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Effects of Different Culture Media Compositions on *In Vitro* Micropropagation from Paradox Walnut Rootstock Nodes

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Abstract

The aim of this study is to establish an effective protocol for *in vitro* micropropagation from node explants of the 'Paradox' (*Juglans regia* x *J. hindsii*) rootstock. In the first stage of the study, the node explants were cultured in semi-solid media after being exposed to different sterilization methods. The most effective sterilization method was observed as ST2 with the lowest darkening rate (4 %). According to the results, SP4 medium provided the highest number of shoots per unit explant (1.05 shoots/explant) and 100 % shoot formation, SP2 medium provided the best mean shoot length (1.6 cm) and the mean number of nodes per unit explant (7.95 nodes/explant). Best root induction was provided from ½ Modified MS medium containing 4 mg/L IBA with 30 g/L sucrose. For root induction explants were retained under dark conditions for 7 days at 24±2ºC. In order to ensure root formation and elongation, shoot explants with root induction cultured in a 16-hour photoperiod (3500 lux) for 21 days in different nutrient media containing 2.4 mg/L Gelrite, hormone-free and mixed with vermiculite (1:1 v/v) and was rooting rate is 45.45 % in ¼DFe medium.

Cite

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1. INTRODUCTION

Systematically, walnut is included in Juglandales (Walnuts) order, Juglandaceae (Walnuts) family, and Juglans (Walnut) genus. Walnut has 2n=32 chromosomes and 25 species, and the most important and first thing that comes to mind is *Juglans regia* L.. This species is known in the world as "Anatolian walnut", "Persian walnut" and "English walnut" and is widely cultivated especially for its fruit (Şen, 2011). Walnuts are consumed a lot, especially in the form of dried fruit. The bark, fruit peel, green fruit peel and leaf parts of the walnut plant are widely used in the pharmaceutical and cosmetic industry, and as a dyestuff in the carpet and textile industry (Yiğit et al., 2009).

The important walnut producing countries in the world are China, the United States of America, Iran and Turkey. All walnut production in the USA is obtained from closed walnut orchards established with standard varieties (Açıksöz, 2020). While our country was at the forefront of walnut production in the world in the 1970s, it fell to the second place in the 1980s, the third in the 1990s, and the fourth in the 2000s, and even the amount of walnuts we produce has become unable to meet the needs of our country (Aslansoy, 2012). The biggest obstacles to walnut production in our country are; high pH of the soil, walnut anthracnose disease and

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indoor worm. Therefore, it is necessary to expand the production and plantations of materials resistant to these stress factors in Turkey (Budak, 2010).

As with other fruit species, propagation by seed in walnuts is not a preferred propagation method due to genetic expansion, except for rootstock or seedlings and breeding studies. For this reason, the most frequently used reproduction method is grafting (Budak, 2010). However, grafting processes are dependent on many factors, which reduces the chance of success and alternative methods are sought (Er et al., 2017). Plant tissue culture allows intensive production of desired clones with its advantages. Therefore, it becomes an important option against traditional propagation methods such as seed, cutting, branching and grafting in various plant species. However, micropropagation of fruit species and especially nut species is more difficult than micropropagation of herbaceous plants. For this reason, it is extremely important to choose the appropriate physiological period and explant, to overcome the harmful effects of tannin and other toxic compounds in cultures, to provide an acceptable shoot reproduction rate, to obtain rooted shoots in the micropropagation of selected hard-shelled fruit clones (Şirin, 2014).

Plant tissue culture is a proven method that has long been used to establish and maintain a variety of different plant species. Plant tissue culture techniques cover the methods used to obtain the new plant product aimed the study from cell, tissues and organs transferred to a sterile nutrient medium (Gürel et al., 2013). Plant tissue culture consists of cutting plant tissues and their growth on a nutrient medium (Kumar & Loh, 2012). For this reason, the most important step in plant tissue cultures is the sterilization process (Kyte, 1987). Contaminations may originate from exogenous and endogenous microorganisms. Plant cultures can be contaminated by a wide variety of bacterial, fungal and viral organisms, many of which are species-specific (Varghese & Joy, 2016). These contaminants may show themselves immediately, or they may not show their effects in any way for a long time (Ray & Ali, 2016). Approximately 20-55 % of contamination losses in *in vitro* plant cultures are due to bacteria (Tekielska et al., 2019). It is aimed to remove invading microorganisms from the environment with the sterilization process applied during tissue culture studies. In studies carried out with plant tissue collected from the field or with the possibility of microbial contamination, surface sterilization with additional surfactant is required in addition to ethyl alcohol and/or bleach. Concentration and exposure time are determined experimentally depending on the type of explant used (Leelavathy & Sankar, 2016). Fu et al. (2003) in their study on *Carya illinoensis* (pecan) plant, the most appropriate sterilization method is to first soak it in 70 % EtOH and mix it in 0.2 % $HgCl_2$ (with the addition of a few drops of Tween 20) for 15 minutes and then rinsing with sterile distilled water 10 times. They also found that adding penicillin and streptomycin to the nutrient medium prevented contamination (Fu et al., 2003). Leal et al. (2007) stated that internal contamination is an important problem in micropropagation experiments with explants taken from mature walnut trees. In addition, they stated that darkening as a result of oxidation in the tissues during the surface sterilization stage caused a great reduction in shoot formation and this problem could be overcome by using young shoot explants that are more resistant to disinfection but are semi-woody (Leal et al., 2007).

