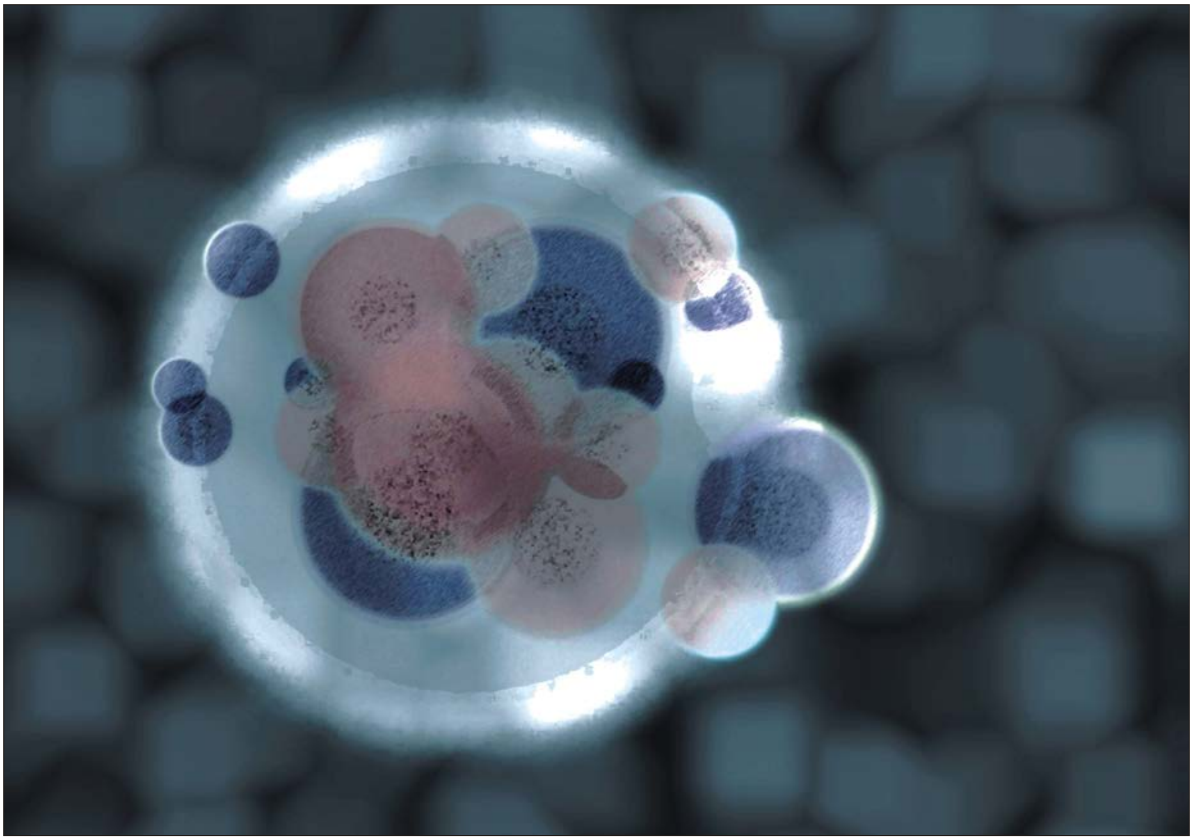

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In vitro Adventitious Shoot Regeneration of *Liquidambar orientalis* Miller

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Abstract: *In vitro* adventitious shoot regeneration of *Liquidambar orientalis* Miller (Anatolian sweet gum) endemic to Turkey was studied using leaf explants to provide an efficient alternative propagation method. Sterile leaf explants, harvested from shoot proliferating cultures, were placed on solidified Woody Plant Medium (WPM) supplemented with 0 or 0.54 μM α-naphthaleneacetic acid (NAA) and various concentrations of 6-benzylaminopurine (BAP). Shoot organogenesis occurred directly, without a distinct callus stage and well-defined shoots were visible between 6th and 9th weeks. The highest shoot numbers were achieved on WPM supplemented with 0.54 μM NAA and 11.1 μM BAP (19.97 shoot/explant). Shoots were elongated on WPM with 3.3 μM BAP. Excised shoots (2-3 cm) were rooted on the WPM with addition of 9.8 μM indole-3-butyric acid (IBA) (83.3%). About 80% of the rooted shoots were established in pots after hardening.

