoots and shoot clusters (three shoots per cluster) (0.5–1.0 cm in length)] each containing one node and 4 leaves were used. Plant tissue Glass Tubes (23/24 × 140 mm, Lab Associates b.v., Oudenbosch, The Netherlands) (GT), 210-cc-capped glass culture jars (6.6 cm in diameter × 8.09 cm long) (210-GCJ), 400-cc-capped glass culture jars (9.4 cm in diameter × 7.95 cm long) (400-GCJ) and Vitro Vents® (115 × 90 × 96 mm) (Duchefa Biochemie B.V., The Netherlands) were used as culture vessels. The GTs were loosely closed with 25 × 25 mm (diam. × h.) transparent polycarbonate caps. Metal screw caps were used for 210-GCJs (6.3 cm in diameter) and 400-GCJs (8.2 cm in diameter). Transparent and specially designed polypropylene caps enabling gas exchange were used for Vitro Vents®. To maintain the explant/nutrient medium proportion; 1 explant was cultured in GTs (10 mL of medium/tube), 3 explants were

Table 1 Effect of media (MS, WPM) supplemented with different concentrations of PGRs (BAP, IBA, BAP + IBA) on shoot regeneration from bud cultures of *L. orientalis* after 45 days of the culturing

Medium	PGR (mg/L)		% Explants producing shoots ± SE	Mean number of shoots/explant ± SE	Mean shoot lengths (mm)/explant ± SE	% Explants producing necrotic shoots ± SE
	BA	IBA				
MS	–	–	86.7 ± 3.3 abc	1.0 ± 0.1 i	15.3 ± 0.1 ab	16.7 ± 3.3 bc
	0.5	–	93.3 ± 3.3 ab	1.0 ± 0.1 i	6.7 ± 0.1 e	16.7 ± 3.3 bc
	1.0	–	90.0 ± 5.8 abc	1.9 ± 0.2 hi	7.7 ± 0.1 de	40.0 ± 0.0 a
	2.0	–	86.7 ± 3.3 abc	0.9 ± 0.1 i	14.3 ± 0.2 abc	46.7 ± 3.3 a
	–	0.5	73.3 ± 8.8 c	2.1 ± 0.3 hi	10.7 ± 0.1 bcde	30.0 ± 0.0 ab
	–	1.0	86.7 ± 3.3 abc	3.6 ± 0.4 fg	13.8 ± 0.1 abcd	40.0 ± 5.8 a
	–	2.0	100.0 ± 0.0 a	2.5 ± 0.2 gh	17.8 ± 0.1 a	46.7 ± 3.3 a
	0.5	0.5	93.3 ± 3.3 ab	3.1 ± 0.3 fgh	10.6 ± 0.1 bcde	20.0 ± 0.0 bc
	1.0	1.0	90.0 ± 5.8 abc	5.9 ± 0.5 cd	8.5 ± 0.1 cde	23.3 ± 3.3 b
	2.0	2.0	80.0 ± 5.8 abc	3.5 ± 0.4 fgh	8.8 ± 0.1 cde	30.0 ± 0.0 ab
WPM	–	–	96.7 ± 3.3 ab	1.1 ± 0.1 i	16.2 ± 0.2 ab	0.0 ± 0.0 d
	0.5	–	76.7 ± 3.3 bc	4.7 ± 0.5 def	11.6 ± 0.1 bcde	0.0 ± 0.0 d
	1.0	–	83.3 ± 3.3 abc	5.5 ± 0.6 cde	11.2 ± 0.1 bcde	0.0 ± 0.0 d
	2.0	–	76.7 ± 3.3 bc	4.5 ± 0.6 ef	10.4 ± 0.1 bcde	3.3 ± 3.3 d
	–	0.5	90.0 ± 0.0 abc	2.4 ± 0.2 gh	13.0 ± 0.1 abcd	3.3 ± 3.3 d
	–	1.0	93.3 ± 3.3 ab	7.0 ± 0.6 c	10.8 ± 0.1 bcde	10.0 ± 0.0 c
	–	2.0	90.0 ± 5.8 abc	3.3 ± 0.3 fgh	11.9 ± 0.1 abcde	20.0 ± 0.0 bc
	0.5	0.5	100.0 ± 0.0 a	13.1 ± 0.6 a	12.1 ± 0.1 abcd	0.0 ± 0.0 d
	1.0	1.0	100.0 ± 0.0 a	14.5 ± 0.9 a	14.5 ± 0.1 ab	0.0 ± 0.0 d
	2.0	2.0	100.0 ± 0.0 a	9.7 ± 0.5 b	10.7 ± 0.0 bcde	0.0 ± 0.0 d

