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ADVENTITIOUS SHOOT FORMATION FROM HYPOCOTYL AND COTYLEDON EXPLANTS OF RELICT ENDEMIC LIQUIDAMBAR ORIENTALIS Miller

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Abstract

In this study, the effect of Plant Growth Regulators (PGRs) [6-Benzylaminopurine (BAP) (0, 1.0, 2.0, 3.0 mg L^{-1}) alone and BAP in combinations with α-Naphthaleneacetic acid (NAA) (0, 0.05, 0.10, 0.15 mg L^{-1}) or Indole-3-butyric acid (IBA) (0, 0.01, 0.05, 0.10 mg L^{-1})] on direct organogenesis from hypocotyl and cotyledon explants of 4-5-week-old sterile seedlings of Liquidambar orientalis (Oriental sweetgum) were investigated. For organogenesis, the addition of BAP to the medium was required, also the combinations of BAP with NAA responded better in terms of the percentage of shoot-forming explants, the number of shoots per explant and the growth of shoots as compared to combinations of BAP with IBA. The highest average percentage of shoot formation (76.64%) was obtained from hypocotyl explants cultured on Woody Plant Medium (WPM) containing 0.10 mg L^{-1} NAA + 3.0 mg L^{-1} BAP. The highest average shoot number per explant (32.20 shoots/explant) was observed in also hypocotyl explants cultured on WPM supplemented with 0.10 mg L^{-1} IBA + 1.00 mg L^{-1} BAP. The combination of 0.05 mg L^{-1} NAA + 1.0 or 2.0 mg L^{-1} BAP was found to be appropriate for both explants.

Keywords: Direct organogenesis, *Liquidambar orientalis,* Oriental sweetgum, Plant Growth Regulators (PGRs)

RELİKT ENDEMİK *LIQUIDAMBAR ORIENTALIS* (Miller)'İN HİPOKOTİL VE KOTİLEDON EKSPLANTLARINDAN ADVENTİF SÜRGÜN OLUŞUMU

Öz

Bu çalışmada, Bitki Büyüme Düzenleyicileri (BBD)'nin [6-Benzil Amino Pürin (BAP) (0, 1.0, 2.0, 3.0 mg L^{-1}) tek başına ve BAP'ın Naftalen Asetik Asit (NAA) (0, 0.05, 0.10, 0.15 mg L^{-1}) veya İndol Bütirik Asit (IBA) (0, 0.01, 0.05, 0.10 mg L^{-1}) ile kombinasyonları] Liquidambar orientalis (Anadolu sığlası)'in 4-5 haftalık steril fidelerinin hipokotil ve kotiledon eksplantlarından direkt organogenez üzerine etkisi araştırılmıştır. Organogenez için ortama BAP ilavesi gerekli bulunmuş ayrıca, BAP'ın NAA ile kombinasyonları IBA ile kombinasyonlarına göre, gerek sürgün oluşturan eksplant yüzdesi gerekse eksplant başına sürgün sayısı ve sürgünlerin gelişimi bakımından daha iyi sonuç vermiştir. Ortalama en yüksek sürgün oluşturan eksplant yüzdesi (%76.64), 0.10 mg L^{-1} NAA + 3.0 mg L^{-1} BAP içeren Woody Plant Medium (WPM)'da kültüre alınan hipokotil eksplantlarından elde edilmiştir. Eksplant başına ortalama en yüksek sürgün sayısı (32.20 sürgün/eksplant), 0.10 mg L^{-1} IBA + 1.0 mg L^{-1} BAP eklenmiş WPM besin ortamında kültüre alınan hipokotil eksplantlarında gözlenmiştir. Her iki eksplant için de 0.05 mg L^{-1} NAA'in 1.0 veya 2.0 mg L^{-1} BAP ile kombinasyonu uygun bulunmuştur.

Anahtar kelimeler: Direkt organogenez, *liquidambar orientalis*, anadolu sığlası, bitki büyüme düzenleyicileri (BBD)

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1. Introduction

The *Liquidambar* genus which was in the Hamamelidaceae family in previous years has been incorporated in Altingiaceae family of the Saxifragales' ordo by plant taxonomists as a result of recent studies (according to the Angiosperm Phylogeny Group III system-APG III). The spread area of *Liquidambar orientalis* Miller (Oriental sweetgum) which is one of four different species of *Liquidambar* genus is in the South-west of Anatolia (Turkey) and on the island of Rhodes

(Greece) [1, 2]. *Liquidambar orientalis* (syn. *Liquidambar imberbe*) is a relict endemic taxon belonging to geological tertiary period as an element of the Eastern Mediterranean [2].