Key words: Adventitious shoot formation, endemic plant, leaf explants, *Liquidambar orientalis* (Anatolian sweet gum)

INTRODUCTION

Liquidambar orientalis Miller (Anatolian sweet gum), an arcto-tertiary relic of Turkish flora forms riverine forests only in southwest of Turkey. The genus *Liquidambar* have only 4 species (including *L. orientalis)* such as *L. styraciflua* L. (in North and Middle America), *L. formosana* Hence (China and Taiwan), *L. altingia* Blume. (in Southeast Asia) around the world. The present distribution of *Liquidambar orientalis* is restricted to southwest of Anatolia and Rhodes (in small stands). The Anatolian sweet gum was abundant on mid-europe before tertiary glaciations. Presently, southwest Anatolia forms a natural refuge for the species.

Among the woody species *Liquidambar orientalis* has priority in Turkey's National Conservation Programme[1]. Conservation programs for the Anatolian sweet gum depends mainly on *in situ* approaches. Various strategies of *ex situ* preservation are available for the conservation programs of endemic or threatened plant species[2]. *In vitro* culture is an efficient method for *ex situ* conservation of plant bio-diversity[3,4]. Many endangered species can be quickly propagated and preserved from a minimum of plant material, rendering low impact on wild populations by using this technology[5]. In many studies on shoot formation from leaves of woody plants show that a separate callus phase occurs before shoot formation[6-9]. As known well, relatively long callus phase cause partial loss of genetic stability and unexpected variations.

For this reason, a propagation protocol without a callus phase is required to obtain a genetically stable resource for further steps of conservation studies using plant tissue culture techniques.

To our knowledge, only one study has been reported to date regarding micropropagation of *Liquidambar orientalis* in which only buds and shoot tips were used as explants[10].

This study describes adventitious shoot formation from leaf tissues of *Liquidambar orientalis* without the formation of a distinct callus stage and aims to contribute to the present knowledge on conservation methods for endemic plant species.

MATERIALS AND METHODS

All explant tissues were harvested aseptically from shoot proliferating cultures initiated from lateral buds of 25-30 year-old trees.

Shoot proliferating cultures were maintained on Woody Plant Medium[11] salts and vitamins supplemented with 3.3 μM 6-benzylaminopurine (BAP), 30 g L^{-1} sucrose and 8 g L^{-1} agar (Agar-agar). pH was adjusted to 5.6 prior to autoclaving.

In organogenesis experiments, only expanding or recently expanded leaves were harvested. The distal ends of palmate leaves were excised and removed, then remaining laminas were placed abaxial side down on culture medium. Margins of explants were sunken 1-2 mm into the culture medium to provide contact with the

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medium (WPM), which was supplemented with 0 or 0.54 µM NAA and various concentrations of BAP, 30 g L^{-1} sucrose, 8 g L^{-1} agar (pH 5.6).

All culture media were sterilized in a autoclave at 121°C for 20 min. Culture vessels were 100 mL erlenmeyer, containing 25 mL medium and sealed with four layers of aluminum foil. The culture environment for all experiments was maintained at 24±2°C with illumination provided by cool white fluorescent lamps at 40 µ E m^{-2} s^{-1} with a 16 h light period. All cultures were transferred on to fresh medium at 3-week intervals.

The experiments were set up as a Completely Randomized Design. Each replicate contained 10 experimental units (3 explant/erlenmeyer). Each replicate was repeated at least three times. The data on number of shoots were subjected to ANOVA and means were compared using Duncan's Multiple Range Test at the 5% level of probability.