Vahdati et al. (2009) stated that the success of tissue culture depends on the chemical composition of the culture medium and the micropropagation of the walnut is partially limited by the lack of a suitable culture medium. It was thought that the minerals present in similar proportions to those found in walnut seeds may provide an optimum environment for shoot micropropagation. The mineral composition of the seeds of five walnut cultivars 'Serr', 'Pedro', 'Lara', 'Hartley' and 'Ron de Montignac' was analyzed by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICPOES). In addition, the effect of Cu and myo-inositol on shoot length and rooting rate of explants was determined. Mineral concentrations in walnut seeds were reported to be 2 to 26 times higher than in Driver and Kuniyuki Walnut (DKW) medium. Based on the minimum mineral concentration determined in the seeds, two new media containing DKW macro and micronutrients at strengths of 1.5 and 2 were formulated. It has been reported that explants cultured in DKW nutrient medium at 2 and 1.5 power form shoots with green leaves, but they produce callus similar to those cultured in DKW nutrient medium. The researchers, who stated that a better growth was observed in DKW medium at 1.5 power, found the growth rates different in all the cultivars they used. They stated that the maximum shoot length and the number of lateral buds were obtained in “Vina” variety at 1.5 DKW. It has been shown that the number of lateral buds per shoot and shoot length is increased with higher Cu and myo-inositol concentrations. It was stated that better rooting (up to 70 %) was obtained when the shoots were placed in media containing 10x Cu and 2x concentrations of myoinositol in nutrient medium. As a result, the researchers stated that some walnut

cultivars should be grown in environments with higher mineral concentrations than DKW nutrient media, and that higher Cu and myo-inositol levels may be effective in increasing the growth rate and rooting rate of walnut explants (Vahdati et al., 2009). In another study conducted by Kepenek and Kolağası (2016) in *J. regia* L., it was determined that the multiplication rate increased depending on the TDZ and IBA concentrations. It was also stated that the highest multiplication number (5.33 shoots) was obtained in NGE medium containing 2.5 mg/L TDZ and 2.5 mg/L IBA (Kepenek & Kolağası, 2016).

One of the main problems with tissue culture techniques in walnut micropropagation is the low propagation rate. It has been reported that the reason for this low rate is the slow growth of the regenerated shoots, the long culture times and the hidden contamination in the culture (Revilla et al., 1989). On the other hand, Fidancı (2005) stated that the most important problem in the culture initiation phase in micropropagation studies carried out in walnuts is contamination and darkening (Fidancı, 2005). They also reported that the propagation of *J. regia* L. varies widely according to the genotype, and slow *in vitro* growth causes low reproduction rates and difficulties encountered when traditional rooting methods are used (Tuan et al., 2017). In countries where walnut cultivation is developed, many studies have been carried out on the reproduction of walnuts by tissue culture and significant knowledge has been obtained. Most of these studies focused on the appropriate growth regulator concentration to be added to the culture medium. Some researchers have stated that one of the most important problems encountered in the reproduction of walnuts by tissue culture is the darkening caused by phenolic substances. It has also been reported that there are significant differences between genotypes in terms of shoot formation rate and shoot length (Şirin, 2014). In micropropagation studies carried out in walnut species, the desired success has not been achieved yet. The main reason for this is that almost all species of walnut plant are resistant to micropropagation. There are some limitations encountered in the micropropagation of walnut and still waiting to be resolved. Factors such as explant-induced internal contamination, darkening, low reproduction and rooting rate limit the micropropagation success of the walnut, and these limitations are thought to arise depending on the genotype. For this reason, there is a need for more detailed research and development of *in vitro* studies, especially in walnut species (Licea-Moreno et al., 2020).

Paradox rootstock is a first generation hybrid (F1) obtained from pollination of *J. hindsii* and *J. regia*. The apical and lateral meristems of the Paradox were discussed by Driver and Kuniyuki to examine the *in vitro* propagation of this rootstock. It has been stated that DKW, a walnut-specific medium, provides optimum multi-shoot growth with the addition of 4.5 µM benzyladenine (BA) and 5 nM indolebutyric acid (IBA), and the multiplication coefficient can be up to five (Driver & Kuniyuki, 1984). On the other hand, Bosela and Michler (2008) observed a lower rate of vitrification in black walnut (*Juglans nigra* L.) environments in high salt environments (DKW and MS) than in low salt environments (WPM and ½ DKW). Shoot regeneration occurred in node explants in all media containing Zeatin, BAP and TDZ, however, the highest shoot length was obtained in nutrient media containing combinations of BAP and Zeatin as a result of the eight-week culture period (Bosela & Michler, 2008).

One of the most important limitations in micropropagation of walnut species is the low rooting rate. Researchers have tried various methods to overcome this problem (Scaltsoyiannes et al., 1997). Jay-Allemand et al. (1992) determined that the use of ¼ strength DKW and vermiculite (250/200 h/h) promoted root elongation and secondary root development in two-week cultures. In the study, it was also determined that rooting increased by 15-50 % and the number of primary roots was 2-6 times higher than the control group. Researchers; also reported that root penetration and aeration of vermiculite were higher than gelrite and weak rooting was observed in the trials in which only distilled water was added to vermiculite (Jay-Allemand et al., 1992). In another study by Caboni and Damiano (2006), the critical factors to obtain a good rooting response are; ½ strength DKW medium containing 10µM IBA and a 10-12 day dark treatment (induction phase) has been reported. It has been stated that it is also important to transfer the microshoots to a hormone-free environment after the induction phase (Caboni & Damiano, 2006). Tuan et al. (2017) used liquid and semi-solid nutrient media prepared with Rugini and DKW media containing different concentrations of IBA and BAP in their study on walnut. As a result of 21 days of culture, while a higher percentage of fresh weight was obtained in Rugini medium, it was determined that solid medium was more suitable for shoot propagation and the best plant growth regulator and concentration was 2.2 µM BA. It was determined that 5 days of darkness was required for rooting in semi-solid media containing 12 µM IBA and there was no significant difference between ¼ DKW + Vermiculite and ¼ MS + Vermiculite media (Tuan et al., 2017). Steven and Pijut achieved

shoot regenerations in semi-solid DKW medium supplemented with 8.9 µM BAP, 0.005 µM IBA, 200 mg/L casein hydrolyzate, 50 mg/L adenine hemisulphate, 2 mL/L PPM™, and 4.1 µM MT. Micropropagation of shoots was achieved when cultured in a 3L polycarbonate Fernbach-style flask in liquid initiator medium on a rotary shaker at 25°C under a 16 hour photoperiod (100 rpm). For rooting, 5-week cultures were carried out in DKW medium containing 0.11 % Phytagel, 50 µM IBA and vermiculite (2:1 v/v) (Stevens & Pijut, 2018).