Briefly, *Liquidambar orientalis* is important for being an endemic species, and also for the oil (styrax) that comes to the end of the damage of the plant stem. However, stem damage applied to obtain sweetgum oil causes the destruction of the tree. Moreover, because of the reasons such as urbanization and/or lands opened for agriculture, sweetgum forest areas have been disappearing [3]. *L. orientalis* forests are ecologically and economically important [2]. For these reasons, Oriental sweetgum has been accepted as a species that will be protected throughout Europe after it was incorporated in the group of Noble Hardwoods in 2001 by EUFORGEN (European Forest Genetic Resources Program) [4].

The production of Oriental sweetgum trees and expanding the forest areas are very important. The propagation of this plant is usually via seed [5]. However, it has been stated that it is possible to produce it with softwood cuttings [2] and production by grafting [6]. It is also stated that it can be produced with tumor-like formations (1-3 cm in diameter) seen in tree stems and this subject should be researched in detail [6]. Moreover, there are also studies on production by tissue culture. Genç (1999)'s study [7] was the first study on the production of *L. orientalis* by tissue culture and this was followed by successful studies such as in vitro shoot regeneration from leaf explants of Erdağ and Emek (2005) [8], adventitious shoot induction from the hypocotyl explants of Özmen (2011) [9], micropropagation through meristematic nodules produced by cultures of primordial shoots of Bayraktar et al. (2015) [10]. There are many studies on the production of American sweetgum (*Liquidambar styraciflua*) by tissue culture [11]. Sommer and Brown (1980) [12] established callus culture first on solid medium and then they placed this culture in liquid medium to obtain differentiated adventitious embryos. There are studies on the micropropagation of the American sweetgum (*Liquidambar styraciflua*) with hypocotyl segments [13, 14], shoot tips of one-year-old seedlings and mature trees [15], leaf and petiol segments in mature-phase plants [16, 17], somatic embryogenesis from different explanted tissues (staminate inflorescence explants) [18], embryogenic culture [19], stem segments [20] and axillary buds of 4-year-old plants [21]. These studies have proven that tissue culture techniques can be used in clonal propagation of American sweetgum.

In the present study, the effects of BAP and auxin (NAA and IBA) combinations at different concentrations on the multiple shoot production from hypocotyl and cotyledon explants of *L. orientalis* were tested.

2. Material and Methods

2.1. Obtaining Sterile Seedlings and Culture Establishment

Liquidambar orientalis Miller seeds, a relict endemic taxon, were collected from seed plantation forest in Fethiye-Muğla. The sizes of seeds are 1.77 ± 0.09 mm width and 5.05 ± 0.10 mm length and 1000-grain weigth is 2.576 ± 0.05 g [22].

The seeds were dipped into 70% ethyl alcohol for 1-minute, then 30-31% H_2O_2 for 15 minutes, and then washed in sterile distilled water 3-4 times. Surface-sterilized seeds were cultured on ½ Murashige-Skoog (MS) medium (1962) [23] without plant growth regulator to obtain sterile seedlings. Culture tubes were incubated for germination and seedling growing under photoperiod 16-hour light (the 27.5 µE/m^2/s light) / 8-hour dark at 22 ± 1 °C temperature [22]. For the purpose of micropropagation, the hypocotyl (3-5 mm) and cotyledon (2 x 5 mm) segments of 4-5-week-old sterile seedlings were used as explants. Hypocotyl and cotyledon explants were cultured on Woody Plant Medium (WPM) (Lloyd and McCown, 1980) [24] supplemented with alone 6-Benzylaminopurine (BAP) (cytokinin) (0, 1.0, 2.0, 3.0 mg L^{-1}) and combination with α-Naphthaleneacetic acid (NAA) (auxin) (0, 0.05, 0.10, 0.15 mg L^{-1}) or Indole-3-butyric acid (IBA) (auxin) (0, 0.01, 0.05, 0.10 mg L^{-1}).

30 g L^{-1} sucrose was added as a carbohydrate source, and pH of medium was adjusted to 5.8 and then this medium was solidified with agar (8 g L^{-1}). Culture vessels dispense medium and autoclaving at 121 °C, 1.2 atm. pressure for 20 min.

