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Research Article
(Araştırma Makalesi)

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Effects of charcoal and physical state of medium on micropropagation of black mulbery (*Morus nigra* L.)

Aktif karbon ve kültür ortamının fiziksel halinin Urmu dutu (*Morus nigra* L.) nun mikroçoğaltımı üzerine etkisi

* This article has been summarized from the second author's master thesis.

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ABSTRACT

Objective: The purpose of this research was to investigate the effects of charcoal and physical state of culture medium on growth of *Morus nigra.* L.

Material and Methods: For the study tree mature black mulbery (*M. nigra*) genotypes A5, T6 and T8 grown in vitro were used as the explant source in this study. To find out the possible effects of charcoal and culture physical state (culture medium) on culture growth used four) Liquid Nas and Read Medium (NRM) + 0.5 gl^{-1} activated charcoal, Liquid NRM containing no charcoal, Gelled NRM + 0.5 gl^{-1} activated charcoal, agar-gelled NRM containing no charcoal) medium were used in the study to find out the possible effects of the presence of charcoal and physical state of the culture medium on culture growth.

Results: Medium physical state and presence of activated charcoal in the medium greatly affected mean shoot number per cultured explants. Mean shoot numbers obtained per explants varied between 1.1 and 5.8. Medium containing no charcoal produced more shoots compared to medium containing charcoal and agar - gelled medium produced more shoots compared to liquid medium.

Conclusion: Agar- The gelled medium with agar gave better results for black mulbery (*M. nigra*) than the liquid medium.

ÖZ

Amaç: Bu çalışmanın amacı *Morus nigra* L. nin in vitro mikro çoğaltımı üzerinde aktif kömür ve kültür ortamının fiziksel halinin araştırılmasıdır.

Materyal ve Yöntem: Çalışmada Urmu dutun (*M. nigra*) 3 genotipi (A5, T6, T8) explant olarak kullanılmıştır. Kültür ortamında kömürün varlığı ve ortamın fiziksel halinin (sıvı veya jel) incelenmesi için sıvı Nas ve Read medium (NRM)+0,5 gr L^{-1} aktif kömür, aktif kömürsüz sıvı NRM ortamı, katı NRM ortamı+0,5 gL^{-1} aktif kömür ve aktif kömürsüz NRM ortamı kullanılmıştır.

Araştırma Bulguları: Kültür ortamında kömürün varlığı ve ortamın fiziksel hali (sıvı veya jel) bir eksplantten elde edilen ortalama sürgün sayısını önemli derecede etkilemiştir. Bir eksplantten elde edilen ortalama sürgün sayısı 1.1 ile 5.8 arasında olmuştur. Aktif kömür içermeyen ortam üzerinde daha fazla sürgün elde edilmiş ve sıvı ortama kıyasla jelleştirilmiş ortam üzerinde elde edilen sürgün sayısı daha yüksek olmuştur.

Sonuç: Urmu dutunun İn vitro mikroçoğaltımında agar ile katılaştırılmış NRM ortamı sıvı NRM ortamından daha iyi sonuç vermiştir.

INTRODUCTION

The family Moraceae includes the genus *Morus*, which includes the black mulberry (*Morus nigra* L.) Approximately 10-16 species of mulberry are cultivated worldwide. In commercial mulberry cultivation, three main types of mulberry, white (*Morus alba* L.), red (*Morus rubra* L.), and including black (*M. nigra*) are generally used for fruit production. Black mulbery (*M. nigra*) is also known as "amoreira'', "amorapreta'' or "negra'' (Ercisli & Orhan, 2007; Wasano et al., 2009; Hussain et al., 2020). It is a woody perennial tree or shrub. It is an economically important plant used for its fruit and is vital to sericulture industry. Mulberry plants are also grown in many countries for silkworm (*Bombyx mori* L.) production (Wang et al., 2022).