In this study, it was aimed to establish an effective micropropagation protocol by providing complete plant regeneration in Paradox ~~variety~~, which is one of the important walnut rootstocks. In addition, it is aimed to apply different sterilization methods in order to prevent darkening and contamination problems that cause difficulties in micropropagation of walnut species. After determining the most suitable sterilization method, experiments were carried out under semi-solid culture conditions in order to ensure shoot induction and determine the maximum multiplication coefficient. It is based on the establishment of a suitable micropropagation protocol in this walnut variety, which is generally known to be resistant to *in vitro* culture by transferring the obtained shoots to rooting media.

2. MATERIAL AND METHOD

2.1. Material

As the material, node explants of "Paradox" walnut rootstock obtained from the greenhouse in Menemen/Yahseller region by ULU CEVİZ Tarım Ürünleri Sanayi ve Ticaret Anonim Şirketi were used.

2.2. Method

Semi-solid media composition

In the study, DKW (Driver & Kuniyuki, 1984), Rugini (Rugini, 1984) and MS (Murashige & Skoog, 1962) nutrient media were prepared to be used in different micropropagation stages, with full power, half power, modified form and different doses of plant growth regulators (Table 1, Table 2).

Sterilization

The fresh shoots of Paradox walnut rootstocks taken from the greenhouse were brought to the laboratory in a humid environment immediately after they were cut from the tops in spring and summer. The explants separated from their leaves were cut with one or two nodes in each piece. The cut explants were rinsed with detergent water for 10 minutes. It was then kept under running water for 10 min. After the pre-sterilization process, the explants were subjected to 4 different sterilization processes in a laminar flow cabinet (Table 3). After deciding on the best nutrient medium and sterilization process, attempts were made to establish node cultures.

Shoot induction

In the sterilization trials, it was decided that the most successful sterilization method determined by statistical analysis was ST2. Node explants sterilized by the ST2 method continued to be cultured. For shoot induction experiments, node explants were cultured in three replications with 10 explants in each replication in glass tubes containing DKW and Rugini-based nutrient media, whose contents were given in Table 2, at 1/2 strength, as well as modified and/or plant growth regulator added versions of these media.

In vitro rooting

Rooting experiments were carried out with propagated shoots. The media and contents used for rooting experiments are given in Table 4. Rooting experiments were carried out in 2 stages (Caboni & Damiano, 2006; Peixe et al., 2015). In the first stage, 2-3 cm long micro-sprouts transferred to different medium (Table 4) were kept under dark conditions for a period of 7 days to ensure root induction. After root induction was achieved, it was transferred to different rooting media (Table 5) in a 21-day period. Attempt; randomized plots were established according to the experimental design with 3 replications and 10 explants in each replication. Culture dishes were stored in a photoperiod of 16 hours light/8 hours dark, at 24±2°C and 3500 lux light intensity with white LED illumination.

Table 1. *The main nutrient media ingredients used in the research (pH:5.8)*

Compounds (mg/L)	DKW (Driver & Kuniyiki, 1984)	Modified DKW (MDKW) (Fidancı, 2005)	Rugini (Rugini, 1984)	MS (Murashige & Skoog, 1962)	Modified MS medium
NH_4NO_3	1416.00	708	412.00	1650	1650
H_3BO_3	4.80	4.8	12.40	6.2	6.2
$CaCl_2.2H_2O$	147.00	74.5	440.00	440	440
$Ca(NO_3)_2.4H_2O$	1960.00	984	600.00	-	-
KNO_3	-	1100.00		1900	1900
KCl	-	500.00		-	-
KI	-	-	-	0.83	0.83
$CuSO_4.5H_2O$	0.25	0.25	0.25	0.025	0.025
$MgSO_4.7H_2O$	740	335	1500.00	370	720
$MnSO_4.H_2O$	33.50	33.5	16.90	16.9	16.9
KH_2PO_4	259.00	132.5	340.00	170	265
K_2SO_4	1560.00	779.5	-	-	-
$Na_2MoO_4.2H_2O$	0.39	0.39	0.25	0.25	0.25
$ZnSO_4.7H_2O$	17.00	17.00	14.30	8.6	17
$CoCl_2.6H_2O$	-	-	-	0.025	0.025
$FeSO_4.7H_2O$	33.40	16.9	27.80	27.8	27.8
Na_2EDTA	44.70	22.7	37.30	37.3	37.3
Myo-inositol	1000.00	100	100.00	100	100
Tiamin-HCL	2.0	2.0	0.50	0.1	0.1
Nicotinic acid	2.0	1.0	5.0	0.5	0.5
Pyridoxine-HCl	-	-	0.50	0.5	0.5
Glycine	2.0	2	2.0	2.0	2
Biotin	-	-	-	-	
Folic acid	-	-	-	-	
Glutamine	-	-	2190	-	
Sucrose	30000	30000-40000	30000	30000	30000-40000

Statistical analysis

In vitro applications within the scope of the project were carried out in three replications according to the randomized plot design. The data obtained from the applications were evaluated using the Minitab® 17 statistical program.

Table 2. *Media composition*

	Basal medium	Plant growth regulators (mg/L)	Other added chemicals (mg/L)
DKW	DKW	-	-
D1B	DKW	1 BAP	-
D2B	DKW	2 BAP	-
D4B	DKW	4 BAP	-
SP	DKW	-	200 casein hydrolyzate + 50 adenine hemisulphate
SP1	DKW	1 BAP + 0.001 IBA	200 casein hydrolyzate + 50 adenine hemisulphate
SP2	DKW	2 BAP + 0.001 IBA	200 casein hydrolyzate + 50 adenine hemisulphate
SP4	DKW	4 BAP + 0.001 IBA	200 casein hydrolyzate + 50 adenine hemisulphate
Ru	Rugini	-	-
Ru1	Rugini	1 BAP	-
Ru2	Rugini	2 BAP	-
MRu0,5	Modified Rugini	0.001 IBA	200 casein hydrolyzate + 50 adenine hemisulphate
MRu1	Modified Rugini	0.001 IBA	200 casein hydrolyzate + 50 adenine hemisulphate

Table 3. *Sterilization process*

Sterilization code	Sterilization process	Medium
ST1	1 min 70 % EtOH + 5 min 0.1 % $HgCl_2$	DKW
ST2	5 min 70 % EtOH + 5 min 0.2 % $HgCl_2$ (Tween 20 added)	DKW
ST3	5 min 70 % EtOH+3 min 20 % bleach	D1B
ST4	1 min 70 % EtOH + 5 min 0.1 % $HgCl_2$ +5 min 12 % bleach	DKW