In each column, mean ± SE followed by the same letter was not significantly different ($p = 0.05$) according to Student–Newman–Keuls test

cultured in 210-GCJs (30 mL of medium/glass jar); 5 explants were cultured in 400-GCJs (50 mL of medium/glass jar) and 15 explants were cultured in Vitro Vents® (150 mL of medium/Vitro Vent®). The explants were cultured on SPM. The experiments were conducted in three replications, 15 explants were used for each replication. Forty-five explants were tested in total per treatment. The data were recorded 45 days after culture initiation (Table 2).

Shoot elongation and rooting

Shoots raised in vitro (approximately 1.5-cm-long, 6 well-developed leaves) were transferred to the Vitro Vent® culture vessels containing 150 mL of WPM containing 15 g/L sucrose, 30 g/L sucrose, 15 g/L sucrose + 120 mg/L ethylenediamine di-2-hydroxyphenyl acetate ferric (Fe-EDDHA—Duchefa Biochemie B.V., The Netherlands) or 30 g/L sucrose + 120 mg/L Fe-EDDHA with the range of IBA or IAA (0.5–4.0 mg/L) (Table 3). The Fe-EDDHA was in addition to the iron already contained in WPM. All of the media were solidified with 0.65 % agar. The experiments were conducted in three replications and 15 shoots were used for each replication. Forty-five explants were tested in total per treatment. The data were recorded 45 days after culture initiation.

Table 2 Effect of various culture vessels and explant types on shoot multiplication of *L. orientalis* on WPM with 1.0 mg/L BAP and 1.0 mg/L IBA after 45 days of the culturing

Type of culture vessel	Mean number of shoots/explant ± SE	
	Single shoot	Shoot cluster
Culture tube	6.5 ± 0.4 f	14.2 ± 0.6 c
210-cc glass culture jars	5.2 ± 0.3 f	9.5 ± 0.3 e
400-cc glass culture jars	5.5 ± 0.4 f	12.0 ± 0.4 d
Vitro Vent®	17.4 ± 0.7 b	40.5 ± 0.9 a

In each column, mean ± SE followed by the same letter was not significantly different ($p = 0.05$) according to Student–Newman–Keuls test

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

Table 3 Effect of the medium (WPM) supplemented with various concentrations of sucrose, IAA, IBA and Fe-EDDHA on shoot elongation and root formation of *L. orientalis* after 45 days of the culturing