With the increase in demand for quality mulberry fruits due to the properties of antioxidants it contains, it has become an increasingly important fruit due to the important nutritional content nowadays. Mulberries are a good source of vitamins and minerals (Gerasopoulos & Stavorulakis, 1997; Wang et al., 2022). In addition, mulberry fruits have a wide range of biochemical activities that are very important for human health (phenolic acids, flavonoid, antioxidant, anti-hyperlipidemia, anti-cancer) (Dairas et al., 2003; Ouyang et al., 2005; Bae & Suh, 2007; Chen et al., 2012). Fruits are used as worming agent, for dysentery and as a laxative, odontalgic, anthelmintic, expectorant, hypoglycemic and emetic (Baytop, 1996, 1999). The fruits of mulberry activate kidney energy and thus are used as antichloristic.

Mulberry is propagated by different methods nowadays. The method of propagation varies according to the variety. However, it is generally propagated by conventional methods such as cuttings, grafting, air layering, and seed (Lu, 2002). Propagation by seed is not desirable due to the heterogeneous nature of the seedlings become of cross pollination (Das, 1983; Hossain et al., 1992). In some countries such as Bangladesh propagation of mulberry through cuttings is restricted to a single season (September-October) (Zaman *et al*., 1997). Developing inbred mulberry is not easy due to a longer juvenility period, high inbreeding depression and the dioecious nature of the plant (Vijayan, 2010).

Black mulberry (*M. nigra)* is much more difficult to propagate than the white mulberry type because grows much slower than white ones (Skrovankova et al., 2022). Black mulberry are commercially propagated by stem cuttings, seeds and grafting. Often cuttings are difficult to root. It is a difficult to propagate species, as it tends to bleed heavily when cut for grafting. Successful rooting ability of stem cuttings depends on the propitious genotype and environmental conditions. Also, the success of grafting depends on cambium activity and compatibility. Although it can be reproduced quickly by seed but seed reproduction is not applied because homogeneity is not achieved due to open pollination. (Pati et al., 2006). Furthermore, stem cuttings cannot be used for reproduction of economically important new mulberry varieties (Kapur et al., 2001). However, the plants can be propagated quickly by in vitro culture methods (Hepaksoy, 2017; Zainal & Hepaksoy, 2018; Taha et al., 2020; Doğan, 2022). Propagation of black mulberry via conventional propagation techniques is not satisfactory. Micropropagation seems to be an attractive alternative propagation (Altman, 2000; Adelderg at al., 2000) but there are only a few studies that report in vitro propagation of different *Morus* species. Moreover, the capacity to transfer the plants outside of culture is essential to the long-term success of mulberry in vitro multiplication on a commercial scale, at a cheap cost, and with good survival rates (Chandra et al., 2010). To reach this objective the possible effects of charcoal and culture physical state (culture medium) on culture growth of black mulberry were investigated.

MATERIAL and METHODS

The present study was carried out in the ÜSKİM plant tissue culture laboratory, Kahramanmaras Sutcu Imam University, Turkey, between October 2013 and August 2014.

Black mulberry (*M. nigra)* axillary buds were used as the experimental materials. Culture of mature Black mulbery genotypes A5, T6 and T8 grown in vitro were used as the explant source.

Culture medium and culture conditions

The medium used for culture growth was Nas and Read (NRM) (2004) medium. The cultures were maintained in a growth chamber at 24±1^{o}C with a 16/8 h (light/dark) photoperiod under cool-white, fluorescent light at 80 µmol m^{-2} s^{-1}.

Determining the effects of charcoal and medium physical state on culture growth

To find out the possible effects of charcoal and culture physical state (culture medium) on culture growth the following culture media were used:

1. Liquid Nas and Read Medium (NRM) + 0.5 gL^{-1} + activated charcoal.

2. Liquid NRM containing no charcoal.

3. Gelled NRM + 0.5 $gL^{-}1$ + activated charcoal.

4. Gelled NRM containing no charcoal.

Nas and Read (2004) medium containing 0.01 mgL^{-1} Indol-3-Butryric Acid (IBA), 0.25 mgL^{-1} Benzyl adenine (BA), and 30 gL^{-1} sucrose was used as the culture medium. If charcoal was to be added to a medium, first 0.5 gL^{-1} charcoal was added to the medium, and then the PH was adjusted to 5.5. To gelled (solidify) medium, 5.5 gL^{-1} Merck microbiological agar was added. The media were autoclaved at 121^{o}C and 1.2 kgf cm^{-2} for 15min. After autoclaving, the medium was distributed to 15 x 250 mm glass-test tubes.