Cultures were incubated at 22 ± 1 °C temparature under 8-hour dark 16-hour light photoperiod of 50 µE/m^2/s during 8 weeks.

At the end of the 8 weeks, the percentages of shoot formation from explants and the number of shoots per explant were determined. The experiments were conducted in two replicates, total 28-30 explants (14-15 explants in one replicate) were cultured for each plant growth regulator concentration and combination. Shoot formation data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test ($P < 0.05$). For this purpose, the SAS Institute (1985) package software was used.

3. Results

In the present study, organogenesis was not observed in both hypocotyl and cotyledon explants on WPM medium without plant growth regulator. Adventitious shoot regeneration was accomplished in all medium which was added BAP, it was determined that alone NAA or IBA was not sufficient for direct shoot regeneration. Shoot formation was observed in both explant types on medium supplemented with alone BAP (1.0, 2.0 and 3.0 mg L^{-1} BAP) (Figures 1A and B). Increasing BAP concentration increased the percentage of shoot producing explant in both explant types (Table 1). The shoot formation started to appear after 22 days following the culturing of explants and the first one occured in

cotyledon explant in medium with 0.05 mg L^{-1} NAA + 1.0 mg L^{-1} BAP or 2.0 mg L^{-1} BAP combinations.

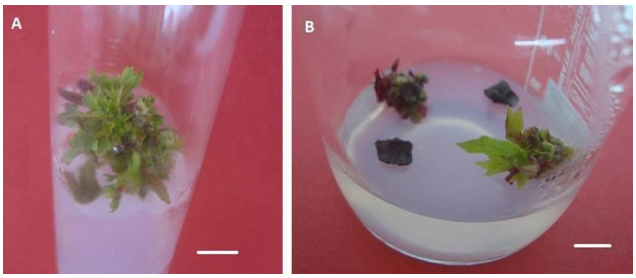

Figure 1. The shoot formations from hypocotyl (A) and cotyledon (B) explants after 8 weeks of culture on the medium added 1.0 mg L^{-1} BAP (Bar: 1 cm)

The combinations of BAP and NAA gave better results in both explant types in terms of production of direct shoot when compared to the BAP and IBA combinations. The percentage of shoot producing explant was generally over 50% in combinations BAP and NAA (Table 1). The highest percentage of shoot producing explant (76.64%) was obtained from hypocotyl explants on medium added 0.10 mg L^{-1} NAA + 3.0 mg L^{-1} BAP. However, in 0.05 mg L^{-1} NAA + 1.0 mg L^{-1} BAP medium similar percentage shoot formation was observed (75.00%) (Table 1, Figure 2A). The best shoot formation in cotyledon explants was obtained on the medium containing 0.05 mg L^{-1} NAA + 2.0 mg L^{-1} BAP (72.91%), also shoot formation on medium supplemented with 0.15 mg L^{-1} NAA + 3.0 mg L^{-1} BAP and 0.10 mg L^{-1} NAA + 2.0 or 3.0 mg L^{-1} BAP were found statistically significant (71.91%, 68.03% and 67.20%, respectively) (Table 1) (Figure 2B).

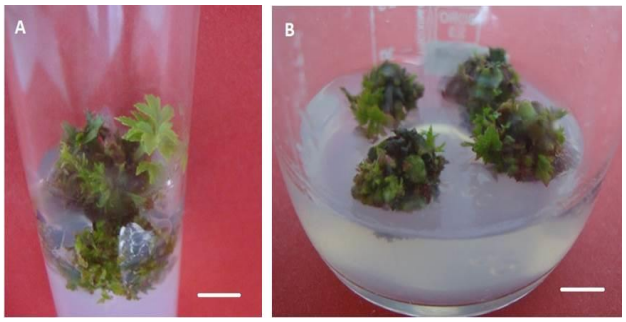

Figure 2. Direct shoot formation from explants after 8 weeks of culture on medium supplemented with BAP and NAA. (A): Shoots from hypocotyl explants on medium added 0.05 mg L^{-1} NAA + 1.0 mg L^{-1} BAP, (B): Shoots from cotyledon explants on medium added 0.10 mg L^{-1} NAA + 2 mg L^{-1} BAP (Bar: 1 cm)

Generally low percentage shoot formation was observed in BAP and IBA combinations. When BAP and IBA used together in medium, 40.00-67.56% and 7.33-32.00% shoot formation obtained from hypocotyl and cotyledon explants, respectively (Table 1).

