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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Influence of $FeSO_4.7H_2O$, Indole-3-Butyric Acid and Different Nutrient Medium on *In Vitro* Sapling Propagation and Micrografting of Walnut (*Juglans Regia L.*)

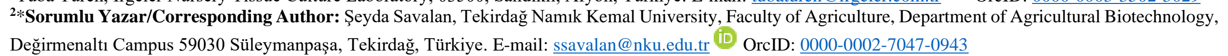
$FeSO_4.7H_2O$, İndol-3-Bütirik Asit ve Farklı Besin Ortamlarının *In Vitro* Ceviz (*Juglans Regia* L.) Fidanı Çoğaltımı ve Mikroaşılma Üzerine Etkisi

Tuba TÜREN[1], Şeyda SAVALAN[2*], Elif Ceren PEHLİVAN[3]

Abstract

Walnuts are considered a functional food and play a significant role worldwide in people's regular diets. The cultivation and trade of walnut rootstocks and saplings are crucial agricultural activities globally and in Türkiye. Rootstocks are used for grafting and propagating different walnut varieties, and they are essential for tree development, fruit yield, product quantity, and most importantly, their ability to adapt to various ecological conditions and resist diseases and pests. Fruit growing and production of fruit rootstocks of deep-rooted plants in horticulture is given high importance in many countries. However, poor rooting and slow-growing sapling prevent establishing high-yield clonal production. Especially walnut sapling propagation is more complicated and time-consuming than other woody plants. Our research aims to grow high-quality saplings by using biotechnological techniques. This study used modifications to the current *in vitro* basal medium and evaluated the effects on *in vitro* rooting and micrografting of walnut species. The optimal medium for shoots induction (2.93±0.90) of the plant was determined as 4 number medium (Murashige and Skoog (MS) nutrient medium with 5.4 µM NAA + 6.9 µM TDZ + 0.6 µM GA3 and solidified with 0.7% agar). In trials to develop sapling, the 10.33 and 6 rooted (2.5cm root length) shoots were obtained from MS nutrient medium with 14.7 and 19.6 µM of IBA and 348.42 µM of FeSO4. 7H2O, and two micrografted plants in MS medium with 19.6 µM of IBA and 348.42 µM of FeSO4.7H2O survived. After three months in the growth chamber, six saplings were ready for transfer to the field. Our findings suggested that FeSO4.7H2O and high dose IBA treatment in MS medium is efficient for *in vitro* rooting and obtaining *in vitro* micrografted saplings.

Keywords: Clonal production, Micropropagation, Plant growth regulator, Plant tissue culture, Woody plants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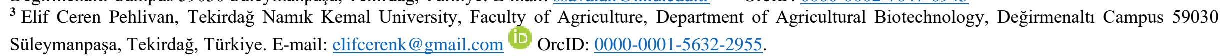
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Öz

Dünya genelinde Düzenli beslenme alışkanlığında önemli bir yere sahip olan ceviz, fonksiyonel gıda olarak tanımlanmaktadır. Ceviz bitkisinin adaptasyon kabiliyetinin yüksek olması, kaliteli yağ ve protein içerdiğinden besin değerinin yüksek olması, sanayide kullanımı ve bunlara bağlı ekonomik getirisi nedeniyle üzerinde durulması gereken bir tür konumundadır. Ceviz bitkisinde anaç ve fidan yetiştiriciliği Dünya'da önemli tarım faaliyetlerinden biri sayılır. Ancak çelik köklendirme ve fidanların büyüme süresinin uzun ve zor olması, yüksek verimli klonal üretim tesislerinin kurulmasına bir engeldir. Özellikle sağlıklı ve kaliteli ceviz fidanı üretim süreci diğer odunsu bitkilere göre daha karmaşık ve zaman alıcıdır. Bu araştırmada biyoteknolojik teknikler kullanılarak kısa zamanda kaliteli fidanların yetiştirilmesi amaçlanmıştır. Denemelerde genel olarak *in vitro* mikroçoğaltım tekniklerinde kullanılan besiyeri üzerine modifikasyonlar yapılmıştır ve bu modifiye besi yerlerinin, *in vitro* şartlarında yetişen sürgünlerin köklendirmesi ve *in vitro* mikroaşılama üzerindeki etkileri değerlendirilmiştir. Çalışmanın sonucuna bakıldığında ortalama sürgün (2.93±0.90) oluşumu için en uygun ortam 4 numaralı (5.4 µM NAA + 6.9 µM TDZ + 0.6 µM GA3 içeren Murashige ve Skoog) besin ortamından elde edilmiştir. Sürgün köklendirmeye yönelik denemelerde 14.7 ve 19.6 µM IBA ve 348.42 µM FeSO4. 7H2O içeren MS besin ortamından 10.33 ve 6 köklü (2.5cm kök uzunluğu) sürgünler elde edilmiştir. Mikroaşılama çalışmalarında 19.6 µM IBA ve 348.42 µM FeSO4.7H2O içeren MS ortamında iki mikro aşılanmış fide elde edilmiştir. Elde edilen 6 fidan 3 ay tam kontrollü yetiştirme odasında alıştırma süresini atlattıktan sonra bahçeye transfer edilmiştir. Çalışma sonucunda besi yerine uygulanan FeSO4.7H2O ve yüksek doz IBA *in vitro* köklenme ve *in vitro* mikroaşılı fidan elde etmede etkili olduğu gözlemlenmiştir. Çalışmanın sonuçları diğer odunsu bitkilerde yapılan klonal çoğaltım çalışmalara yardımcı olacaktır.