For each genotype 10 replicate test tubes were used, with one explant per tube (each explant containing 1 axillary bud). Then culture vessels were randomly placed on the growth shelves. were first maintained in the dark for 1 week at 24±1^{o}C. Then, they were subjected to a 16/8 h (light/dark) photoperiod under cool-white, fluorescent light at 80 µmol $m^{-2}s^{-1}$ in the same growth chamber for 4 weeks. All experiments wer e repeated at least twice time.

Rooting stage

At this stage, shoots obtained on multiplication medium and are ≥ 2cm were used for rooting experiments. To induce rooting microshoots obtained on multiplication medium were cultured on gelled (NRM containing 0, 0.5, 1.0 or 1.5 mgL^{-1} IBA). Four replications (Magenta vessels containing 5 microshoots) of each genotype were applied to each IBA level. Microshoots transferred to rooting medium were first exposed to the dark medium for one week at 24 °C, and then were subjected to a 16/8 h (light/dark) photoperiod for three weeks. Data of rooting percentage (%), average number of roots and average root length were recorded after 4 weeks. At the end of 4 weeks, rooted microsprouts from which rooting data were obtained were washed in tap water to remove the agar at the bottom. The microshoots cleared from the agar were transferred to plastic cups containing a mixture of 60% peat and 40% perlite. To prevent moisture loss in the glasses, they are covered with a transparent plastic cup. The humidity of the plastic cups was checked daily, and after 1 week the cups were perforated (about 1 cm^{2}) and plants were kept covered for another week. The micro shoots were tried to acclimate gradually in this way for 2 weeks and at the end of 2 weeks, the transparent cub was removed. Than plants were left to grow under 16/8 h (light/dark) photoperiod under cool-white fluorescent light at 80µmol m^{-2} s^{-1} for two weeks. At the end of the fourth week, the acclimatized plants were transplanted into bigger pots containing the same mixture described above. Then, after one month, the plants were shifted to the greenhouse (shaded with a mesh blocking 50% of the light) and left to grow under uncontrolled temperature (min. 18^{o}C - max. 27^{o}C) and relative humidity conditions (Nas et al., 2012). For each treatment and genotype there were three replicate jars with 5 shoots per jar, and all experiments were repeated three times.

Statistical Analysis

All statistical analysis were performed using SPSS 22 (IBM). Comparison of treatment means was done using LSD at P ≤ 0.05 used. Data for explants in a culture vessel were divided by the number of explants, and the mean shoot number and number shoot length were used for statistical analysis.

RESULTS and DISCUSSION

Shoot multiplication

Chemical composition of culture medium (with or without charcoal) and medium physical state (liquid or gelled) had a great influence on the number of shoots obtained per cultured explant. Similar to mean shoot number per cultured explant, chemical composition of culture medium (with or without charcoal) and medium physical state (liquid or gelled) influenced mean shoot length of all three black mulbery (*M. nigra*) genotypes used in this study (Table 1).

Table 1. The effect of charcoal and medium physically on mean shoot number and shoot length (cm) ± std. dev. (cm) of *Morus nigra*

Çizelge 2. *Aktif kömür ve kullanılan ortamın fiziksel durumunun Morus nigra nin ortalama sürgün sayısı ve uzunluğu (cm) üzerine etkisi*