For shoot elongation, shoot clumps with thiny shoots (0.5-1 cm) were transferred on to WPM supplemented with 3.3 µM BAP, 30 g L^{-1} sucrose and 8 g L^{-1} agar (pH 5.6).

Elongated shoots in 2-3 cm height were excised and cultured for rooting on WPM supplemented with IBA (3.7 or 9.8 µM), 30 g L^{-1} sucrose and 6 g L^{-1} agar. Each shoot was transferred to test tubes (25x150 mm) containing 10-12 mL of medium. The test tubes were sealed with their original lids. Environmental conditions were the same as shoot organogenesis experiments. Rooting experiments were tested with at least 12 shoots and all experiments were repeated three times.

After 9 weeks of rooting *in vitro*, the plantlets were removed from culture tubes, washed thoroughly with sterile distilled water and transplanted in 10 cm diameter plastic pots containing autoclaved sand: Perlite (3:1 v/v) mixture. To maintain initially the plantlets at high humidity, pots were covered with clear plastic bags. Meanwhile, the plantlets were kept in culture room and watered with WPM salts for 2 weeks. Plantlets were acclimatized to a reduced relative humidity by gradually opening the plastic cover. After two weeks the plantlets were completely uncovered and transferred in plastic pots containing soil:sand (1:1 v/v) mixture.

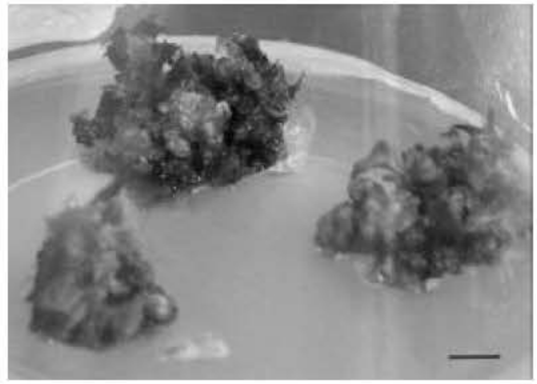

Fig. 1: Shoots and shoot buds in greenish-white colour after four weeks of incubation in the WPM containing 0.54 µM NAA and 11.1 µM BAP. Scale bar: 1 cm

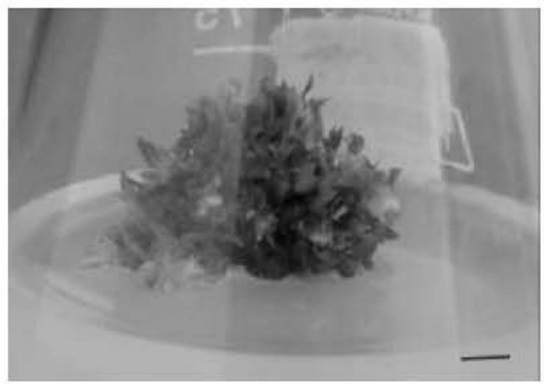

Fig. 2: Adventitious shoots from leaf explants after nine weeks in the WPM containing 0.54 µM NAA and 11.1 µM BAP. Scale bar: 0.5 cm

Table 1: Effect of NAA and BAP on shoot regeneration from leaf explants of *Liquidambar orientalis* Miller (only shoots greater than 5 mm in length were counted after 9 weeks of incubation)

NAA (µM)	BAP (µM)	No. of shoots/explant
0	2.2	1.20e
0	4.4	4.57d
0	11.1	11.43b
0	22.2	7.53c
0.54	2.2	1.80e
0.54	4.4	6.97c
0.54	11.1	19.97a
0.54	22.2	8.00c

* Values followed by the same letter(s) within columns are not significantly different at the 0.05 level (Duncan's Multiple Range Test)

RESULTS AND DISCUSSION

To obtain direct shoot organogenesis from leaf explants (with no distinct callus stage) of Anatolian sweet gum, 0 or 0.54 µM α-naphthaleneacetic acid (NAA) and various concentrations of 6-benzylaminopurine (BAP) were experimented. Leaf explants, obtained from shoot proliferating culture were excised at their distal ends and the remaining laminas were placed onto medium with their abaxial side down. Two weeks after the culture initiation, shoot buds in greenish-white colour were observed at the margins and also on lamina in the media containing growth regulators. Shoot buds transformed to shoots at the 4th week of the culture initiation (Fig. 1).