Anahtar Kelimeler: Klonal çoğaltım, Mikroçoğaltım, Bitki büyüme düzenleyicisi, Bitki doku kültürü, Odunsu bitkiler

1. Introduction

Rapid, large-scale, and sustainable propagation of high quality, disease-free, and homogenous plant materials using plant tissue culture techniques is highly desired. These techniques use induce regeneration from cells, tissues, and organs under aseptic conditions in a solid, liquid, or semi-solid nutrient medium (Chandra et al., 2010; Deb and Imchen, 2010). The Walnut (*Juglans regia* L.) is a precious cultivated dry fruit species of economic significance for producing nuts.

Sapling cultivation is among the essential agricultural activities. Principally, the prominent method of vegetative proliferation of walnuts is grafting. However, low proliferation and rooting rates are among the factors that prevent the propagation of walnut. (Martinez et al., 2010; Paunović et al., 2013; Xiaoying et al., 2014)

There is trend to propagate these plants through tissue culture to avoid intense labor force, loss of time, and high cost through conventional propagation methods on a large scale (McGranahan et al., 1988; Scaltsoyiannes et al., 1997). Rodriguez (1982a, 1982b) and Rodriguez and Sanchez-Tames (1981) were among the first to report the improvement of walnut cultures *in vitro* and describe the development of shoots or roots from the cultured walnut embryos. Later, a full of work was conducted on various walnut species using several types of explants, media especially DKW medium developed by Driver and Kuniyuki (1984), rooting metods with satisfying outcomes(Jay-Allemand et al., 1992). *In vitro* vegetative propagation of Juglans sp. is highly significant during these days with fast growth of human population (Land and Cunningham, 1994; Scaltsoyiannes et al., 1997).

Micrografting was first described by Murashige et al. (1972) and later by many researchers in woody (Ribeiro et al., 2022; Sammona et al., 2018) and herbaceous plants (Khawar and Özcan, 2002) as a tool to root and recover difficult to root recalcitrant plant species and produce virus-free plants. Therefore, this technique has been used for many different intentions. Exemplarily, propagation through micro cuttings from adult tree-derived shoots could be employed to produce true-to-type plants. Micrografting strongly depends on the formation of a vascular connection between the scion and the rootstock (Ribeiro et al., 2015) to elucidate plant systemic signaling (Tsutsui and Notaguchi, 2017) of different species (Estrada-Luna et al., 2002). *In vitro* micrografting can be envisaged as an alternative technique for reproducing walnut species. The main objective of this study was to research the benefit of different concentrations of PGRs (Plant Growth regulators) on shoot regeneration, as well as to investigate the effects of $FeSO4.7H2O$ and indole-3-butyric acid on the rooting and micrografting ability of walnut.

2. Materials and Methods

2.1. Plant Material

Chandler, Yalova-1, and Vlach walnut saplings were obtained from a sapling vendor in Tekirdağ, Türkiye, and kept in a fully controlled growth chamber (*Figure 1*). All treatments for propagation and rooting experiments were carried out in the Department of Agricultural Biotechnology Plant Biotechnology Laboratories in Tekirdağ Namık Kemal University.

Figure 1. Rootstocks from which explants are provided for in vitro sapling propagation

2.2. Explant Sterilization

Soon after procurement of the rootstocks, they were kept in the fully controlled growth chamber for 60 days. The diseased leaves if any were removed from the plants; followed by cutting 2 cm long fresh apical and lateral buds. These were disinfected and cleaned, followed by rinsing with antibacterial liquid detergent, and brushed under running tap water for 3 hours. These explants were further treated with a dip in 70% ethyl alcohol for 2 minutes and rinsed with distilled water (Pehlivan et al., 2017) The following stages of sterilization were performed in a laminar flow sterile cabinet using two techniques described below:

(a) The apical and lateral buds were sterilized for 5 minutes with 70% (v/v) Actijen ensured by 2×5 times rinsing with bidistilled sterilized water.

(b) In another sterilization method, explants were sterilized in 30 and 50% commercial bleach for five min ensured by 3×5 times rinsing with bidistilled-sterilized water (Daneshvar, 2019).