Medium	Genotype					
	A5		T6		T8	
	M.S No	M.S L	M.S No	M.S L	M.S No	M.S L
Agar + charcoal	1.0 ± 00 d	1.1 ± 0.2 d	1.1 ± 0.1 c	1.2 ± 0.4 d	1.2 ± 0.2 c	1.0 ± 0.0 d
Agar + no charcoal	5.5 ± 1.5 a	2.2 ± 1.8 b	5.8 ± 1.3 a	2.1 ± 0.4 b	5.2 ± 0.4 a	2.0 ± 0.0 b
Liquid + charcoal	1.1 ± 0.1 c	2.1 ± 1.0 c	1.1 ± 0.2 c	1.9 ± 0.1 c	1.2 ± 0.1 c	2.2 ± 1.1 a
Liquid + no charcoal	4.3 ± 0.3 b	2.6 ± 0.2 a	3.9 ± 1.2 b	2.5 ± 0.8 a	2.5 ± 0.1 b	1.9 ± 0.5 c

M.S. No: ortalama sürgün sayısı M.S.L: Ortalama sürgün uzunluğu, Farklı harfler önemli farklılıkları gösteriri (p<0.05) M.S No: mean shoot numer, M.S. L: mean shoot lenght, Diferent letters indicate significant diferences (p < 0.05).

Mean number of shoots obtained per explants are shown in Table 1. Clearly medium containing no charcoal was superior to medium containing charcoal as it produced more shoots. Moreover, gelled medium containing no charcoal was better than liquid medium containing no charcoal. The highest numbers of shoots per cultured explant of genotype A5 (5.5 shoot/explant), genotype T6 (5.8 shoot / explant) and genotype T8 (5.2 shoot / explant) were obtained on agargelled medium containing no charcoal. On agar-gelled medium containing 0.5 gL^{-1} charcoal mean numbers of shoots obtained per cultured explants were 1.0, 1.1 and 1.2 for genotype A5, genotype T6 and genotype T8, respectively.

Mean shoot number per explant obtained on liquid medium was significantly lower than that obtained on agar-gelled medium. Moreover, mean shot number obtained on liquid medium containing no charcoal was higher than that on liquid medium containing charcoal. Mean shoot numbers on liquid medium containing no charcoal were 4.3, 3.8 and 2.5 for genotype A5, genotype T6 and genotype T8, respectively. On liquid medium containing 0.5 gL^{-1} charcoal mean shoot numbers were 1.1, 1.1 and 1.2 for the same genotypes, respectively. Our results showed that the fortified NRM agar-gelled medium with 0.01 mgL^{-1} Indol-3-Butryric Acid (IBA), 0.25 mgL^{-1} Benzyl adenine (BA) finding is more suitable for in vitro propagation of mulberry. In some studies, the best shoot number was obtained at high concentrations, while in our study, BA (0.25 mgL^{-1}) at low concentration gave very good results. Ohyama (1987) was demonstrated that the MS medium containing 2 mgL^{-1} 6-benzylamino purine (BAP) was the best for shoot multiplication (Bhau & Wakhlu, 2001, 2003; Lalitha et al., 2013). This is close and in agreement with the Taha et al. (2020).

The mean shoot length of the three genotypes is shown in Table 1. In contrast to the mean shoot length, the mean shoot lengths on liquid medium were higher or similar to those on gelled medium. Gelled medium containing no charcoal and liquid medium containing both charcoal and no charcoal produced longer shoots than gelled medium containing 0.5 gL^{-1} charcoal. Shoot length on liquid medium with and without charcoal was similar (Table 1 and Figures 1&2).