Sucrose (g/L)	Concentration (mg/L)			Mean shoot lengths (mm) ± SE	Percentage of shoots rooted ± SE	Mean number of roots/rooted shoot ± SE	Mean root lengths (mm)/rooted shoot ± SE	Meristemoid cluster at the base of shoots
	Fe-EDDHA	IAA	IBA					
15	–	–	–	29.9 ± 0.2 fgh	62.2 ± 8.0 cd	2.3 ± 0.4 o	14.2 ± 0.1 jkl	–
		0.5	–	26.1 ± 0.1 ghi	100.0 ± 0.0 a	4.3 ± 0.3 klm	19.8 ± 0.1 fg	–
		1.0	–	29.3 ± 0.1 fgh	100.0 ± 0.0 a	4.0 ± 0.3 klm	22.3 ± 0.1 efg	–
		2.0	–	21.0 ± 0.0 jkl	100.0 ± 0.0 a	6.9 ± 0.4 hi	13.9 ± 0.1 jkl	–
		4.0	–	39.1 ± 0.2 cde	100.0 ± 0.0 a	8.4 ± 0.3 gh	31.5 ± 0.2 c	–
		–	0.5	16.1 ± 0.0 m	86.7 ± 3.9 a	2.7 ± 0.2 no	5.8 ± 0.1 n	+
		–	1.0	17.0 ± 0.0 m	60.0 ± 0.0 cd	5.0 ± 0.4 jkl	5.5 ± 0.0 n	+
		–	2.0	29.5 ± 0.1 fgh	80.0 ± 3.9 ab	6.7 ± 0.4 hi	9.0 ± 0.1 m	+
		–	4.0	20.9 ± 0.0 jkl	20.0 ± 0.0 f	3.1 ± 0.1 mn	4.0 ± 0.0 o	+
	120	–	–	33.1 ± 0.1 ef	66.7 ± 3.9 bc	3.8 ± 0.3 lm	19.0 ± 0.2 ghij	–
		0.5	–	30.4 ± 0.1 fg	100.0 ± 0.0 a	5.1 ± 0.3 jkl	23.4 ± 0.1 def	–
		1.0	–	41.7 ± 0.2 cd	100.0 ± 0.0 a	8.4 ± 0.3 gh	31.0 ± 0.1 c	–
		2.0	–	43.4 ± 0.3 cd	100.0 ± 0.0 a	11.3 ± 0.7 ef	29.0 ± 0.2 cd	–
		4.0	–	43.7 ± 0.2 bc	100.0 ± 0.0 a	10.5 ± 0.4 f	30.3 ± 0.1 c	–
		–	0.5	35.5 ± 0.1 cde	100.0 ± 0.0 a	5.7 ± 0.3 ij	17.7 ± 0.1 ghij	–
		–	1.0	36.3 ± 0.1 cde	100.0 ± 0.0 a	8.8 ± 0.4 g	25.0 ± 0.1 cde	–
		–	2.0	43.5 ± 0.2 bc	100.0 ± 0.0 a	21.0 ± 0.7 c	64.8 ± 0.2 a	–
		–	4.0	37.7 ± 0.2 cde	100.0 ± 0.0 a	14.6 ± 0.8 d	46.5 ± 0.2 b	–
30	–	–	–	22.1 ± 0.1 jkl	57.8 ± 9.7 cd	3.7 ± 0.3 lm	19.1 ± 0.1 ghi	–
		0.5	–	19.0 ± 0.1 klm	40.0 ± 3.9 e	4.2 ± 0.4 klm	19.9 ± 0.1 fg	–
		1.0	–	19.8 ± 0.1 klm	66.7 ± 7.7 bc	3.8 ± 0.3 lm	14.8 ± 0.1 ijkl	–
		2.0	–	19.0 ± 0.1 klm	51.1 ± 5.9 d	4.2 ± 0.3 klm	19.2 ± 0.2 fgh	–
		4.0	–	16.0 ± 0.0 m	37.8 ± 5.9 e	5.5 ± 0.4 jk	12.8 ± 0.1 jkl	–
		–	0.5	18.3 ± 0.1 lm	93.3 ± 3.9 a	5.3 ± 0.5 jkl	14.6 ± 0.1 ijkl	+
		–	1.0	18.6 ± 0.1 lm	80.0 ± 3.9 ab	7.3 ± 0.5 ghi	13.3 ± 0.1 jkl	+
		–	2.0	18.9 ± 0.1 klm	93.3 ± 3.9 a	14.2 ± 0.7 d	12.7 ± 0.1 kl	+
		–	4.0	39.7 ± 0.2 cde	100.0 ± 0.0 a	12.2 ± 0.4 def	17.7 ± 0.1 ghij	+
	120	–	–	25.5 ± 0.1 hij	62.2 ± 5.9 cd	4.6 ± 0.2 jkl	19.5 ± 0.1 fg	–
		0.5	–	23.0 ± 0.1 ijk	88.9 ± 2.2 a	7.5 ± 0.5 ghi	17.0 ± 0.1 ghijk	–
		1.0	–	24.6 ± 0.1 ij	93.3 ± 3.9 a	7.1 ± 0.4 ghi	13.5 ± 0.1 jkl	–
		2.0	–	26.5 ± 0.1 ghi	100.0 ± 0.0 a	12.4 ± 0.4 de	16.8 ± 0.1 hijkl	–
		4.0	–	22.0 ± 0.1 jkl	86.7 ± 3.9 a	13.6 ± 0.5 d	12.5 ± 0.1 l	–
		–	0.5	26.3 ± 0.1 ghi	100.0 ± 0.0 a	8.4 ± 0.3 gh	29.4 ± 0.1 c	–
		–	1.0	51.6 ± 0.1 a	100.0 ± 0.0 a	24.2 ± 1.0 b	41.0 ± 0.1 b	–
		–	2.0	34.9 ± 0.2 de	100.0 ± 0.0 a	13.7 ± 0.4 d	30.4 ± 0.1 c	–
		–	4.0	48.3 ± 0.2 ab	100.0 ± 0.0 a	29.7 ± 1.2 a	46.2 ± 0.2 b	–