Table 1. PGRs combinations and concentrations in MS medium for shoot regeneration

Medium Number	BAP	TDZ	NAA	GA3	2.4-D
	Medium for Shoot Regeneration (µM)				
1	8.9	-	2.7	0.6	-
2	6.7	-	2.7	0.6	-
3	-	9.1	5.4	0.6	-
4	-	9.6	5.4	0.6	-
5	-	4.6	5.4	0.6	-

2.4. Shoot Regeneration

Apical and lateral bud's explants (Kefayeti et al., 2019) were cultured on semi-solid [gelled with 0.65% Plant agar (Duchefa)] MS medium and 3% sucrose (C12H22O11, Duchefa) containing five different concentrations of Cytokines (TDZ and BAP), Auxin (NAA), Gibberellin (GA3) and control MS medium for shoot regeneration (*Table 1*) (Royandazagh and Pehlivan, 2016)

2.5. Shoot Rooting

All rooting treatments were performed on shoots of an average of 8 cm length. The rooting study was carried out in DKW, MS, ½×MS basal medium with 4.90, 9.8, 14.7, and 19.6 µM IBA and 174.20 µM, 348.42 µM, and 522.62 µM $FeSO_4.7H_2O$. After the root culture with three replications that were kept in the dark for four days, they were held in the plant growth chamber at 24 ± 2 °C under the red and white LED light (luxury) for 16 hours of light and 8 hours of dark photoperiod

2.6. In Vitro Micrografting

Before *in vitro* micrografting, the leaves and cotyledons of 6 seedlings of Vlach species were removed followed by a vertical cut of 0.5 cm made at the apical end. Chandler species *in vitro* shoots arising from apical and lateral buds micropropagation was used as a source of scions. *In vitro* grafting was performed by inserting the wedge-shaped end of scions into the vertical cut of rootstocks, and the grafts were made by placing rootstocks and scions together in one place followed by wrapping them together using a sterilized transparent tape after *in vitro* grafting. The plantlets were cultured for 63 days (3×subculture) on MS, ½×MS medium with 4.90, 9.8, 14.7, and 19.6 µM IBA and 174.20 µM, 348.42 µM, and 522.62 µM $FeSO_47H_2O$.

2.7. Transfer Plantlets to Ex Vitro Condition and Acclimatization

The micropropagated walnut plantlets (rooted shoot with 8-12 cm in length) and micrografting plantlets were washed with tap water without harming the leaves and roots. These were transferred to sterilized peat moist in jars. These pots were covered with transparent polythene bags and were left to grow in a growth chamber. The bags were gradually perforated at the end of the second week to increase air circulation and reduce humidity to 50% at 24 ± 2 °C. This app was done to adapt the plants to external conditions. The polythene bags were removed entirely

later, and the plants were left to grow and harden in the growth chamber. These plants were watered with liquid fertilizer (water-soluble) once a month.

2.8. Statistical Analysis

All treatments of regeneration experiments used 15 explants which were divided equally into three replicates containing five explants treatments were repeated twice afterward. The data were subjected to a one-way analysis of variance (ANOVA, IBM® SPSS® statistics 24.0 for Windows), and the post hoc tests were performed using either Tukey's b or Least Significant Difference (LSD) test.

3. Results and Discussion

3.1. Explant Sterilization

The decontamination of micro-organisms from the explants has great importance for *in vitro* tissue cultures of plants; therefore, in this study, surface sterilization of apical and lateral bud's explants isolated from 3 different walnut species was used Actigen at 70-90% and NaOCl at 30-50% concentrations for 5 minutes. In the sterilization process performed with commercial bleach, the proportion of necrosis and contamination was determined at a higher level than Actijen, as well as necrosis was observed in the sterilization process performed for 5 minutes in a 90% solution of the Actijen. According to the results of the trials, the best sterilization and explant vitality result (100%) was obtained from a 70% Actijen solution. During tissue sterilization for control of necrosis and contamination, as suggested by (Pehlivan et al., 2017), 70% Actijen solution was used to disinfect, effectively controlling the contamination and vitality of explants. Additionally, in clematis, plants were used with 70% ethyl alcohol (for 1 min), 0.3-0.4% Cl2 (15 min), and 1% thimerosal (10 min) for explant sterilization (Mitrofanov et al., 2021), Dong et al. (2007) and Yulan et al. (2004) stated that walnut explants were kept in 70% ethyl alcohol for a while, followed by sterilization with $HgCl_2$. Also, in most studies, sodium hypochlorite was used as a disinfectant to sterilize these plant explants. For example, the microcuttings in Chandler, Franquette, and Jupaneşti cultivars were sterilized by washing carefully with a regular detergent and water, followed by the washing with ethyl alcohol hypochlorite 5% and sterile distilled water apart from NaOCl (Gotea et al., 2012).

3.3. Shoot Regeneration

Tissues from woody plant species are somewhat challenging to grow and differentiate *in vitro* (Jain et al., 2012). The apical and lateral bud's explants (Onay, 2000; Rathore et al., 2004; Fidancı, 2005; Royandazagh, 2019) were transferred to an MS medium containing different concentrations and combinations of BAP, TDZ, NAA, and GA3. After 3×subcultures (63 days), the data were collected by testing the growth of the propagated shoots. Although the analysis of variance results showed significant differences among treatments, mean in terms of shoot induction per explant, shoot length, and the number of leaves per shoot, these analysis results showed no significant difference between species. The shoot propagation was noted on all explants regardless of their concentrations and combinations of BAP, TDZ, NAA, and GA3. The maximum number of shoot induction per explant was obtained as 2.93±0.90 on 4 number medium (MS medium containing 6.9 µM TDZ + 5.4 µM NAA + 0.6 µM GA3) in Vlach species. In terms of the longest shoots were obtained as 5.03±0.60 on 3 number medium (MS medium containing 9.1 µM TDZ + 5.4 µM NAA + 0.6 µM GA3) and the maximum number of leaves per shoot was obtained as 5.66±0.00 on 4 number medium (MS medium containing 6.9 µM BAP+ 5.4 µM NAA + 0.6 µM GA3) in Yalova species (*Table 2* and *Figure 2*).