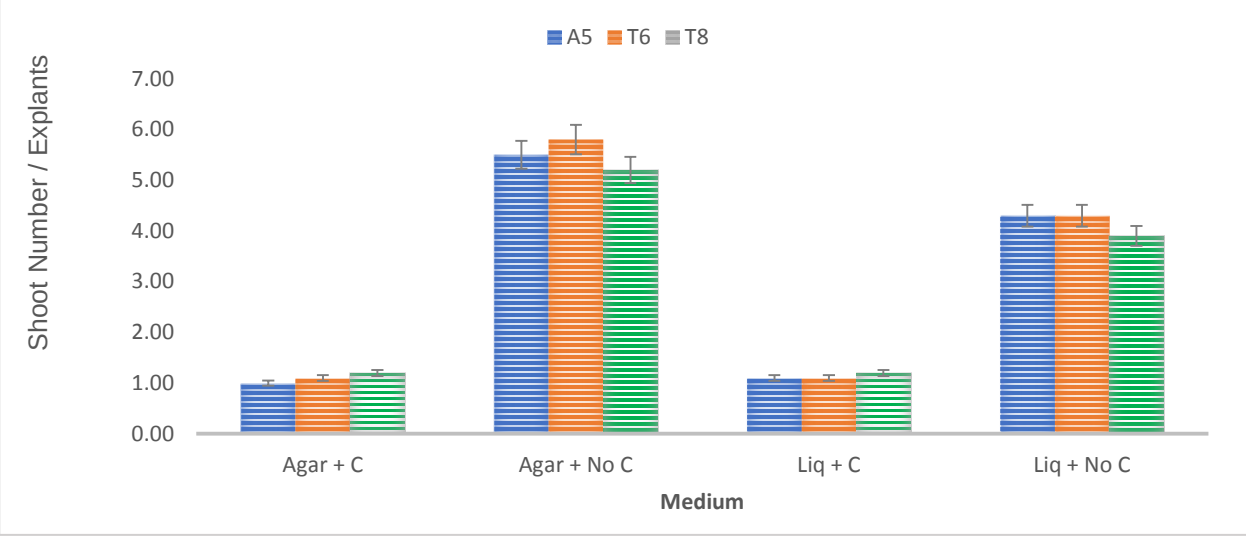

Figure 1. Mean number of shoots of *Morus nigra.* genotypes obtained on liquid or gelled.

Şekil 1. *Morus nigra*'nın aktif kömür ve aktif kömür içermeyen sıvı ve katı ortamda oluşan ortalama sürgün sayısı.

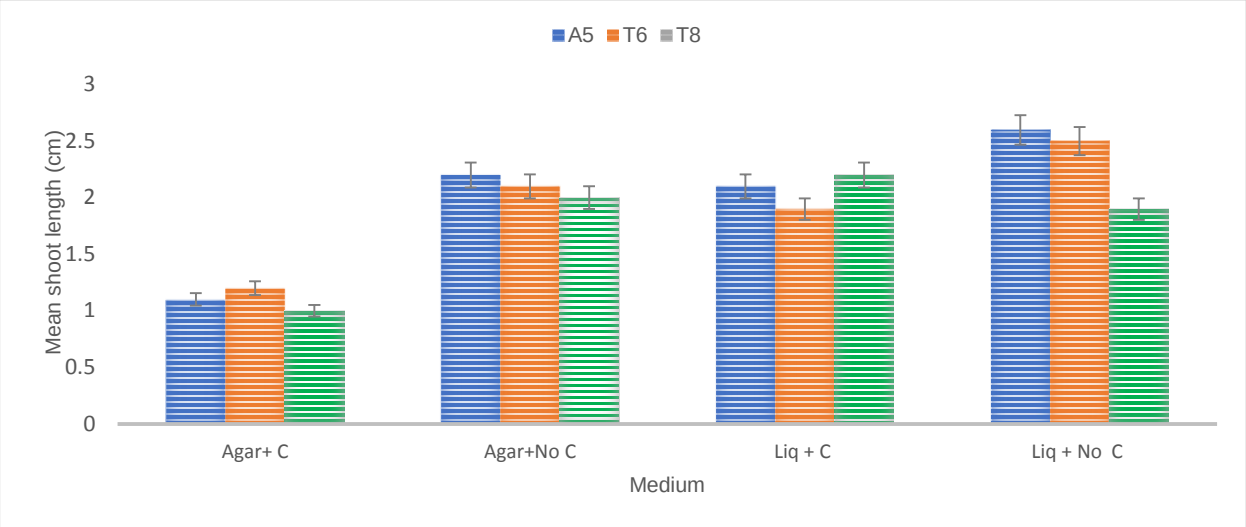

Figure 2. Mean shoot lengths of *Morus nigra* genotypes obtained on liquid or gelled medium with/without charcoal.