Among the IBA and BAP combinations, the highest shoot formation percentage was determined on medium supplemented with 0.10 mg L^{-1} IBA + 2.0 mg L^{-1} BAP (32.00%) in cotyledon explants. In hypocotyl explants, 0.10 mg L^{-1} IBA + 1.0 mg L^{-1} BAP (67.56%), 0.05 mg L^{-1} IBA + 2.0 mg L^{-1} BAP (65.00%) and 0.05 mg L^{-1} IBA + 3.0 mg L^{-1} BAP (55.00%) combinations gave significantly higher shoot formation percentages when compared other BAP and IBA combinations (Table 1).

The highest average shoot number per explant was observed from hypocotyl explants when used 0.10 mg L^{-1} IBA + 1.0 mg L^{-1} BAP combination (32.20 shoots/explant) followed by 0.05 mg L^{-1} IBA + 3.0 mg L^{-1} BAP combination (31.00 shoots/explant) (Table 1). In cotyledon explants, the highest average shoot number was obtained (30.89 shoots/explant) in 0.10 mg L^{-1} NAA + 1.0 mg L^{-1} BAP combination (Figures 3A and B). The highest shoot formation was detected on the medium containing 0.10 mg L^{-1} NAA + 3.0 mg L^{-1} BAP (76.64%) where average shoot number per explant were found 19.50 from hypocotyl explant (Table 1). Shoots formed in combinations with NAA of BAP grow better than the combinations with IBA of BAP (Figures 3A, B and C).

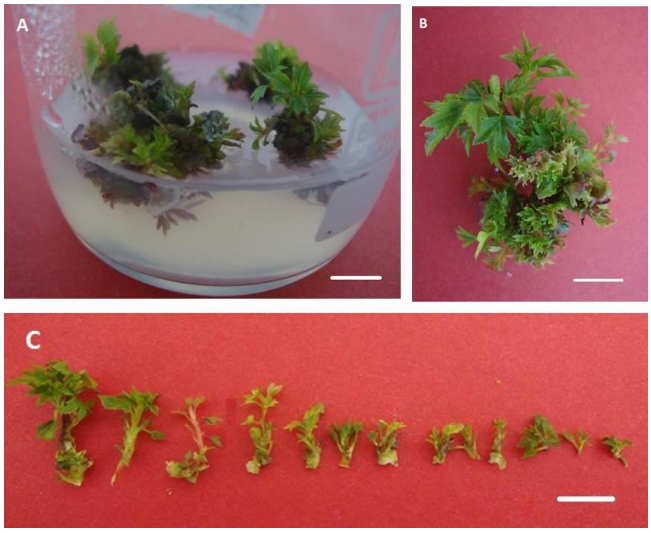

Figure 3. The shoots obtained from the cotyledon explants on medium supplemented with NAA and BAP combination in 8-week culture. (A) and (B): Shoots observed on medium added 0.10 mg L^{-1} NAA + 1.0 mg L^{-1} BAP, (C): Shoots separated from each other whose obtained on medium containing 0.15 mg L^{-1} NAA + 1.0 mg L^{-1} BAP (Bar: 1 cm)

Table 1. The effect of BAP and NAA or IBA combinations on direct organogenesis from hypocotyl and cotyledon explants of *L. orientalis* on WPM medium