For shoot regeneration in walnuts, Al-Mizory and Mayi (2012) explained best results were obtained from the combination of nodal explants and 1 mg/L KIN + 2 mg/L BAP. On the other hand, Sekmen et al. (2017) has been indicated that the best micropropagation result is 45% shoot growth in DKW-C nutrient medium in which 1 mg/L GA3 + 0.5 mg/L IBA + 1 mg/L BAP and activated charcoal was added. According to recent studies, Mitrofanova et al. (2021) in clematis plants explained that most adventitious micro shoot regeneration without any morphological abnormalities was obtained on the medium supplemented with BAP or TDZ. The best shoot propagation was determined on 1-cm long segments with a single node and basal MS medium supplemented with 4.40 µM BAP or 6.0 µM TDZ.

Table 2. Effects of different concentrations and combinations of PGRs on regeneration of shoots after 63 days of culture from apical and lateral buds of walnut species

Medium Number (Table 1)	**1**	**2**	**3**	**4****	**5**
			Number of shoot induction per explant		
Chandler	1.60±0.20bc	1.00±0.00cd	2.50±0.42ab	2.90±0.80a	0.70±0.60d
Yalova	1.80±0.20bc	1.06±0.93dc	2.50±0.42ab	2.90±0.23a	0.90±0.23d
Vlanch	1.50±0.30bc	1.30±0.30bc	2.33±0.60ab	2.93±0.90a←	0.70±0.56c
			Average shoot length		
Chandler	3.11±0.20b	2.60±1.40b	5.03±0.60a←	4.22±1.20ab	0.90±0.60c
Yalova	1.80±0.85ab	1.11±0.20cb	1.60±0.21b	2.70±0.60a	0.60±0.21c
Vlanch	1.40±0.40b	1.60±0.21b	1.70±0.34b	2.70±0.60a	0.51±0.90c
			Number of leaves per shoot		
Chandler	2.70±0.58b	3.00±0.00b	3.00±0.00b	5.00±0.00a	2.00±0.00c
Yalova	3.00±0.00b	3.00±1.00b	4.00±0.00b	5.66±0.60a←	3.33±0.60b
Vlanch	2.00±0.00b	3.00±1.00b	5.00±0.00a	4.66±0.60a	2.66±0.60b

**Means shown by different small letters in a single column are statistically different at 0.01 level of significance using Duncans multiple range test

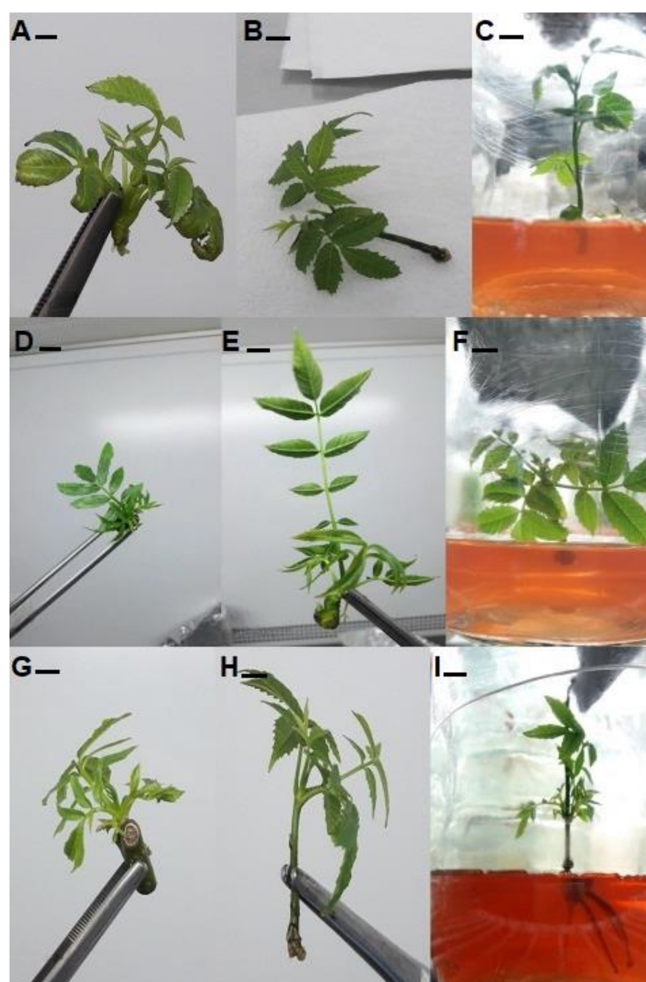

Figure 2. In vitro shoot regeneration from apical and lateral bud's explants of chandler (A) Single shoots elongated as a result of 3 subcultures of chandler (B) Yalova1 species (C) Vlach (D and E) Yalova1 species (H) Rooting of in vitro regenerated shoots using MS medium with 19.61µM of IBA and 348.42 µM of $FeSO_4 7H_2O$ in Yalova1(I). Scale bar =0.5 cm

