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## In vitro Plantlet Regeneration from Mature Embryos of Linden (*Tilia platyphyllos* Scop.) and Multiplication of its Buds

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**Abstract:** In this study, the effects of different sucrose, BAP and Kinetin concentrations in MS medium on plantlet formation, bud formation on the plantlets and development of embryos isolated from the seeds of *Tilia platyphyllos* under in vitro conditions were investigated. In addition, the effects of different culture times on the development of embryos and plantlet formation were studied. The results indicated that the cultured embryos on the media supplemented with sucrose (10, 20, 30, 40, 50 and 60 g/l) developed plantlets with epicotyls and roots. The best results were obtained on the medium supplemented with 30 g/l of sucrose. Epicotyls of the embryos developing roots and shoots on the media with sucrose were transferred without roots to the medium of MS supplemented with BAP (0.2, 0.5, 1.0, 2.0 mg/l) or Kinetin (0.2, 0.5, 1.0, 2.0 mg/l). The average number of buds formed on the excised epicotyl explants on the media with BAP was higher than that with Kinetin. The best hypocotyl and root length growths were observed on the embryos which were cultured one month after harvesting the seeds on the medium supplemented with 30 g/l of sucrose. Some of the plantlets were then transferred into a mixture of 2 : 1 : 1 of peat, sand and perlite media, respectively, and were kept in greenhouse conditions. The results indicated that it is possible to germinate the embryos of the mature linden seeds on media supplemented with sucrose and then by using plantlet epicotyls taken into culture, multiplication of the buds can be realised.

**Key Words:** *Tilia platyphyllos*, embryo culture, mature embryo, tissue culture, plantlet regeneration

### Büyük Yapraklı İhlamur (*Tilia platyphyllos* Scop.)'un in vitro Koşullarda Embriyo Kültürü ile Bitki Regenerasyonu ve Tomurcuklarının Çoğaltılması

**Özet:** Bu çalışmada, *Tilia platyphyllos*'un olgun tohumlarından izole edilen embriyolar farklı şeker, BAP (6-benzylaminopurine) ve Kinetin dozlarının eklendiği MS ortamında kültüre alınmış ve bu ortamların in vitro koşullarda embriyo gelişimi, bitkicik oluşumu ve tomurcuk oluşumuna olan etkileri araştırılmıştır. Bununla birlikte, farklı kültür tarihlerinin, embriyoların gelişimi ve fidecik oluşumundaki etkileri de araştırılmıştır. Denemeler sonucunda, bitki büyüme düzenleyicileri bulunmayan, sadece şeker ilave edilen ortamlarda (10, 20, 30, 40, 50 ve 60 g/l) embriyolar epikotil ve kök farklılaşması gerçekleştirerek bitkicik oluşturmuştur. En etkili şeker dozu 30 g/l olarak bulunmuştur. Şekerli ortamlarda epikotil ve kök farklılaşması gösteren bitkiciklerin epikotilleri kesilerek farklı BAP (0.2-2.0 mg/l) veya Kinetin (0.2-2.0 mg/l) dozlarını içeren ortamlara aktarılmıştır. Epikotil eksplantları üzerinde tomucuk oluşumu bakımından 1.0 mg/l BAP dozu diğer dozlara göre daha etkili bulunmuştur. Tomurcuk oluşumu bakımından denenen tüm BAP dozları, Kinetin dozlarına göre daha etkili olmuştur. 30 g/l şeker eklenerek farklı tarihlerde kültüre alınan embriyolarda hipokotil ve kök uzunluğu bakımından en iyi gelişme tohumların toplanmasından yaklaşık 1 ay sonra kültüre alınan embriyolarda gerçekleşmiştir. Şekerli ortamlarda epikotil ve kök gelişimi görülen bitkiciklerin bir bölümü kültürden 4 hafta sonra 2 : 1 : 1 oranlarında karıştırılmış turba, kum perlit ortamına şaşırtılarak sera koşullarına alınmışlardır. Araştırma sonuçları, olgun ihlamur tohumu embriyolarının şekerli ortamlarda çimlendirilerek bitki elde edilebileceğini ve buradan elde edilen bitki epikotillerinin kültüre alınmasıyla, tomurcukların çoğaltılabileceğini göstermiştir.