PGR (mg L^{-1})			Explants			
			Hypocotyl		Cotyledon	
IBA	NAA	BAP	% Explants producing shoots ± SE	Number of shoots per explant (Mean ± SE)	% Explants producing shoots ± SE	Number of shoots per explant (Mean ± SE)
0	0	0	0	0	0	0
0	0	1	31.66±1.26 hı	19.49±2.79	7.43±1.44 f	13.33±0.33
0	0	2	34.15±2.20 h	18.33±2.29	11.00±1.00 f	9.00±1.00
0	0	3	41.63±1.62 gh	16.00±5.19	33.00±1.52 d	25.00±2.52
0	0.05	0	0	0	0	0
0	0.05	1	75.00±2.64 ab	17.23±1.66	54.07±3.15 bc	25.20±4.00
0	0.05	2	55.00±2.11 cdef	26.50±2.72	72.91±0.58 a	16.33±3.00
0	0.05	3	65.00±2.64 abcd	24.57±3.45	51.66±1.66 bc	29.25±3.90
0	0.10	0	0	0	0	0
0	0.10	1	63.26±1.90 bcde	19.60±3.98	49.00±2.23 c	30.89±3.53
0	0.10	2	65.00±2.88 abcd	22.22±3.24	68.03±0.70 a	15.90±1.35
0	0.10	3	76.64±4.40 a	19.50±0.67	67.20±3.99 a	19.75±2.22
0	0.15	0	0	0	0	0
0	0.15	1	59.00±2.65 cdef	26.33±2.64	64.72±1.21 ab	19.43±2.11
0	0.15	2	51.66±1.63 efg	29.00±3.00	53.77±0.61 bc	16.33±1.28
0	0.15	3	60.00±2.89 cdef	14.00±0.57	71.91±4.62 a	20.16±2.84
0.01	0	0	0	0	0	0
0.01	0	1	43.38±2.00 gh	26.50±3.10	15.33±0.88 ef	-
0.01	0	2	42.31±1.45 gh	13.66±1.66	8.00±1.15 f	-
0.01	0	3	42.62±1.45 gh	18.00±6.11	7.66±1.45 f	-
0.05	0	0	0	0	0	0
0.05	0	1	40.00±2.88 gh	17.66±3.48	7.83±1.48 f	-
0.05	0	2	65.00±1.78 abcd	16.33±3.93	10.66±0.66 f	-
0.05	0	3	55.00±2.88 def	31.00±7.24	11.33±1.33 f	-
0.10	0	0	0	0	0	0
0.10	0	1	67.56±1.45 abc	32.20±3.80	7.33±1.45 f	-
0.10	0	2	48.33±4.40 fg	2.40±0.74	32.00±1.15 d	-
0.10	0	3	50.00±2.88 fg	21.20±2.03	10.00±0.57 f	-

"-" shoot growing appeared very poor or did not grow during culture. Different letters are significantly different from each other at $P < 0.05$ according to Duncan's multiple range test. The data are the means ± SE.

4. Discussion

In the present study, the cotyledon and hypocotyl segments of sterile seedlings were cultured on basal WPM medium because of WPM medium's have been succesfull in various in vitro culture studies on *Liquidambar* species [10, 15] and the usage of this medium in most of sweetgum cultures [8, 16, 17, 20, 21, 25, 26]. The effects of BAP as a cytokinin alone or combinations in different concentrations with two different auxins (NAA and IBA) of BAP on direct organogenesis were determined. In both explants, organogenesis couldn't be started when plant growth regulator was absent in medium. Brand and Lineberger (1988) [16] stated that the combination of BAP + NAA was effective in maintaining high explant viability and NAA alone did not affect organogenic capacity; however, BA was necessary for shoot formation in *L. styraciflua*. Xu et al. (2007) [26] also reported that organogenesis was induced by using NAA and regeneration efficiency was increased up to 2- to 4-fold when the combination of TDZ + NAA used in *L. formosana*. Similarly, in our study, organogenesis was observed well on media containing cytokinin and auxin combination in *L. orientalis*. The highest organogenesis response

(76.64%) was in hypocotyl explants cultured on WPM supplemented with 0.10 mg L^{-1} NAA + 3.0 mg L^{-1} BAP combination and the highest average shoot number per hypocotyl explant were determined as 32.20 shoots in 0.10 mg L^{-1} IBA + 1.0 mg L^{-1} BAP combination. However, in this explant, the maximum shoot number per explant was observed as 47. In cotyledon explants, the maximum shoot number per explant was found 56 in 0.05 mg L^{-1} NAA + 1.0 mg L^{-1} BAP combination (the average shoot number per explant was 25.20 in this combination). Bayraktar et al. (2015) [10] searched the combinations of IBA and BAP on micropropagation of *L. orientalis* and found that percentage of explants (primordial shoots isolated from axillary buds) producing shoots was 100% in all combinations of BAP + IBA (0.5 + 0.5, 1.0 + 1.0, 2.0 + 2.0 mg L^{-1}). They determined the highest average shoot number as 14.5 shoots per explant on WPM medium supplemented with 1.0 mg L^{-1} IBA and 1.0 mg L^{-1} BAP. In our study, IBA was used at a lower concentration compared to Bayraktar et al. (2015) [10] study. Although the percentage of explants producing shoot obtained from our study was lower than that of these study, the average shoot number per explant obtained from our study was higher. Besides, the explants cultured were different in two studies.