3.4. Rooting and acclimatization

In the propagation of woody plants, the rooting of shoots is often problematic. Rooting the obtained shoots is a time-consuming process. Survival of rooted microcuttings requires that the roots support the plant while new leaves and stems are produced during acclimatization (McClelland, 1990). Losses at this stage have vast economic consequences. Rooting of walnut shoots is more challenging and difficult than other woody plants (Ahuja, 2013). In this study, *in vitro* regenerated 6-8 cm long shoots in 3 species of walnut were transferred to DKW, MS, ½×MS medium basal medium (Saadat et al., 2002) with 4.90, 9.8, 14.7, and 19.6 µM IBA and 174.20 µM, 348.42 µM, 522.62 µM $FeSO_4.7H_2O$. The first indications of rooting appeared after 2×subcultures (42 days) on MS medium containing 14.7 and 19.6 µM IBA and 348.42 $FeSO_4.7H_2O$ with 10.33 rooted shoots obtained in 19.6 µM of IBA and 348.42 $FeSO_47H_2O$ with an average 2.5 cm long roots, and six rooted shoots obtained in 14.7 µM of IBA and 348.42 µM $FeSO_4.7H_2O$ with an average of 2.2 cm root length (*Figure 3*). DKW basal medium with 4.90, 9.8, 14.7, and 19.6 of IBA caused necrosis on the treated shoots. Prolonged culture of the shoot on MS and ½×MS medium with 4.90, 9.8, 14.7, 19.6 µM of IBA and 174.20 µM, 348.42 µM, 522.62 µM $FeSO_4.7H_2O$ tended to induce callus formation that inhibited root growth.

Figure 3. Yalova 1 sapling was transferred to pots in growth chamber (A and B) The saplings are ready for transfer to fields with profuse growth of leaves (C and D). Scale bar=1 cm

It was visible that this basal medium with different concentrations of IBA $FeSO_4.7H_2O$ was unsuitable for rooting. Only 6 rooted plantlets were acclimatized to external conditions after their transfer to pots. After 90 days, rooted plants were available for transfer to the field (Figure 3) (Table 3). These results were supported by Kepenek ve Kolağası (2016), who reported that they obtained the best rooting in walnut from a medium containing 5 mg L^{-1} IBA. Tuan et al. (2016) defined the best root growth in different hybrid walnuts in the culture conditions of vermiculite + gelrite added to a DKW medium containing 12 µM IBA. Zarghami and Salari (2015) evaluated different IBA concentrations for rooting and reported that the medium containing 3 mg L^{-1} IBA gave successful results in Chandler species. Licea-Moreno et al. (2015) substituting ethylenediaminetetraacetate ferric sodium (FeEDTA) with ethylenediamine di-2-hydroxy-phenyl acetate ferric (FeEDDHA) diminished chlorotic symptoms and significantly improved the rooting ability of all genotypes in walnut shoots, with up to 90 % micro shoots with viable developing roots using 6.81 mg L^{-1} Fe_3^+. Considering the results obtained from rooting of other plants, Sotiropoulos et al. (2006) described that explants of pear rootstock 'OHF 333' (*Pyrus communis* L.) supplied with 0.1 mM Fe in the form of (NH_4) (2) $[Fe(SO_4)^2].6H_2O$ had higher rooting percentage, as well as significantly more

number of roots. Trejgell et al. (2012) explained that treatment *of Carlina onopordifolia* shoots with IBA for 5 s (short pulse) and growing them on MS medium supplemented with FeEDTA, increased induction of roots per shoot that were significantly higher than the number of roots on medium containing FeEDDHA. Similarly, Zawadzka et al. (2009) investigated the dependence of *in vitro* rooting and acclimatization to greenhouse conditions on the source of iron used in the shoot multiplication and rooting medium using five raspberries (*Rubus idaeus* L.) cultivars ('Beskid', 'Canby', 'Malling Seedling', 'Norna' and 'Veten'). FeEDDHA in the rooting medium led to higher chlorophyll contents, earlier and more abundant rooting (8.7 vs. 5.3 roots per shoot), 30% higher fresh and dry weights, and thus higher quality micro plants than FeEDTA.

Table 3. Effect of high dose of IBA, $FeSO_4.7H_2O$ and nutrient medium on in vitro rooting number/ acclimatization of walnut shoots

	IBA (µM)			**IBA (µM)**			**IBA (µM)**			**IBA (µM)****		
	4.90			9.82			14.73			19.6 1		
	$FeSO_4.7H_2O$			**$FeSO_4.7H_2O$**			**$FeSO_4.7H_2O$**			**$FeSO_4.7H_2O$**		
	174.2	348.4	522.6	174.2	348.4	522.6	174.2	348.4	522.6	174.2	348.4	522.6
MS medium	-	-	-	-	-	-	-	6/0b	-	-	10.33/6a**	-
½ MS medium	-	-	-	-	-	-	-	-	-	-	-	-
DKW medium	-	-	-	-	-	-	-	-	-	-	-	-

**Means shown by different small letters in a single line are statistically different at 0.01 level of significance using Duncans multiple range test