Table 4. *Medium composition for in vitro root induction**

Code	Basal medium	Plant growth regulators	Carbon sourse
1I30	MMS	4 mg/L IBA	30 g/L sucrose
1I40	MMS	4 mg/L IBA	40 g/L sucrose
½I30	½ MMS	4 mg/L IBA	30 g/L sucrose
½ 30	½ MMS	-	30 g/L sucrose
½ I40	½ MMS	4 mg/L IBA	40 g/L sucrose
MD304	MDKW	4 mg/L IBA	30 g/L sucrose
MD404	MDKW	4 mg/L IBA	40 g/L sucrose
MDFI	MDKW	4 mg/L IBA+119 mg/L FeEDDHA	30 g/L sucrose

*Gelrite: 2.4 g/L

Table 5. *Medium compositions for in vitro root growth (Carbon source: 30 g/L sucrose; Gelrite: 2.4 g/L)*

Code	Basal medium	Medium composition
MDFe	MDKW	119 mg/L FeEDDHA
¼ DFe	¼ DKW	119 mg/L FeEDDHA
MDV	MDKW	1:1 vermiculite
¼ DV	¼ DKW	1:1 vermiculite

3. RESULTS AND DISCUSSION

3.1. Result

Sterilization

The effects of the sterilization experiments on the observation parameters were examined and the data were recorded. Values were calculated as percentages. (Table 6 and Figure 1). According to its effect on contamination, the most successful method was determined as ST1. Contaminations seen in cultured explants were of bacterial or yeast origin. Generally, contaminations were observed 7 to 15 days after the culture period (Figure 2). When considered in terms of darkening effects, the ST4 method was found to be the most unsuccessful with 46.7 % on darkening. No darkening was observed in the explants cultured in the ST1 method. ST1 method was determined to be more successful than other methods in terms of darkening. Darkening problem usually emerged within the first week of node explants. According to the effect on vitrification, no vitrification problem was encountered when ST1 and ST2 methods were used and they were accepted as successful methods. The highest vitrification was recorded in the ST3 method with 33.3 %. The emergence of vitrification in the shoots occurred on the 7th day after the explants were cultured. According to the necrosis effect, ST3 was determined as the most unsuccessful sterilization method with 33.3 % necrosis formation percentage, no necrosis was observed in the ST1 and ST4 sterilization methods and they were considered successful. The emergence of necrosis in the shoots occurred on the 7th day after the explants were cultured. When these data were examined, ST1 was evaluated as a more successful sterilization method. However, due to increased contamination and darkening in the advancing processes of the culture, studies were continued with ST2, a mercuric chloride-based sterilization method containing Tween20®. In all trials after the sterilization trial, the explants were sterilized and cultured using the ST2 method.

Table 6. *Effects of different sterilization methods on darkening, contamination, vitrification and necrosis*

Sterilization code	Darkening (%)	Contamination (%)	Vitrification (%)	Necrosis (%)
ST1	0	13.3	0	0
ST2	12.5	37.5	0	8.3
ST3	4.17	37.5	33.3	33.3
ST4	46.7	20	3	0

Shoot Induction

In the trials where the effects of the medium in which the explants were transferred after sterilization on shoot initiation, the highest darkening was observed in the Ru medium with 72 %, while no darkening was observed in the MRu1 medium. While no vitrification was observed in most of the media (D4B, SP1, SP2, Ru2, MRu0,5 and MRu1), 33.3 % vitrification was detected in the D1B medium. While necrosis was not observed in DKW, D4B, SP, SP1, SP4, Ru1, Ru2, MRu0,5 and MRu1 nutrient media, necrosis occurred at a rate of 33.3 % in D1B media (Table 7 and Figure 3).

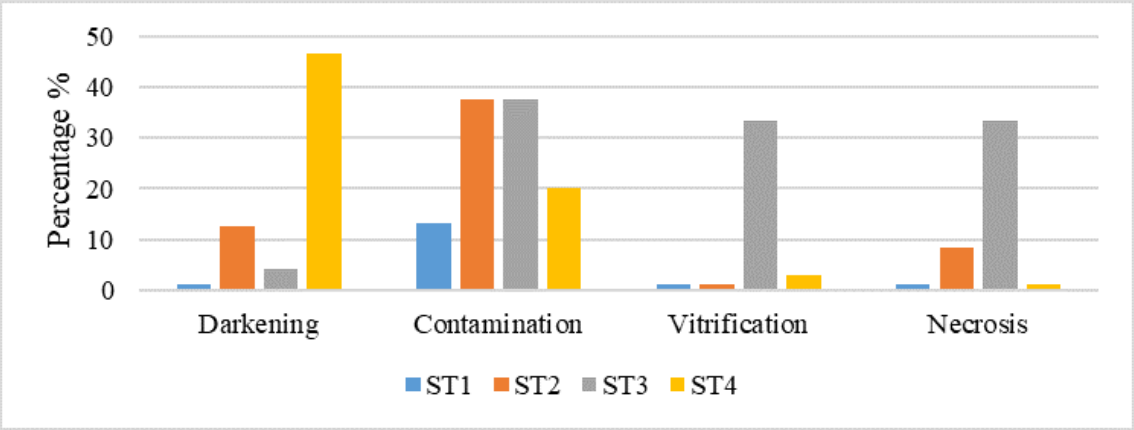

Figure 1. *Evaluation of different sterilization methods according to four observation parameters (darkening (%), contamination (%), vitrification (%), necrosis (%))*