In each column, mean ± SE followed by the same letter was not significantly different ($p = 0.05$) according to Student–Newman–Keuls test

[0.65 % (w/v) agar]. They were autoclaved at 121 °C at 1.04 kg cm^{-2} for 15 min. All the cultures were incubated in a growth room under approximately 25 °C in a cool white fluorescent light (50 μmol $m^{-2}s^{-1}$) and 16-h photoperiod for 45 days.

Screening of meristematic nodule clusters

The meristematic nodule clusters were identified and photographed with a digital camera (Olympus E-330, Olympus Corp. Japan) fitted onto an Olympus compact

stereo microscope (Olympus SZX7 with SZX-LGB DF PLAPO observation tube, Olympus Corp. Japan).

Acclimatization

Plantlets, 3–5-cm long with well-developed roots, were removed from the culture vessels, and the roots were washed with water to remove agar medium residues. The plantlets were transferred to 5 cm in diameter pots containing a 1:3 peat:loam mixture. They were covered with perforated transparent bags to prevent moisture loss and kept in a chamber at approximately 25 °C, 70 % humidity, 50 µmol $m^{-2}s^{-1}$ irradiance (cool white fluorescent light) and 16-h photoperiod. The potted plants were ventilated for 10–15 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 diluted (1:10) WPM salts supplemented with 0.5 mg/L BAP which did not contain sucrose during 4 weeks.

Statistical analysis

The data were recorded 45 days after culture initiation. The experiments were set up in a completely randomized design, and all factors/treatments were replicated three times. For the bud culture experiments (Table 1), each replicate comprised of ten explants. For the culture vessel and explant types (Table 2) and shoot elongation and rooting (Table 3) experiments, each replicate comprised of 15 explants. All data were analyzed using standard ANOVA procedures. The significant differences among the mean values were compared by the Student–Newman–Keuls (SNK) test at $P = 0.05$ using SPSS Version 16.0 (SPSS Inc., Chicago, USA).

Results

Establishment of primordial shoot culture

The effect of two medium formulations and PGRs on shoot regeneration from axillary primordial shoots collected from suckers of mature *L. orientalis* trees was examined (Table 1). High frequencies of shoot regeneration (73–100 %) occurred on both media types (Fig. 1c–f). The regeneration frequency reached 100 % explants (primordial shoots) on the MS supplemented with 2 mg/L IBA and on WPM containing BAP + IBA combinations at the concentrations of 0.5 + 0.5, 1.0 + 1.0, 2.0 + 2.0 mg/L, respectively. The number of shoots per explant showed a significant difference when cultured on WPM containing BAP + IBA combinations compared with the other media. The highest number of shoots (14.5 shoots per explant) was obtained on WPM supplemented with 1.0 mg/L BAP + 1.0 mg/L IBA (Table 1).

The highest mean shoot lengths per explant (17.8 mm) was observed on MS medium containing 2.0 mg/L IBA. Although shoot development occurred on all media, necrosis started in shoot tips cultured on MS medium 3 weeks after the beginning of the culture and reached 46.7 % on the MS medium supplemented with 2.0 mg/L BAP or 2.0 mg/L IBA.