Şekil 2. *Morus nigra*'nin aktif kömür veya aktif kömür içermeyen sıvı ve katı ortamda oluşan sürgünlerin ortalama uzunluğu.

There was a clear inverse relationship between the quantity of shoots per explant and shoot length. Shoot length measured on a liquid medium, with or without charcoal, was comparable to or superior to that measured on a solid medium. The low quantity of shoots obtained on liquid medium may have contributed to the fact that they were longer than those on solid medium.

The effect of IBA concentration on rooting in the in vitro propagation of Black mulberry (*Morus nigra*)

To improve the rooting of *M. nigra* microshoots, the effects of three IBA concentration were tested. The shoots that were new obtained from regenerating explants were cut from the original explants and subcultured on the multiplication medium for 1 mount were cultured on root induction medium (0, 0.5, 1.0, 1.5 mgL^{-1} IBA).

Table 2. Rooting percentages and mean rooting percentage of *Morus nigra* microshoots in the presence of various IBA concentrations

Çizelge 2. *Farklı IBA konsantrasyonlarındaki Morus nigra'nın köklenme yüzdesi ve ortalama kök sayısı*

IBA (mgL^{-1})	Genotype A5		Genotype T6		Genotype T8	
	Rooting percentage (%)	Number of roots per microshoots	Rooting percentage (%)	Number of roots per microshoots	Rooting percentage (%)	Number of roots per microshoots
0.0	13.0 ± 3 a	1.8 ± 1.2 a	--	--	--	
0.5	17.0 ± 3 a	3.7 ± 1.2 a	14.5 ± 4.2 a	2.5 ± 0.9 a	14 ± 0.5 a	2.6 ± 0.0 a
1.0	15.5 ±2.2 a	2.3 ± 0.9 a	16.5 ± 2.2 a	2.7 ± 0.9 a	10 ± 0.9 a	1.8 ± 1.1 a
1.5	14.0 ± 2.2 a	1.9 ± 0.9 a	14.0 ± 2.2 a	1.9 ± 0.9 a	14,5 ± 0.9 a	2.0 ± 0.5 a

Diferent letters indicate significant diferences ($p < 0.05$).

Four weeks after root indication rooting data were recorded. Regardless of IBA concentration and genotype rooting ratios were similar. Depending on IBA concentration and genotype, rooting percentage of microshoots varied between 10% and 17% (Table 2) The number of roots per microshoots varied between 1.2 and 3.7, and IBA concentrations and genotype 2.2 roots per microshoots were obtained (Table 2, Figure 3). It was determined that IBA is a possible auxin that encourages roots in in vitro regenerated shoots (Rajore & Batra, 2005).