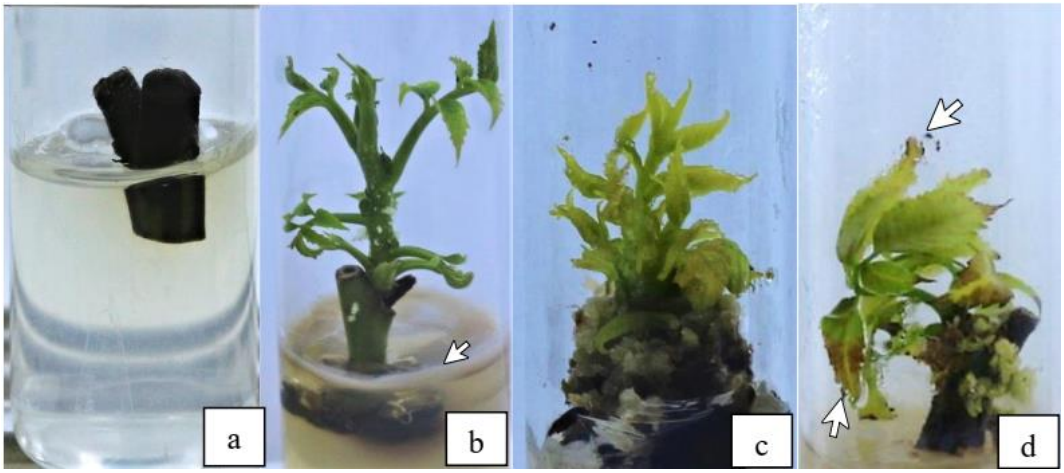

Figure 2. *Examples of problems encountered in walnut culture are* ***a)*** *darkening,* ***b)*** *contamination,* ***c)*** *vitrification,* ***d)*** *necrosis*

Table 7. *The effect of different medium composition on the percentages of darkening. vitrification and necrosis*

Code	Darkening (%)	Vitrification (%)	Necrosis (%)
DKW	46.7	3	0
D1B	4.17	33.3	33.3
D2B	8.3	12.5	4.17
D4B	5.6	0	0
SP	3.3	3.3	0
SP1	23	0	0
SP2	3	0	0
SP4	14	4.6	0
Ru	72	25	50
Ru1	47.3	9.3	0
Ru2	28	0	0
MRu0.5	4.67	0	0
MRu1	0	0	0

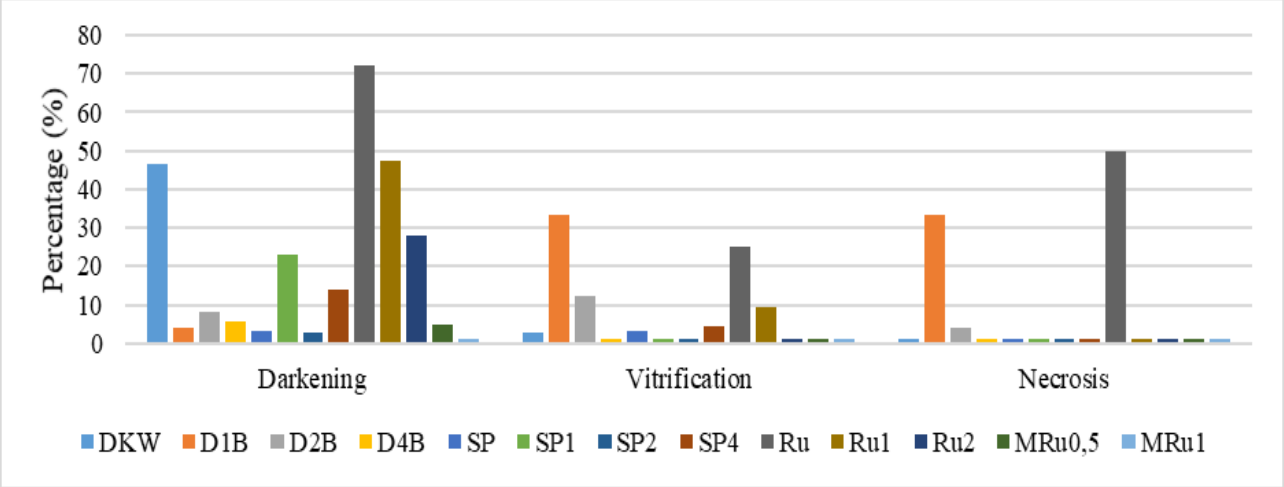

Figure 3. *The effect of different medium composition on the percentages of darkening, vitrification and necrosis*

As a result of the analysis of variance, the effect of the nutrient medium on the number of shoots and the percentage of shoot formation was found to be significant ($p < 0.05$) (Table 8). The most successful nutrient medium was SP4 with 1.05 units/explant and 100 % shoot formation. According to these parameters, the least successful nutrient medium was Ru nutrient medium with 0.57 units/explant and 57 % shoot formation (Table 8, Figure 4 and Figure 5).

Table 8. *Data from shoot initiation experiments**

Code	Average number of shoots obtained per explant (Mean ± S.E.)	Mean shoot length (cm) obtained per explant (Mean ± S.E.)	Percentage of shoot-forming explants (Mean ± S.E.)	Mean number of nodes obtained per explant (Mean ± S.E.)
DKW	0.90 ± 0.07 ab	1.04 ± 0.13 ab	80.00 ± 17.3 abc	4.33 ± 0.66 b
D1B	1.00 ± 0.07 ab	0.68 ± 0.12 bc	93.33 ± 5.77 ab	4.67 ± 0.64 b
D2B	0.90 ± 0.17 ab	0.43 ± 0.08 c	67.00 ± 6.93 abc	1.62 ± 0.34 c
D4B	1.00 ± 0.14 ab	0.41 ± 0.06 c	71.00 ± 6.93 abc	2.69 ± 0.44 bc
SP	0.71 ± 0.10 ab	0.37 ± 0.07 c	63.30 ± 30.6 bc	2.91 ± 0.49 bc
SP1	0.80 ± 0.09 ab	0.37 ± 0.07 c	81.00 ± 8.66 abc	3.95 ± 0.75 bc
SP2	0.95 ± 0.05 ab	1.60 ± 0.19 a	96.67 ± 5.77 ab	7.95 ± 0.69 a
SP4	1.05 ± 0.05 a	0.36 ± 0.09 c	100.0 ± 0.00 a	3.57 ± 0.50 bc
Ru	0.57 ± 0.11 b	0.23 ± 0.10 c	57.00 ± 14.0 c	1.57 ± 0.48 c
Ru1	0.67 ± 0.13 ab	0.63 ± 0.17 bc	61.67 ± 8.08 bc	2.76 ± 0.58 bc
Ru2	0.76 ± 0.10 ab	1.15 ± 0.20 ab	76.00 ± 8.66 abc	3.62 ± 0.58 bc
MRu0.5	0.90 ± 0.07 ab	1.04 ± 0.12 ab	90.67 ± 8.08 abc	3.71 ± 0.40 bc
MRu1	1.00 ± 0.07 ab	1.04 ± 0.12 ab	90.67 ± 8.08 abc	4.10 ± 0.23 bc
p value	0,009	0,000	0,001	0,000