Based on our results, WPM containing 1.0 mg/L BAP + 1.0 mg/L IBA which supported shoot regeneration in 100 % of explants, the highest number of shoots per explants and no shoot tip necrosis was selected as SPM.

Multiplication of shoots

Developing shoots from the primordial shoot explants were transferred to a fresh SPM at 4-week intervals. Although multiple shoot regeneration was observed in the first culture, this high performance did not continue in subsequent subcultures. Therefore, the shoots were adapted to in vitro conditions by subculturing every month in SPM for 18 months. The number of shoots per explant did not exceed 1 in the first 6 subcultures. The number of shoots per explant slowly increased and reached approximately 2.5 at the end of 9 months; approximately 6.5 at the end of 12 months, and 11.5 at the end of 18 months (data not shown).

In the present study, globular, light green and compact nodular clusters began to develop from the cut end of shoots 3 weeks after approximately 12 monthly subcultures (Fig. 2a). These nodular structures showed a meristematic character 4–5 weeks after developing (Fig. 2b) and multiple shoots emerged from these structures (Fig. 2c–e). In our study, both the nodules and the shoots were propagated on SPM.

Culture vessel and explant types

Among the four culture vessels (GT, 210-GCJ, 400-GCJ and Vitro Vent®) and two different explant types (single shoots and shoot clusters consist of three shoots), the highest number of shoots per explant (40.5 shoots) was observed in shoot clusters cultured in Vitro Vent® vessels (Table 2). Shoot clusters were more productive than single shoots in terms of shoot propagation (Table 2). As for the general effect of culture vessels on shoot propagation, Vitro Vent® was superior to the other culture vessels (Table 2). Furthermore, the leaves of the shoots grown in Vitro Vent® vessels showed better development than the shoots grown in other vessels. Shoot tip necrosis only was observed in the shoots grown in 210-GCJs (data not