3.5. In Vitro Micrografting

In vitro micrografting is a technique consisting of an apex taken from a mother plant onto a decapitated young plant grown from a seedling under aseptic conditions or a micro-cutting obtained from *in vitro* micropropagation (Camas et al., 2014). As a result of this study, six seedlings of Vlach species were prepared after removed leaves and cotyledons. The seedlings were decapitated then a vertical cut of 0.5 cm was made from the apical end. Chandler species *in vitro* micropropagated shoots were used as a source of scions. *In vitro* grafting was performed by inserting the wedge-shaped end of scions into the vertical cut of rootstocks, and the grafted place was wrapped

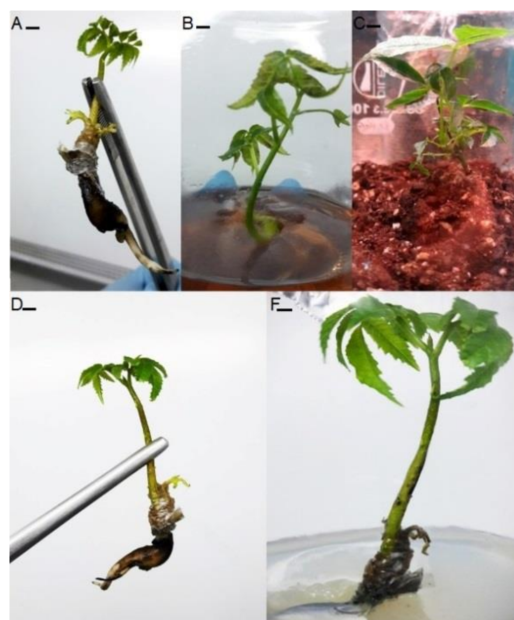

Figure 4. Micrografted sapling Vlach with in vitro micropropagation Chandler shoots (A) Successful micrografted sapling Vlach with in vitro micropropagation Chandler shoot MS medium with 19.6 1 µM of IBA and 348.42 µM of $FeSO_47H_2O$ (B) Micrografted sapling transferred to pots in growth chamber (C) Micrografted sapling Vlach with in vitro micropropagation (D) Could not be survived micrografted sapling in 0MS medium (E). Scale bar=1 cm

using sterilized transparent tape after grafting. Micrografting was performed by using the wedge technique. After the plants were cultured for 63 days (3×subculture) on MS basal medium with 4.90, 9.8, 14.7, and 19.6 µM IBA and 174.20 µM, 348.42 µM, 522.62 µM $FeSO_47H_2O$ two micrografted plants in MS medium survived. No difficulties were observed during the formation of the micrografted plants (*Figure 4*).

Our findings are consistent with those of Koufan et al. (2020) seedlings of *Argania Spinosa* L. to half-strength Murashige and Skoog (½×MS) medium reinforced with 1 mg/L GA3 and 1 mg/L BAP resulted in the highest shoot and root lengths (2.05 and 5.73 cm, respectively). Scions were taken from axillary shoots developed *in vitro* from micro-cuttings of genotype G41. After two months of culture, 65-100% of the micrografted plants survived, explaining similar results in other plant species such as cashew (*Anacardium occidentale L.*). The remarkable growth of micro-scions was observed with hypocotyls rather than with epicotyls. Rooting of micrografted shoots of mature tree origin was poor (13.3%) because the shoots were only partially rejuvenated (Mneney and Mantell, 2001).

4. Conclusions

Based on the results, a challenging-to-generate species of walnut can be produced with the appropriate amount of FeSO4.7H2O, a high dosage of indole-3-butyric acid, a nutrient medium, and an established trial plan. Developing effective shoot propagation, rooting, acclimatization, and micrografting, using apical and lateral buds gave a chance to utilize biotechnological techniques with high potential outcomes for propagation in this plant. The effect is considerable and assigns admissible data on commercial and agricultural proliferation in walnut species. Expansion of this study intentionally may help in the multiplication of different species of walnuts with unlimited consistent and safe access throughout the year.

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Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: Şeyda SAVALAN; Design: Şeyda SAVALAN, Tuba TÜREN; Data Collection or Processing: Tuba TÜREN; Statistical Analyses: Şeyda SAVALAN, Tuba TÜREN; Literature Search: Tuba TÜREN, Elif Ceren PEHLİVAN; Writing, Review and Editing: Şeyda SAVALAN, Tuba TÜREN, Elif Ceren PEHLİVAN.

References

Ahuja, M. R. (2013). Micropropagation of woody plants (Vol. 41). Springer Science Business Media, Netherlands.

Al-Mizory, L. S. M. and Mayi, A. A. (2012). *In vitro* propagation of walnut (*Juglans Regia*) by nodal explants. *Journal of Agricultural Science and Technology,* 2: 665-670.

Camas, R. U. S., Rivera-Solís, G., Duarte-Aké, F. and De-la-Peña, C. (2014). *In vitro* culture: an epigenetic challenge for plants. *Plant Cell Tissue Organ Culture*, 118: 187-201. https://10.1007/s11240-014-0482-8

Chandra, S., Bandopadhyay, R., Kumar, V. and Chandra, R. (2010). Acclimatization of tissue cultured plantlets. from laboratory to land. *Biotechnology Letters*, 32: 1199-1205.