**Anahtar Sözcükler:** *Tilia platyphyllos*, embriyo kültürü, olgun embriyo, doku kültürü, bitki regenerasyonu

### Introduction

*Tilia platyphyllos* is one of three *Tilia* species of the naturally distributed broad-leaved species in Turkey and is found as mixed stands with other tree species. *Tilia*

species' seeds germinate poorly and irregularly because of their hard seed-coats and immature embryos. Embryo culture is considered to be an efficient approach for eliminating dormancy in forest tree species (1), and is a

suitable method for the production of high numbers of plants from a small number of original seeds and a convenient initial source for the establishment of shoot cultures. Thus, seedlings produced with embryo culture can be used later as materials for the micropropagation stage (2-4). Using embryo culture, the dormancy problem has been solved for some forest tree species and plant regeneration realized (5-7). Shoot development on different explants and plantlet regeneration have been achieved for some linden species (8-11). Furthermore, regeneration of *Tilia* and some forest tree plants via somatic embryogenesis was achieved by using immature and mature embryos (12-14).

This study describes in vitro plantlet regeneration from mature embryos of *Tilia platyphyllos* and multiplication of axillary buds on epicotyls.

## Materials and Methods

### Plant Material

The seeds were harvested from 6 trees on the campus of Karadeniz Technical University on 15-18 August 1998. The seeds of open pollinated linden trees were stored at +4°C until the end of the experiment. Seeds of *Tilia platyphyllos* were washed thoroughly in running tap water. Endosperms including the embryo were later soaked in 70% alcohol and then 3% NaOCl for 15 minutes. Subsequently, they were washed with sterile distilled water three times. After surface sterilisation, endosperms were cut and the isolated embryos (Figure 1) were placed horizontally in the test tubes containing 10 ml of nutrient media. The embryos developing plantlets were then transferred to larger culture jars containing 25 ml nutrient medium. Each treatment involved 25-30 embryo explants and was repeated twice.

### Culture media and conditions

Isolated embryos were cultured on MS medium (15). The media were solidified with 7 g/l Difco-Bacto agar and adjusted to pH 5.8 before sterilisation by autoclaving at 121°C for 20 minutes. The cultures were grown at 25±1°C, under a 16 h photoperiod of cool white fluorescent light with illuminance of 4-5 flux.

### Plantlet regeneration

#### Effect of sucrose

In order to determine the effect of sucrose on the development and regeneration of plantlets, 6 different

amounts of sucrose (10, 20, 30, 40, 50 and 60 g/l) were added to the media without plant growth regulators. Four weeks later, the roots of the plantlets with roots and epicotyls were cut and the epicotyls were transferred to a different medium.

#### Effect of culture time

In order to determine the effects of storage time at +4°C on the regeneration of plantlets, the seed embryos of linden were cultured at seven different times three months after harvesting. Sucrose was added to these media at 30 g/l. The epicotyls and root lengths of plantlets were measured 30 days after each culture and the mean values calculated.

#### Effect of plant growth regulators

To determine the effects of cytokinin on the formation of axillary buds on excised epicotyl explants, BAP and Kinetin were separately added to the media in 4 different amounts (0.2, 0.5, 1.0 and 2.0 mg/l). Sucrose was added at 30 g/l. All data were tested by analysis of variance (ANOVA) and significant treatment means were determined by using the Newman-Keuls's multiple range test.

## Results

### Plantlet regeneration

#### Effect of sucrose

Cotyledons became greenish and root growth began after one week in isolated and cultured embryos in 6 different sucrose concentrations. Two weeks after culture, root and hypocotyl growth occurred and epicotyl growth was observed in all samples (Figure 2). Four weeks later, in all sucrose media, plantlet formation occurred and root length and number of buds on epicotyls were counted (Table 1). According to the results, the best mean for root length and number of buds was found with the medium of 30 g/l sucrose (Figure 3).

#### Effect of culture time

The development progress of embryos isolated and cultured at different times and on different media is shown in Table 2. Epicotyls and root differentiation were observed in all media. Hypocotyls were observed swollen and short on the medium in which embryos were cultured for the first time right after the seed collection. Root growth was not satisfactory; however, epicotyl development was better. The best development in terms



Figure 1. Isolated *Tilia platyphyllos* embryos.