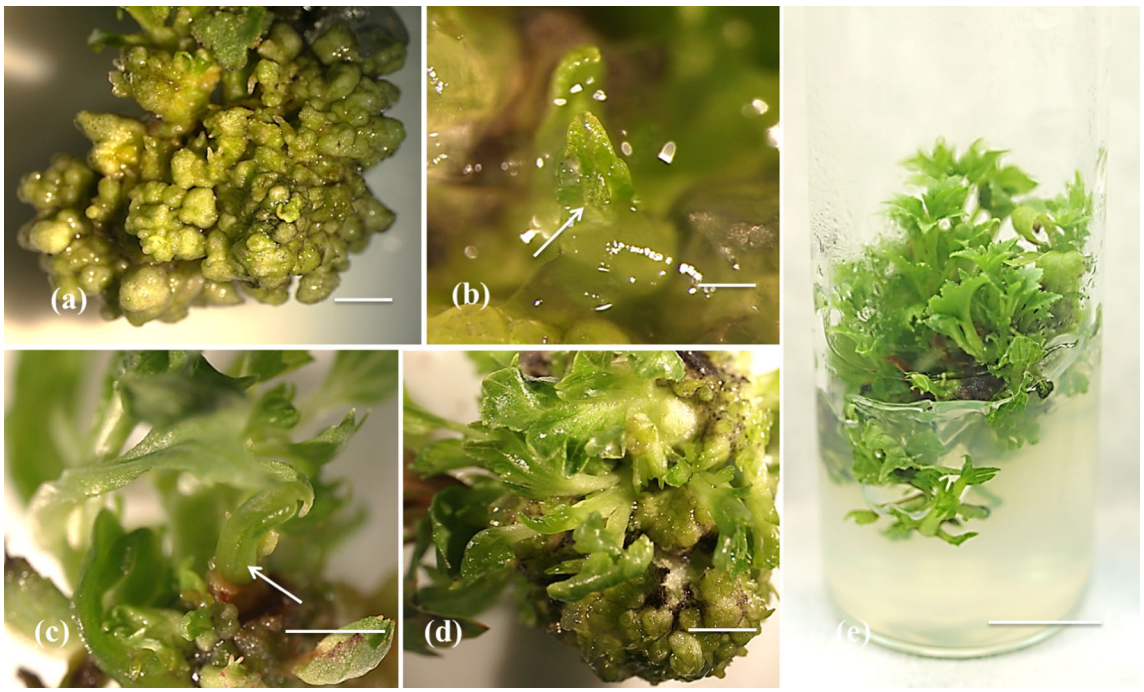

Fig. 2 *Liquidambar orientalis* meristematic nodule culture and multiple adventitious shoot regeneration on WPM containing 1 mg/L BAP and 1.0 mg/L IBA: **a** yellowish nodule clusters induced from the basal part of shoot or shoot clusters after 3 weeks of the culture (*bar* 2.0 mm); **b** shoot meristems arising from a nodule (see *arrows*, *bar* 700 μm); **c** shoot initiation from nodule after 4 weeks of culture (see *arrow*, *bar* 2.0 mm); **d** multiple adventitious shoots regeneration from nodules after 6 weeks of the culture (*bar* 2.0 mm); **e** adventitious shoot mass production from nodules or shoots after 8 weeks of the culture (*bar* 1.0 cm)

shown). Explant type and culture vessel were observed to have no effect on shoot length. The mean shoot lengths ranged between 10 and 18 mm without significant differences among treatments.

Shoot elongation and rooting

Single shoots of approximately 1.5 cm in length were separated from shoot clusters and cultured on rooting media (Table 3). Although the shoots cultured on the media containing 15 g/L sucrose were generally longer than those containing 30 g/L sucrose, the longest shoots (51.6 mm) were obtained on WPM supplemented with 30 g/L sucrose, 120 mg/L Fe-EDDHA and 1.0 mg/L IBA. It was clear that Fe-EDDHA positively affected the mean shoot length (Table 3). The first root formation occurred after 11 days on rooting medium. High frequencies of rooting were observed (Fig. 3a, b; Table 3). The root regeneration rate in the media containing 120 mg/L Fe-EDDHA was higher at both sucrose concentrations. The highest frequencies of rooting (100 %) were observed on WPM containing 15 g/L sucrose, 120 mg/L Fe-EDDHA either with IAA or IBA (Table 3).

The number of roots in the medium containing 30 g/L sucrose was generally higher than in the medium containing 15 g/L sucrose (Table 3). Adding 120 mg/L Fe-EDDHA to the media increased the number of roots. In all the media containing IAA, the roots developed directly from the basal parts of shoots. However, a meristematic nodule developed roots from the basal parts of the shoots on medium containing IBA with no Fe-EDDHA. Adding Fe-EDDHA to the media containing IBA prevented the formation of meristematic nodules. The highest number of roots (29.7) was obtained on WPM supplemented with 30 g/L sucrose, 120 mg/L Fe-EDDHA and 4.0 mg/L IBA, while the longest root length was observed on WPM containing 15 g/L sucrose, 120 mg/L Fe-EDDHA and 2.0 mg/L IBA. In shoots with the highest number of meristematic nodules, the root length was generally lower than the shoots that formed direct roots.

Acclimatization

Three- to five-cm-long plantlets that were rooted in vitro were transferred to soil and were acclimatized over a period of 3 months (Fig. 3c). Forty-five acclimatized plants were transferred to field conditions at the end of 3 months (Fig. 3d).

Discussion

Several micropropagation studies have been described in *Liquidambar* species. In a study performed by Ďurkovič

Fig. 3 **a**, **b** Rooted plantlets of *L. orientalis* on WPM containing 30 g/L sucrose, 120 mg/L Fe-EDDHA and 4.0 mg/L IBA after 45 days of the culture (*bars* 2.0 cm); **c** acclimatized plantlets (80 days) (*bar* 2.0 cm); **d** hardened plants in the garden of Ege Forestry Research Institute (*bar* 10.0 cm)