Erdağ and Emek (2005) [8] cultured leaf explants of *L. orientalis* on WPM medium supplemented with 0.54 µM NAA + 11.1 µM BAP to regenerate direct shoot organogenesis and obtained the highest average shoot number (19.97 shoot/explant) in this medium. A similar result was observed in our study. We obtained 19.60, 22.22, 19.50 shoots per hypocotyl explants cultured on WPM containing 0.1 mg L^{-1} NAA combined with 1.0, 2.0, 3.0 mg L^{-1} BAP, respectively. In cotyledon explants, while similar results were obtained in medium supplemented with 2.0 and 3.0 mg L^{-1} BAP (respectively, 15.90 and 19.75 shoots/explant), the average shoot number per explant on medium supplemented with 1.0 mg L^{-1} BAP was found quite high (30.89 shoots/explant). On the other hand, Özmen (2011) [9] obtained the highest average shoot number (the average shoot number longer than 1 cm was 1.66 shoots/explant) on SH medium in cultures of LO, MS, SH and WPM media supplemented with 0.1 mg L^{-1} NAA + 2.5 mg L^{-1} BAP from hypocotyl explants of *L. orientalis*. Our study and also other in vitro culture studies of this species [8, 10] were found higher average shoot number than Özmen (2011)'s [9] study.

The best shoot number (final shoot number 8.4) was obtained from the culture of 1.0-1.5 cm shoot tips of *L. styraciflua* on WPM medium supplemented with 0.05 mg L^{-1} NAA + 1.0 mg L^{-1} BAP [15]. We found that the average shoot number was 17.23 in hypocotyl explants and 25.20 in cotyledon explants of *L. orientalis* in the same medium (WPM medium supplemented with 0.05 mg L^{-1} NAA + 1.0 mg L^{-1} BAP). In a different study in which mature-phase leaf and petiole explants of *L. styraciflua* were cultured, obtaining 7-8 shoot per explant was possible by adding 0.1 mg L^{-1} NAA + 2.5 mg L^{-1} BA on WPM medium [16]. Ďurkovič and Lux (2010) [21] obtained the highest shoot multiplication rate (5.9 shoot) per explant in *L. styraciflua* on the medium supplemented with 0.7 mg L^{-1} BAP + 0.01 mg L^{-1} IBA and also reported that IBA addition to medium did not significant effect shoot number. In our study, we obtained better results in the average shoot number (26.5 shoots/explant) from hypocotyl explants in combination with 1.0 mg L^{-1} BAP with the same IBA concentration (0.01 mg L^{-1}). Kim et al. (1997) [14] stated that the lower concentrations of TDZ induced the adventitious shoot producing from hypocotyl explants of *L. styraciflua* and they obtained the best result with the average shoot number of 6 per explant on the medium supplemented with 0.01 mg L^{-1} 2,4-D and 0.1 mg L^{-1} TDZ. Merkle et al. (1998) [18] have also suggested 0.01 or 0.1 mg L^{-1} TDZ addition for the highest induction frequency from different tissues obtaining somatic embriyogenesis. However, Özmen (2011) [9] found that adventitious shoot induction is more successful on medium (LO, MS, SH and WPM media) supplemented with BAP when compared to TDZ in *L. orientalis*. Similarly, Ďurkovič and Lux (2010) [21] stated that TDZ alone or with BAP combination does not support significantly the rate of propagation in shoot tip cultures of *L. styraciflua*.

5. Conclusion

In the present study, a successful shoot formation was achieved from hypocotyl and cotyledon explants of *L. orientalis*. The highest shoot formation (76.64%) was found in hypocotyl explants cultured on WPM containing 0.10 mg L^{-1} NAA + 3.0 mg L^{-1} BAP combination. The average shoot number increased when BAP and NAA used together and it was lower in IBA and BAP combinations. The highest average shoot number per explant (32.20 shoots/explant) was observed in hypocotyl explants cultured on WPM supplemented with 0.10 mg L^{-1} IBA + 1.0 mg L^{-1} BAP combination. However, shoots grew better in NAA + BAP combinations rather than IBA + BAP combinations.

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