*Applications were made in triplicate and 10 explants were used for each replication. The differences between the mean values shown with different letters in the same column are significant at the P≤0.01 level according to the Tukey multiple comparison test. SE: Standard Error

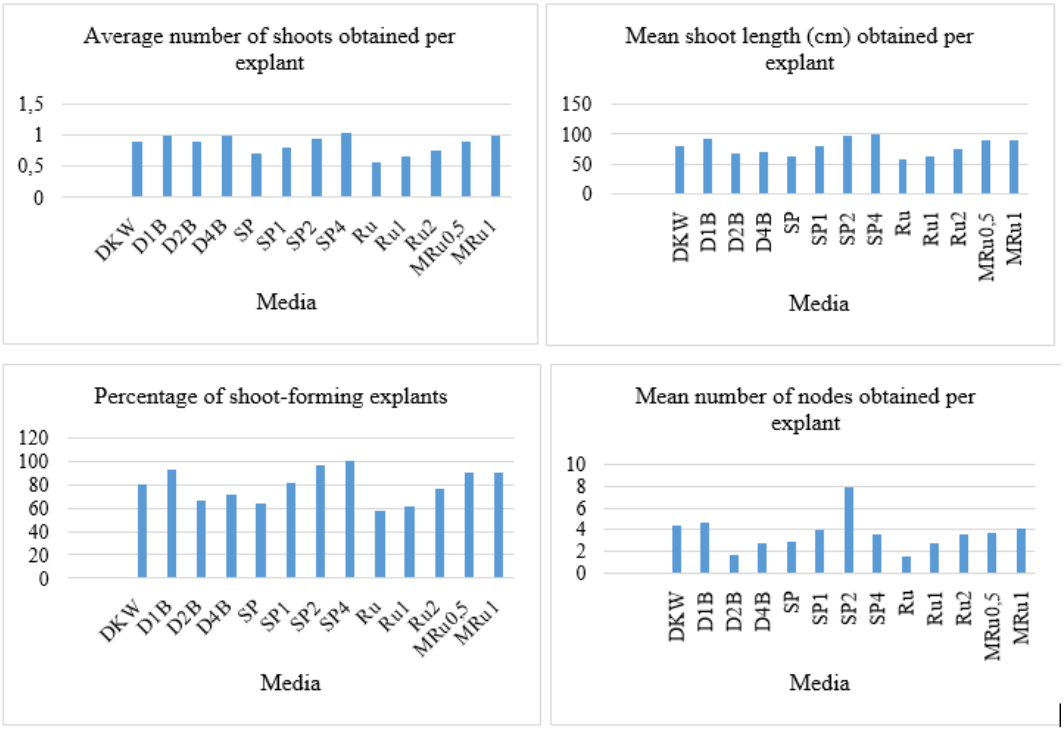

Figure 4. *Graphics from shoot initiation experiments*

Figure 5. *Shoot regeneration from each medium* ***a)*** *DKW* ***b)*** *D1B* ***c)*** *D4B* ***d)*** *SP1* ***e)*** *SP2* ***f)*** *SP4* ***g)*** *Ru* ***h)*** *Ru1* ***i)*** *Ru2* ***j)*** *MRu0,5* ***k)*** *MRu1*

In vitro rooting

Rooting was achieved in two stages in the study. Root induction was performed in the first stage, and the development of these roots was ensured in the second stage of the study. In the light of these data, the nutrient media that initiate the most effective induction process in rooting were determined as ½ I30, ½ I40, 1I30, MD304, ½ 30 and MDFI. The most successful root induction medium was determined as ½ I30 medium (33.3%) containing 4 mg/L IBA (Figures 6 and 7).

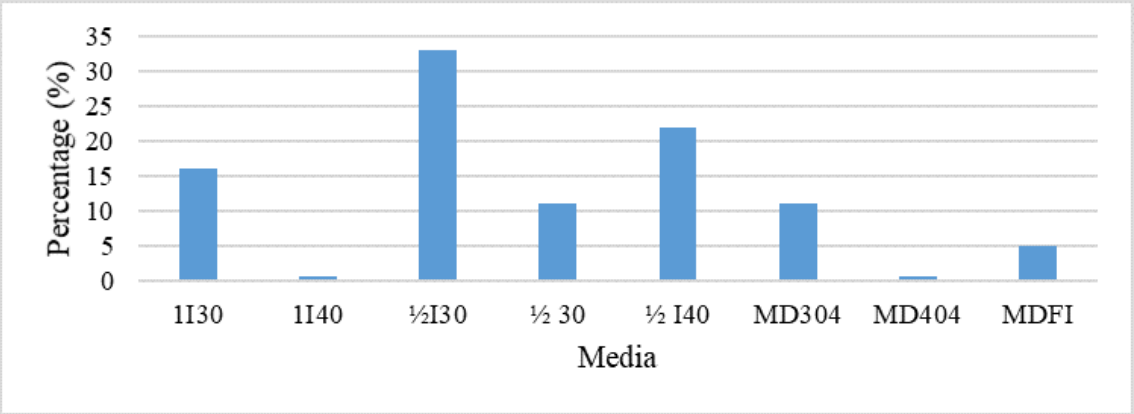

Figure 6. *Effect rates of root induction media on the initiation of root formation (%)*