and Lux (2010) on *L. styraciflua*, shoot tips were cultured on WPM containing BAP or BAP + IBA combinations, the number of shoots did not significantly vary when IBA was added to the medium. Their opposite results might be due to the high concentrations of IBA (0.5, 1.0 or 2.0 mg/L) used in our study with *L. orientalis* which were much higher, 50–200-fold, than the one used by Ďurkovič and Lux (2010). Unlike Ďurkovič and Lux (2010), Sutter and Barker (1985) obtained higher number of shoots per explant with WPM fortified with 0.2 mg/L BA and 0.05 mg/L naphthaleneacetic acid (NAA) in *L. styraciflua*. The positive effect of the BAP + IBA combination at different concentrations on shoot propagation has been observed in many woody plant species, for example, in *Amygdalus communis* L. (0.1 mg/L IBA + 1.0 mg/L BAP) (Gürel and Gülşen 1998), *Jatropha curcas* L. (13.32 µM BAP + 4.92 µM IBA) (Imtiaz et al. 2014), and *Hagenia abyssinica* (4.4 µM BAP + 0.49 µM IBA) (Feyissa et al. 2005).

In our study, shoot tip necrosis was observed in shoots developed from explants on MS medium, which was subsequently deemed unsuitable. Shoot tip necrosis in vitro condition has been attributed to salt strength of different media. There is a general agreement that the high salt concentration of MS medium can cause shoot tip necrosis. However, shoot tip necrosis has been overcome in some studies using a medium with low salt concentration or by reducing the concentrations of some compounds in a medium (Bairu et al. 2009).

In this study on *L. orientalis*, globular, light green and compact nodular clusters formed from the cut end of shoots after approximately 12 monthly subcultures. Nodules are defined as cell clusters that show a consistent internal cell and tissue differentiation pattern (McCown et al. 1988; Teng 1997). Nodule cultures display a developmental pathway in parallel to the embryogenic developmental pathway (McCown et al. 1988; Trindade and Pais 2003). It has been reported that the nodular structures of poplar and *Sansevieria cylindrica* plants had vascular elements (McCown et al. 1988; Shahzad et al. 2009). Furthermore, two cell types (nodular meristematic cells and plastid-dense parenchyma) and two cell layers (epidermal and internal cortex/vascular) were distinguished in poplar. In previous studies, these meristematic masses were termed *meristematic nodules* (Aitken-Christie 1988). Although nodular structures have been randomly observed in many

plants, they are mostly seen in cultures of woody species (McCown et al. 1988) such as observed in this study. Nodular structures can occur from callus, for example in *L. styraciflua* (Kim et al. 1999), or directly from explants, like in *Eucalyptus globulus* (seeds, cotyledons or hypocotyls and juvenile leaves) (Trindade and Pais 2003). A great number of small compact daughter nodules can be produced from the nodules, which tend to develop into shoots. These characteristics provide an attractive alternative to existing micropropagation techniques and mass production can be realized using these cultures (McCown et al. 1988; Kongbangkerd et al. 2005). In this study, both the meristematic nodules and shoots were propagated on SPM.

In the present study, shoot clusters were more productive than single shoots in terms of shoot propagation. A similar result was observed by Aggarwal et al. (2012). They obtained a greater number of shoots in *Eucalyptus tereticornis* Sm. when larger shoot clumps (15–20 shoots) were cultured, compared with smaller shoot clumps (4–5 shoots). While no rooting occurred in single shoots in *Bambusa balcooa* Roxb., rooting frequency, number of roots and root length were found to be better in clusters with 5–8 shoots than in the clusters with 3–4 shoots (Negi and Saxena 2011). It is suggested that there is a synergistic effect among the shoots within the shoot clusters that results in a greater proliferation compared with single shoots in culture.

In vitro growth can be affected by factors such as culture vessel types, headspace, permeability of the caps, and number of explants per culture vessel (Islam et al. 2005). Not only shoot propagation, but also the leaves of the shoots grown in Vitro Vent® vessels showed better development than the shoots grown in other culture vessels. This is in accordance with the results found by McClelland and Smith (1990), who reported that the leaf size of five woody plant species increased as the volume of the culture vessel increased.