Figure 3. Plantlet regeneration on MS medium with 30 g/l sucrose after four weeks of the culture.

of hypocotyls and root length growth was observed on the media where embryos of the seeds were cultured about one month after harvesting (Table 2). In development of embryos cultured up to a month after the harvesting of seeds, hypocotyl and root length growth increments were high, but decreased thereafter.

#### Multiplication of buds on epicotyls

##### Effect of BAP and Kinetin

The epicotyls of plantlets which developed epicotyls and root differentiation in sucrose media were cut and

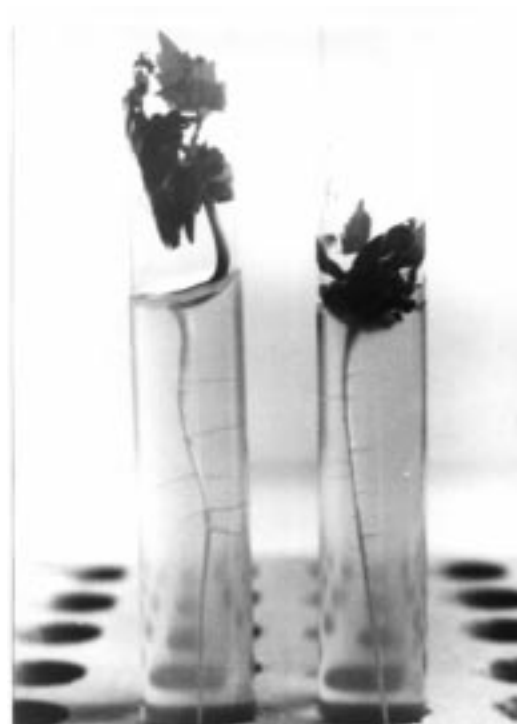


Figure 2. Epicotyl and root differentiation on 30 g/l sucrose medium after two weeks of the culture.

Table 1. Development of embryos in MS media with different sucrose dosages (Numbers followed by the same letter are not significantly different at  $P < 0.05$ ).

Sucrose dosages (g/l)	Mean bud number	Mean root length (cm)
10	3.25 ab	5.50 a
20	4.40 b	5.91 a
30	6.06 c	8.39 b
40	3.91 ab	6.00 a
50	2.80 ab	5.25 a
60	2.20 a	5.20 a

cultured as explants on different BAP and Kinetin media. The number of buds was found to be highest after 4 weeks in the culture medium containing 1.0 mg/l BAP (Table 3 and Figure 4). Although decreasing or increasing the BAP concentration to 2.0 mg/l decreased bud formation, there were no significant differences in other BAP concentrations. However, the best bud formation was observed on the media in which 0.2 mg/l Kinetin was used. The number of buds decreased gradually with increasing concentrations but these were not significant. In terms of bud formation, all BAP concentrations were

Table 2. Development of embryos cultured at different times (Numbers followed by the same letter are not significantly different at P<0.05).

Culture time	Mean hypocotyl height (cm)	Mean root length (cm)
18.08.1998	2.00 a	5.00 ab
01.09.1998	2.23 a	6.57 bc
18.09.1999	3.15 b	7.35 c
04.10.1999	2.41 a	5.36 b
21.10.1998	2.20 a	5.14 ab
07.11.1998	2.16 a	3.68 a
21.11.1998	1.38 a	3.25 a

Table 3. Effect of BAP and Kinetin on bud formation (Numbers followed by the same letter are not significantly different at P<0.05).

Concentration of growth regulator (mg/l)	Mean number of axillary buds	
	BAP	Kinetin
0.2	8.47 a	4.22 b
0.5	9.46 a	2.50 a
1.0	13.56 b	1.87 a
2.0	8.33 a	1.50 a

found to be more effective than Kinetin concentrations (Table 3).

Some of the plantlets were taken out from sucrose cultured media and transferred to 2 : 1 : 1 peat, sand and perlite mixture, respectively, and the plantlets were initially kept under high humidity for 15 days and, once acclimatisation was accomplished, the plants were transferred to the greenhouse under natural daylight conditions (Figure 5). Thus many plantlets were produced.