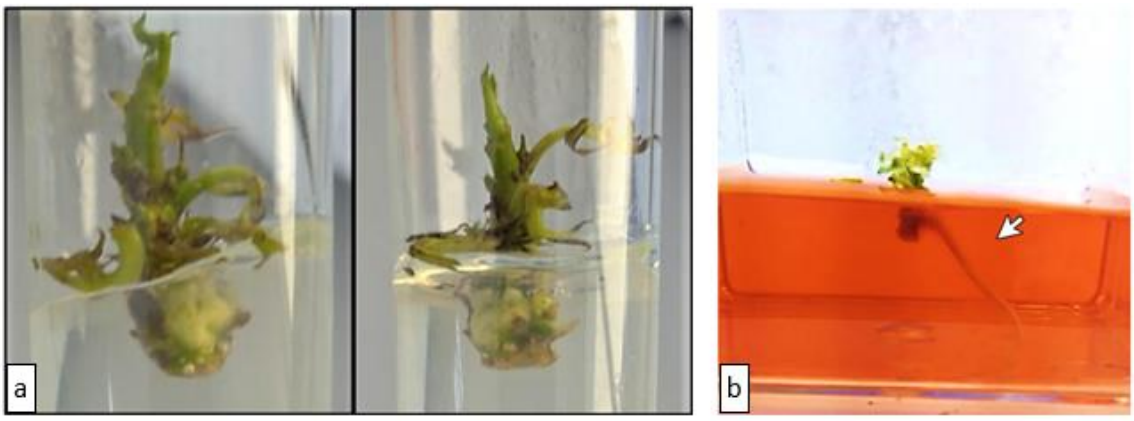

Figure 7. *In vitro rooting with two steps* ***a)*** *Root induction in MD304 medium,* ***b)*** *Root regeneration in ¼ DFe medium*

Root-induced shoots were cultures in different medium composition in Table 4 for root development during 21 days, 16 hours light/8 hours dark photoperiod, 24±2°C and 3500 lux light intensity with white LED illumination. The effects of the medium composition used in the experiments on the average root length (cm) and root number (number) were recorded. Observation data gave statistically insignificant differences (Table 9). It was determined that the most successful rooting medium was ¼ DFe. According to the results obtained from the study, the most successful rooting rate was recorded as 45.45 %. Depending on the nutrient media used, callus formations occurred at rates ranging from 0 to 77.7 % on the shoot bases (Figure 7). In root development experiments, callus formation occurred at the leaf tip of the growing shoot in the culture dish containing ¼ DFe medium. It has been determined that the adventitious root formation also consists of this callus tissue (Figure 8).

Table 9. *The effect of medium compositions on parameters in rooting experiments*

Rooting medium	Rooting regeneration ratio (%)	Longest root lenght (cm)	Average root lenght (cm)	Average root number (number)	Callus regeneration ratio (%)
MDFe	33.3	1	0.34	1	77.7
¼ DFe	45.45	2.5	1.22	2.4	54.5
MDV	11.1	2	2	1	11.1
¼ DV	0	0	0	0	0

Figure 8. *Callus regeneration on explants* ***a)*** *Callus and root structure formed in MDFe medium* ***b)*** *Callus and root formed at the leaf tip of the shoot developed in ¼ DFe medium*

3.2. Discussion

The first stage carried out within the scope of this study is the surface sterilization of the node explants. Elimination of internal contamination sources from walnut plant explants is the most important step in micropropagation studies. Because the darkening and contamination problem encountered in micropropagation studies with walnut varieties and the results of which cannot be clarified yet, should be eliminated. In tissue culture studies performed on all species of Juglans plant, it has been determined that they are quite stubborn against micropropagation. Darkening due to oxidation of the phenolic component in explants, internal contamination sources in explants, difficulties in adaptation to the culture medium, rooting and transfer to the soil are the most important problems of walnut micropropagation. Many scientific studies on walnut micropropagation have been published since the 1980s, but there is still not enough information to establish an accurate micropropagation protocol depending on the species (Kepenek & Kolağası, 2016; Licea-Moreno et al., 2020). Fidancı (2005) stated in his research using Şebin and KR-2 walnut varieties that the most important problem encountered during the cultivation phase of micropropagation is contamination and darkening.

In our study, it was thought that the most important problem to be solved was contamination and darkening, and studies were carried out in this direction. In the different sterilization trials, the highest contamination rate (37.5 %) was recorded in ST2 and ST3 trials, while the lowest contamination rate (13.3 %) was recorded in ST1. Similarly, while the highest darkening (46.7 %) was recorded in ST4, the lowest darkening (0 %) was recorded in the ST1 sterilization method. As a result of the trials carried out by applying different sterilization protocols, the sterilization method in which darkening (4 %) and contamination were minimized was determined as ST2 (5 min. 70 % EtOH + 5 min. 0.2 % $HgCl_2$-Tween 20 added). Dong et al. (2007) reported that for successful sterilization in regeneration experiments of walnut explants, the explants were kept in 70 % EtOH for a while, followed by sterilization with 0.1 % $HgCl_2$, and then rinsed with sterile distilled water. Şirin (2014), in order to develop a sterilization protocol in the axillary buds of Kaman-1 and Kaman-5 walnut varieties, exposed the explants to 70 % ethyl alcohol for 1 minute and then sterilized them in 0.2 % HgCl2 for 5 minutes. In order to prevent the intense darkening in walnut regeneration studies, it was tried to be prevented by adding 100 mg/L ascorbic acid and 100 mg/L citric acid, and it was reported that the study was continued by subculture at 3, 24 and 48 hour intervals.

In the study carried out by Scaltsoyiannes et al. (1997), to sterilize the embryos, the fruits were cracked and kept in 70 % EtOH for 5 minutes and then in 0.1 % NaOCl for 5 minutes, rinsed three times with sterile distilled water and the embryos were cultured. Sterilization success was determined as 0-59 % in the study. When compared with the study carried out by us, it is observed that the results show suitability.

In a study by Tarinejad (2013) on the sterilization of node planes in walnuts, it was determined that 70 % EtOH for 2 minutes, 5 % NaOCl for 10 minutes, followed by 0.7 % $HgCl_2$ for 3 minutes are required for sterilization.

In addition, it was determined that internal contaminations were revealed in the cultures that progressed in the trials in which antibiotics were not used. On top of that, it was determined that the sterilized explants should be cultured in media containing 12.5 mg/L gentamicin and 100 mg/L vancomycin to prevent this. The sterilization method was recommended by us is a more usable method because it includes the use of fewer sterilization agents and the application is shorter and more practical. In addition, a cheaper and commercially preferable method has been proposed due to the use of sterilization without the use of antibiotics.