Shoot tip necrosis of *L. orientalis* was observed in the shoots grown in 210-GCJs (data not shown). The gaseous atmosphere in vitro, and thus the quality of micropropagated plants can be significantly affected by the types of caps used in culture vessels (Lentini et al. 1988; McClelland and Smith 1990). Sufficient ventilation of the culture vessels used for micropropagation depends on their type of closure. The closures that permit gaseous exchange prevent ethylene accumulation and provide CO_2 for assimilation. These conditions enhance photosynthetic capacity, the propagation and survival rate of the plants during acclimatization. Increased losses occur during acclimatization due to high humidity in culture vessels with no gaseous exchange. Conversely, water loss occurs in ventilated vessels and acclimatization is more successful due to the decreased relative humidity within the vessel (Hazarika 2006; Mohamed and Alsadon 2010). 210-GCJ and 400-GCJ were tightly capped vessels and thus did not have good gaseous exchange. While the medium used per explant was standardized, the air ratio to volume per explant differed due to varying culture vessel volumes. The shoot tip necrosis observed in cultures grown in 210-GCJs vessels might be due to the accumulation of harmful gases such as ethylene. Since 400-GCJs have a larger volume, these harmful gases would not have reached higher concentration as in 210-GCJs. As for the GTs, due to a longer distance between the developed shoot and the cap, harmful gases accumulated on top, and this was probably the reason why plant growth was not affected. In addition, GTs permit ventilation as they have loose caps. Vitro Vent® culture vessels are closed with specially designed filtered caps allowing the constant gas exchange.

Erdağ and Emek (2005) obtained the highest number of shoots (20 shoots) in WPM containing 0.54 μM NAA and 11.1 μM BAP in leaves they collected from in vitro shoots of *L. orientalis*. In the present study, the number of shoots approximately doubled (40.5 shoots) by applying various strategies such as different explant type, culture vessels and PGRs.

The use of Fe-EDDHA in the media was reported to increase in vitro rooting, somatic embryogenesis, and the multiplication of axillary shoots (Christensen et al. 2008; Trejgell et al. 2012). The results of the present study were in accordance with the previously published data; adding 120 mg/L Fe-EDDHA to a medium not only had a positive effect on rooting percentage, but also on number and length of roots. Some researchers have reported that peroxidase has an important role in the regulation of auxin content during rooting (Garcia-Gómez et al. 1995; Molassiotis et al. 2004). Molassiotis et al. (2004) reported the variations in peroxidases and catalase activities during in vitro rooting in GF-677 (*Prunus amygdalus* × *P. persica*) and determined two isoenzymes of peroxidase in non-rooted stems (treated with Fe-EDTA) and four isoenzymes of peroxidase in rooted stems. Therefore, it is inferred that Fe-EDDHA provides or increases rooting by affecting peroxidase activity and thus auxin regulation during rooting.

In a previous study on *L. orientalis*, 83 % rooting was obtained from in vitro shoots cultured on WPM supplemented with 30 g/L sucrose and 9.8 μM IBA (Erdağ and Emek 2005). In the present study, the percentage of rooting was 93 % in the same medium. In a study on *L. formosana*, 100 % of shoots rooted on WPM containing 9.8 μM IBA (Xu et al. 2007). Shoots of *L. styraciflua* rooted on WPM containing 0.5–1.0 mg/L IBA (Sutter and Barker 1985).

One of the most important stages of micropropagation is rooting of in vitro shoots and transfer of plants to external conditions. Plant acclimatization after rooting was largely successful in the present study, compared with 80 % that

has been reported by Erdağ and Emek (2005). In another study on *L. orientalis*, due to the poor root development, the plantlets survived only for 1 month (Genç 1999).

Conclusions

In the present study, an efficient in vitro propagation method was developed from axillary buds that were collected from suckers of five mature trees of the *L. orientalis* which is a relict endemic species with a narrowed and damaged biotope. In vitro-regenerated plantlets were acclimatized in a field. In summary, an efficient production procedure was developed that will be utilized commercially in the plant tissue culture industry. This study can contribute to sustainability of the *L. orientalis* ecosystem, and its genetic heritage future for generations.

Author contribution statement Dr. Meltem Bayraktar made all experiments, statistical analysis and tables and wrote this paper. Dr. Sadiye Hayta established initial experiments, reviewed and edited manuscript. Dr. Salih Parlak collected the plant material. Prof. Dr. Aynur Gurel supervised the investigation and edited and reviewed this manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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