Daneshvar, R. (2019). Efficient approaches to *in vitro* multiplication of *Lilium candidum* L. with consistent and safe access throughout year and acclimatization of plant under hot-summer Mediterranean (Csa Type) climate. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 47: 734-742.

Deb. C. R. and Imchen, T. (2010). An efficient *in vitro* hardening technique of tissue culture raised plants. *Biotechnology*, 9: 79-83.

Dong, P., Lichai, Y., Qingming, W. and Ruisheng, G. (2007). Factors affecting rooting of *in vitro* shoots of walnut cultivars. *The Journal of Horticultural Science and Biotechnology,* 82: 223-226.

Driver, J. A. and Kuniyuki, A. H. (1984). *In vitro* propagation of Paradox walnut rootstock. *Horticultural Science*, 19: 507-509.

Estrada-Luna, A. A., López-Peralta, C. and Cárdenas-Soriano, E. (2002). *In vitro* micrografting and the histology of graft union formation of selected species of prickly pear cactus (*Opuntia* Spp.). *Scientia Horticulturae,* 92: 317-327.

Fidancı, A. (2005). Şebin ve K2 ceviz çeşitlerin *in vitro*'da hızlı çoğaltılma tekniklerinin belirlenmesi. *Bahçe*, 34: 239-245.

Gotea, R., Gotea, I., Radu, E. S. and Vahdat, K. (2012). *In vitro* propagation of several walnut cultivars. *Bull UASVM Horticulture*, 69: 167-171.

Jain, S. M. and Ishii, K. (Eds.) (2012). Micropropagation of woody trees and fruits (Vol. 75). Springer Science Business Media, Netherlands.

Jay-Allemand, C., Capelli, P. and Cornu, D. (1992). Root development of *in vitro* hybrid walnut microcuttings in a vermiculite-containing gelrite medium. *Scientia Horticulturae*, 51 (3-4): 335-342.

Kefayeti, S., Kafkas, E. and Ercisli, S. (2019). Micropropagation of 'Chester thornless' blackberry cultivar using axillary bud explants. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* 47(1): 162-168.

Kepenek, K. and Kolağasi, Z. (2016). Micropropagation of walnut (*Juglans regia L.*). *Acta Physica Polonica A.,* 130(1): 150-156.

Khawar, K. M. and Özcan, S. (2002) High frequency shoot regeneration from cotyledonary node explants of different lentil (*Lens culinaris* Medik) genotypes and *in vitro* micrografting. *Biotechnology & Biotechnological Equipment,* 16: 12-17.

Koufan, M., Mazri, M. A., Essatte, A., Moussafir, S., Belkoura, I., El Rhaffari, L. and Toufik, I. (2020). A novel regeneration system through micrografting for *Argania Spinosa* (L.) skeels and confirmation of successful rootstock-scion union by histological analysis. *Plant Cell Tissue Organ Culture,* 142(2): 369-378.

Land, S. B. and Cunningham, M. (1994) Rooted Cutting Macropropagation of Hardwoods, In Foster G, Diner AM, eds, Applications of Vegetative Propagation in Forestry, Proc of The Southern Regional Information Exchange Group Biennial Symposium On Forest Genetics, Southern Forest Experiment Station New Orleans, Louisiana pp. 75-96.

Licea-Moreno, R. J., Contreras, A., Morales, A. V., Urban, I., Daquinta, M. and Gomez, L. (2015). Improved walnut mass micropropagation through the combined use of phloroglucinol and FeEDDHA. *Plant Cell Tissue Organ Culture,* 123(1): 143-154.

Martinez. L. M., Labuckas, D. O., Lamarque, A. L. and Maestri, D. M. (2010). Walnut (*Juglans regia* L.): Genetic Resources, Chemistry, By-Products. *Journal of the Science of Food and Agriculture,* 90: 1959-1967.

McClelland, M. T., Smith, M. A. L. and Carothers, Z. B. (1990). The effects of *in vitro* and *ex vitro* root initiation on subsequent microcutting root quality in three woody plants. *Plant Cell Tissue Organ Culture,* 23: 115-123.

Mcgranahan, G., Leslie, C. A. and Driver, J. A. (1988). *In vitro* propagation of mature Persian walnut cultivars. *Horticulture Science*, 23: 220.

Mitrofanova, I., Ivanova, N., Kuzmina, T., Mitrofanova, O. and Zubkova, N. (2021). *In vitro* regeneration of clematis plants in the nikita botanical garden via somatic embryogenesis and organogenesis. *Frontiers in Plant Science,* 12: 1-18.

Mneney, E. E. and Mantell, S. H. (2001). *In vitro* micrografting of cashew. *Plant Cell, Tissue Organ Culture,* 66: 49-58.

Murashige, T., Bitters, W. P., Rangan, T. S., Nauer, E. M., Roistachek, C. N. and Holliday, P. B. (1972). A technique of shoot apex grafting and its utilization towards recovering virus-free citrus clones. *Horticulture Science,* 7: 118-119.

Onay, A. (2000) Micropropagation of pistachio from mature trees. *Plant Cell Tissue Organ Culture,* 60: 159-163.

Paunović, S. M., Miletić, R., Janković, D., Janković, S. and Mitrović, M. (2013). Effect of humisol on survival and growth of nursery grafted walnut (*Juglans regia L.*) plants. *Horticultural Science*, 40: 111-118.