**Discussion**

According to the results, all embryos were germinated and plantlet formation was observed in all different sucrose concentrations (10-60 g/l). However, the best embryo development and plantlets growth occurred with

30 g/l sucrose. The highest number of buds and root length were also observed with this concentration. It is stated that the growth and survival of cultured embryos mainly depend on carbohydrate addition to the media (1). Similar studies on different species have also confirmed the importance of sucrose on embryo development and plant growth. Epicotyl and root formation were obtained with the addition of sucrose to media for *Juglans regia* embryos, but the best sucrose concentration was determined to be 20 mg/l (6). For use in the multiplication phase as a explant resource, *Morus alba* seeds were germinated on MS medium in which 30 g/l sucrose was added (4). In the same study, a 30 g/l sucrose concentration was found to be more effective in terms of shoot length and number of nodes in a constant BAP media. The best epicotyl and root growth for *Tilia rubra* embryos was reported to be on 30 g/l sucrose media (7).

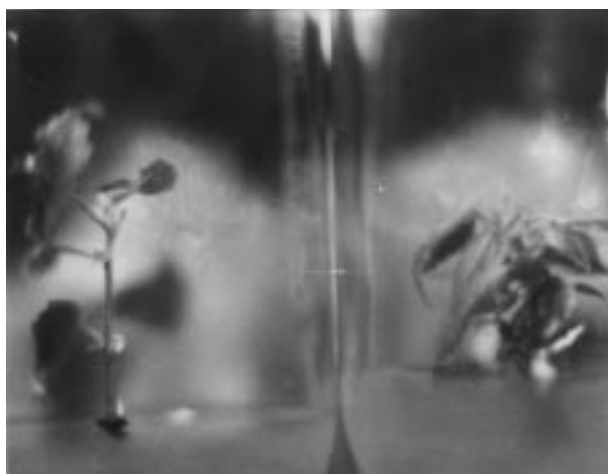


Figure 4. Bud multiplication after four weeks on MS medium with 1.0 mg/l BAP (epicotyls were cut and cultured).



Figure 5. Plants (2 months old) regenerated on sucrose media and transferred to pots.

In studies to determine the effects of time on embryo development and plantlet formation, hypocotyl height and root length growth were not found to be very favourable for the embryos cultured at the first time right after the seed collection, but dense callus formation was observed. In embryos cultured one month after harvesting, the best hypocotyl and root length growth were observed. This may have resulted from differences in the physiological conditions between immature and mature stages and within a month the embryos become mature. As a matter of fact, it was explained that there are two types of germination difficulty in seeds of linden species (16).

The roots of plantlets with shoot and root formation in sucrose media were cut and epicotyl explants were cultured in BAP or kinetin media. Four weeks later, the highest number of buds were determined in 1.0 mg/l BAP. Changing BAP concentrations from 1.0 mg/l to 0.2 mg/l decreased the number of newly formed buds. Although 0.2 mg/l Kinetin media was more effective than the other Kinetin media, its effect was less than all BAP dosages. The type and amount of plant growth regulators have different effects on bud and shoot formation. BAP or Kinetin are generally widely used cytokinins (17). The

dosage effects of cytokinin on bud and shoot multiplication of *Tilia* species were also studied. In a study on *Tilia cordata*, explants from the nodes of 2-24 month old plantlets were used. BAP concentrations of 0.2-1.0 mg/l were found to be more effective than Kinetin, and the shoot growth of buds was the best on 0.2-1.0 mg/l BAP dosages (8). The BAP dosages of 1.0-5.0 mg/l have been found to be effective for shoot growth and multiplication of bud explants taken from old trees of the same species (10). However, when buds of *Tilia amurensis* plantlets were cultured, a 1.0 mg/l BAP dosage was observed to be more effective than the other dosages in terms of shoot multiplication (11). When BAP dosages are closely examined, it may be concluded that differences result from physiological conditions between juvenile and mature stages.

In this study, new plants were produced by the multiplication of plantlets' buds from mature embryos of *Tilia platyphyllos* under in vitro conditions as in plant regeneration by somatic embryogenesis from cultured immature embryos of *Tilia cordata* (12). We conclude that *Tilia* embryos can be germinated in vitro on media not containing any growth regulators but containing sucrose.

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