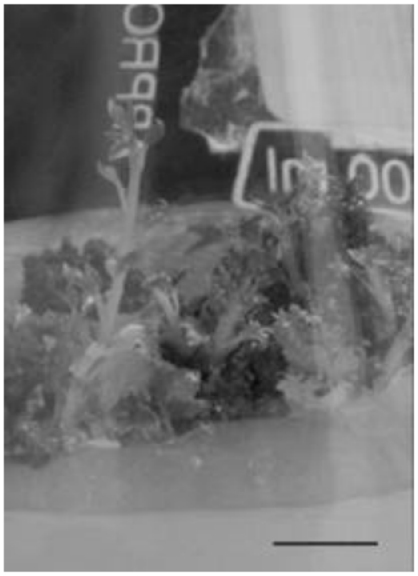

Fig. 3: Elongated shoots in the shoot proliferating medium. Scale bar: 1 cm

Fig. 4: An acclimatized plantlet

No organogenic response was obtained from leaf explants cultured in the medium without growth regulators. An increase in the number of shoots per explant was observed parallel to the increase in BAP concentration of the medium up to 11.1 µM. However, the continued increase in the BAP concentration to 22.2 µM caused a decrease in the number of shoots per explant (Table 1). Similar results were reported for *Liquidambar styraciflua* L.[12].

There was no significant increase in the number of shoots per explant when 0.54 µM of NAA was added to the media containing 2.22 µM BAP and 22.2 µM BAP. However, significant increases were observed with the addition of NAA 0.54 µM to the media containing 4.4 µM BAP and 11.1 µM BAP. These results can be attributed to the synergestic effect of BAP and auxins as reported by Khan *et al.*[13] for an endemic woody plant *Syzygium alternifolium* (Wight) Walp.

In conclusion, the highest number of shoots was obtained on medium containing 0.54 µM NAA and 11.1 µM BAP (Fig. 2). Therefore, the use of NAA and BAP at these concentrations is recommended for the direct shoot organogenesis from leaf explants of *Liquidambar orientalis*.

For shoot elongation, shoot clumps with thiny shoots (0.5-1 cm) were transferred to the shoot proliferating medium. Some of the shoots, which were developed in this medium, were taken for rooting experiments while the remaining shoots were used for plantlet regeneration. Elongated shoots (longer than 2-3 cm) (Fig. 3) were excised and transferred to WPM supplemented with 3.7 or 9.8 µM IBA, 30 g L^{-1} sucrose and 6 g L^{-1} agar for rooting (pH 5.6). The shoots of *Liquidambar orientalis* rooted on medium containing 3.3 µM IBA with a very low percentage (32%). This value is significantly lower in comparison with *Liquidambar styraciflua*[14]. Furthermore, the roots were very thin and weakly developed. When 9.8 µM IBA instead of 3.7 µM IBA concentrations were used, the percentage of rooted shoots increased (83.3%). The roots were morphologically healthy in terms of their length, branching and also colour. Fossard and Bourne[15] emphasized that similar auxin concentration (9.8 µM) were for rooting *Eucalyptus ficifolia* F. Muell. shoots.

The plantlets with well developed root were transferred *ex vitro* conditions described as previously. Percentage of survival of shoots was approximately 80%. All plantlets developed into normal plants (Fig. 4).

The present paper reports for the first time *in vitro* differentiation and whole plant regeneration of *Liquidambar orientalis* Miller by using leaf explants without any distinct callus stage providing a reduced risk of genetic variation. The outlined procedure also offers a potential system for rapid clonal propagation and conservation of this arcto-tertiary relic endemic.

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