Pehlivan, E. C., Kunter, B. and Royandazagh, S. (2017) Choice of explant material and media for *in vitro* callus regeneration in Sultana grape cultivar (*Vitis vinifera L.*). *Journal of Tekirdag Agricultural Faculty*, Spec Issue: 30-34.

Rathore, J. S., Rathore, V., Shekhawat, N. S., Singh, R. P., Liler, G., Phulwaria, M. and Dagla, H. R. (2004). Plant Biotechnology and Molecular Markers, In A Narula, S Srivastava, eds, Micropropagation of Woody Plants, Anamaya Publishers, New Delhi, India, pp 195-205.

Ribeiro, H., Ribeiro, A., Pires, R., Cruz, J., Cardoso, H., Barroso, J. M. and Peixe, A. (2022). *Ex vitro* rooting and simultaneous micrografting of the walnut hybrid rootstock 'Paradox' (*Juglans hindsi*× *Juglans regia*) cl.'Vlach'. *Agronomy,* 12: 595.

Ribeiro, L. M., Nery, L. A., Vieira, L. M. and Mercadante-Simões, M. O. (2015). Histological study of micrografting in passion fruit. *Plant Cell Tissue Organ Culture,* 123: 173-181.

Rodriguez, R. (1982a). Callus initiation and root formation from *in vitro* culture of walnut cotyledons. *Horticultural Science,* 17: 195-196.

Rodriguez, R. (1982b). Stimulation of multiple shoot-bud in walnut seeds. *Horticultural Science*, 17: 92.

Rodriguez, R. and Sanchez-Tames, R. (1981). Cultivo detejitos y differentiation en nogal. *Revista de la Facultad de Ciencias Universidad de Oviedo Ser Biologia*, 22: 21-28.

Royandazagh, D. S. (2019). Potential of flow cytometry in sex determination and *in vitro* micropropagation of *Laurus nobilis* L. *Applied Ecology and Environmental Research,* 17: 5953-5964.

Royandazagh, S. D. and Pehlivan, E. C. (2016). In vitro Micropropagation of *Lilium candidum* L. and alkaloids. *Journal of Tekirdag Agricultural Faculty,* 13(3): 100-110.

Saadat, Y. A. and Hennerty, M. J. (2002). Factors affecting the shoot multiplication of Persian walnut (*Juglans regia* L.). *Scientia Horticulturae,* 95: 251-260.

Sammona, O. S., Abde Elhamid, N. A. and Samaan, M. S. F. (2018). Effect of some factors on the micropropagation and micrografting of some grape rootstocks *in vitro. Arab Universities Journal of Agricultural Sciences*, 26: 133-146.

Scaltsoyiannes, A., Tsoulpha, P., Panetsos, K. P. and Moulalis, D. (1997). Effect of genotype on micropropagation of walnut trees (*Juglans regia*). *Silvae Genetica*, 46: 326-332.

Sekmen, Ş., Sesli, Y. and Sarısu, H.C. (2017). Establishing *in vitro* Propagation Protocol for Chandler Walnut Cultivar. *Bahçe*, 46: 169-174.

Sotiropoulos, T. E., Almaliotis, D., Papadakis, I., Dimassi, K. N. and Therios, I. N. (2006) Effects of different iron sources and concentrations on *in vitro* multiplication, rooting and nutritional status of the pear rootstock 'OHF 333'. *European Journal of Horticultural Science*, 71 (5): 222-226.

Trejgell, A., Libront, I. and Tretyn, A. (2012). The effect of Fe-EDDHA on shoot multiplication and *in vitro* rooting of *Carlina onopordifolia* Besser. *Acta Physiologiae Plantarum,* 34: 2051-2055.

Tsutsui, H. and Notaguchi, M. (2017). The use of grafting to study systemic signaling in plants. *Plant and Cell Physiology,* 58: 1291-1301.

Tuan, P. E., Meier-Dinkel, A., Holtken, A. M., Wenzlitschke, I. and Winkelmann, T. (2016). Paving the way for large-scale micropropagation of juglans × intermedia using genetically identified hybrid seed. *Plant Cell Tissue Organ Culture,* 126: 153-166.

Xiaoying, M., Yufei, H. and Guogang, C. (2014). Amino acid composition, molecular weight distribution and gel electrophoresis of walnut (*Juglans regia L.*) proteins and protein fractionations. *International Journal of Molecular Sciences*, 15:2003-2014.

Yulan, F., Feng, G. and Wie, W. (2003). Sterilization of *Carya illinoensis* explants in tissue culture. *Journal of Anhui Agricultural University*, 31: 169-172.

Zarghami, R. and Salari, A. (2015). Effect of different hormonal treatments on proliferation and rooting of three persian walnut (*Juglans regia L.)* genotypes. *Pakistan Journal of Biological Sciences,* 18: 260-266.

Zawadzka, M. and Orlikowska, T. (2009). Influence of FeEDDHA on *in vitro* rooting and acclimatization of red raspberry (*Rubus idaeus L.)* in peat and vermiculite. *The Journal of Horticultural Science and Biotechnology,* 84: 599-603.