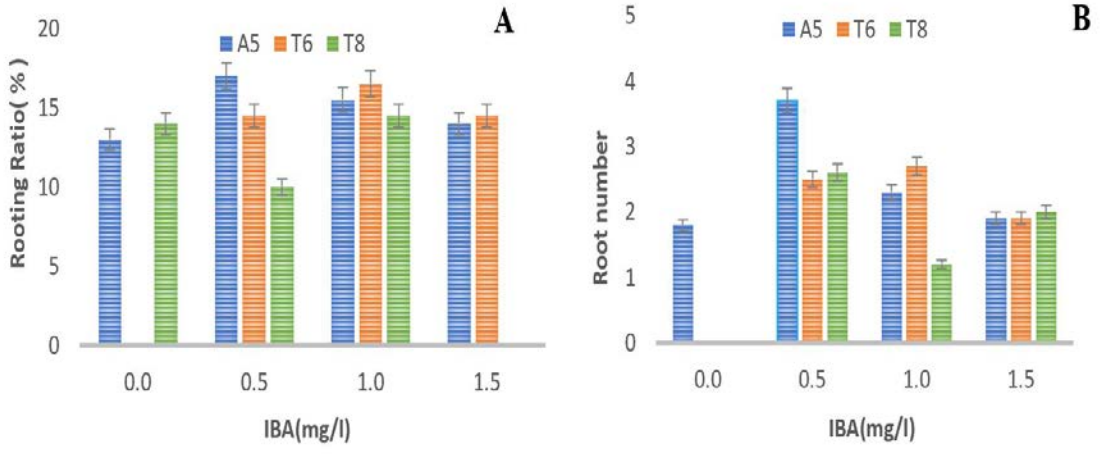

Figure 3. Mean rooting percentage (A) and Mean number of roots (B) of *Morus nigra* genotypes obtained on medium containing different concentrations of IBA (mg/l).

Şekil 3. Farklı IBA konsantrasyonlarında *Morus nigra*'nın köklenme yüzdesi (A) ve mikrosürgün başına ortalama kök sayısı (B).

Rooted microshoots were removed from culture vessels and roots were washed with tap water to remove the agar. After removal of agar from roots, rooted microshoots were transferred to pots. Then the plantlets were subjected to the gradual acclimatization procedure explained in materials and methods (Figure 4). Over 90% of rooted microshoots survived after acclimatization stage (Figure 4).

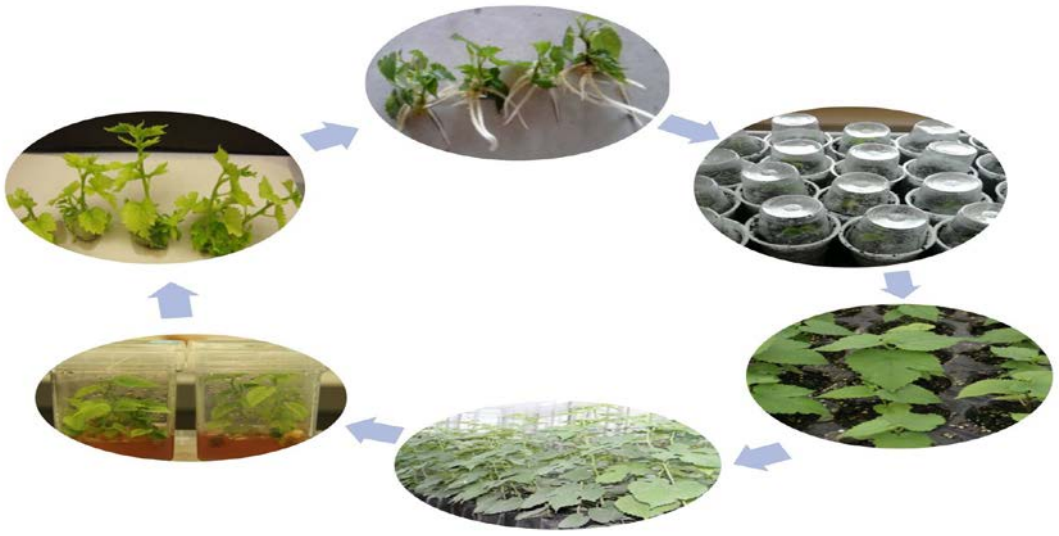

Figure 4. Micropropagation and acclimatization stages of *Morus nigra*.

Şekil 4. *Morus nigra'nın in vitro çoğaltma ve aklimatizasyon aşamaları.*

CONCLUSIONS

Traditional propagation methods of black mulberry are not satisfactory for commercial propagation. In this study the best in vitro Propagation capacity was observed when using axillary buds explants of black mulberry. If shoot length is concerned, first a desired shoot number should be obtained on agar - gelled medium then shoots should be cultured on a liquid medium to obtain longer shoots. Charcoal in the culture medium was inhibitory for culture growth and its use in micropropagation of black mulberry is not recommended. Although the rooting of microshoots need to be improved, once microshoots are rooted, they can successfully be transplanted ex vitro and a reasonable number of clone plants can be distributed to the growers in a relatively short period of time.

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