One of the problems encountered in the studies carried out on walnut has been stated as the low reproduction rate. The reason for this was thought to be the long culture period and latent contaminations as a result of the slow growth of regenerated shoots (Revilla et al., 1989). Lone (2017) stated that woody species had a weaker *in vitro* micropropagation response than herbaceous plants, and this was due to darkening and explant necrosis. Necrosis can occur when plants do not have enough nutrients. Extremely low amounts of phosphorus initially cause the leaves of the plant to appear bright green, but older leaves become necrotic. Other nutrients that cause necrosis are potassium, nitrogen, boron, iron and nickel (Schader, 2019). In our study, the rate of necrosis and especially darkening was observed to be higher in Rugini environments in general. On the other hand, it was observed that the darkening decreased in the modified Rugini media created by adding casein hydrolyzate and adenine hemisulphate based on Rugini. It can be thought that the low salt concentration of Rugini media compared to MS and DKW media may cause darkening and necrosis, and the casein hydrolyzate and adenine hemisuflate explants added to the medium protect from this condition. In the light of this information, it can be stated that the sterilization method we carried out in the 'Paradox' walnut rootstock species is compatible with the literature and can help prevent darkening and contamination problems in walnut species.

In the study carried out by Tetsumura et al. (2002) on different walnut varieties, the highest shoot number was obtained as 1.7, while the highest shoot length was determined as 1.97 cm. In another study carried out by Kepenek and Kolağası (2016), the highest number of shoots obtained per explant was found to be 5.32, while the shoot length was 3.88 cm. In our study node explants with surface sterilization were transferred to the specified media for shoot induction. According to the results obtained at the end of 21 days; the highest number of shoots per explant (1.05 shoots/explant) was obtained in semi-solid SP4 (DKW containing 4 mg/L BAP + 0.001 mg/L IBA + 200 mg/L casein hydrolysate + 50 mg/L adenine hemisulphate) nutrient medium. In addition, the most successful medium was semi-solid SP2 (DKW containing 2 mg/L BAP + 0.001 mg/L IBA + 200 mg/L casein hydrolyzate+50 mg/L adenine hemisulphate) in terms of the highest number of nodes (7.95 nodes/explant) and shoot length (1,6 cm) per explant.

Scaltsoyiannes et al. (1997) in their regeneration study on *Juglans regia* L., it was found that the best axillary shoots were in DKW nutrient medium containing 4.44 µM BAP and 0.005 µM IBA, and the shoot length was maximum in culture medium containing 2.22 µM BAP. Saadat and Hennerty (2002), was stated that it gave better results in DKW medium than MS and WPM environments, and the use of Phytagel as a gelling agent was recommended. Tetsumura et al. (2002) obtained successful results in DKW nutrient medium with 5 µM BAP and 0.05 µM IBA. Fidancı (2005) tested MS, DKW, WPM media in Şebin and KR-2 walnut cultivars and the best growth was observed in DKW medium containing 1.0 mg/L BAP and 0.01 mg/L IBA. In our study, successful results were obtained in the formation of shoots in the presence of BAP from the node explants cultured in semi-solid DKW medium.

In their study, Meier-Dinkel and Wenzlitschke (2015) cultured nodal segments in DKW and ROM media containing BAP and IBA, and higher reproduction rate (7.59) and shoot length (2.1 cm) were achieved in ½ ROM media. In our study, Rugini and DKW media and their modifications were tested. DKW, which we tried at full strenght, gave higher shoot number than Rugini medium. In their study by Bosela and Michler (2008), vitrification of black walnut explants was observed at frequencies of 60-100 % in WPM and ½ DKW environments, compared to frequencies of 10-40% in high-salt environments (DKW and MS). In our study, more vitrification was found in DKW media than in Rugini media.

After the shoot induction and propagation stages, the shoots reaching 3-5 cm in length were taken and transferred to the specified rooting media. Rooting experiments were carried out in two stages. In the first stage, shoot explants taken into nutrient media for root induction were kept in dark conditions for 7 days. In this induction stage, the most successful medium (33 %) was ½I30 nutrient medium, whereas root induction

did not occur or explants died in nutrient media with the codes 1I40, ½30, MD404. In the next stage, shoot explants taken from dark conditions were transferred to rooting media containing different nutrient compositions in order to achieve root formation, and they were maintained in a light 16 hours light/8 hours dark photoperiod in a 21-day period. At the end of this period, the explants in ¼ DFe coded medium were most successfully rooted (2.4 cm root length, 1.22 root number). After root induction is achieved in shoots kept in dark conditions for a while, there is a lot of literature information about promoting root elongation under light conditions (Kamali et al., 2001; Fu et al., 2003; Caboni & Damiano, 2006; Tuan et al., 2017). Same as our study, in their study, Caboni and Damiano (2006), determined that the dark application was effective on adventitious rooting, which not only increased IBA uptake but also affected the concentration of endogenous auxin in free and conjugated forms. In Caboni and Lauri (1995) and Ripetti et al. (1994) studies, they stated that for the initiation of rooting in walnuts, the explants should be taken into an environment containing IBA first, and then the subculture should be made in a fresh hormone-free medium for the elongation of the roots.

4. CONCLUSION

The problems encountered in walnut production necessitated the preference of alternative methods. In this case, micropropagation studies have become an important alternative as they both enable clonal reproduction and enable seasonal production in a short time in a narrow space. In this study, micropropagation studies were carried out on the "Paradox" variety, which is the most demanded rootstock. When the given information is examined, the best media for shoot induction and also growth were found as SP4 and SP2 media based DKW medium. Rooting in two stages as root induction and elongation increased the rooting efficiency in the study. The development of these methods with future studies is of great importance for the development of walnut agriculture.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Açıksöz, U. (2020). *Bandırma İlçesindeki Ceviz Fidanı Üretiminin Mevcut Durumunun İncelenmesi*. MSc Thesis, Bursa Uludağ University.

Aslansoy, B. (2012). *Sultandağı (Afyon) yöresi cevizlerinin (Juglans regia L.) seleksiyon yoluyla ıslahı üzerine araştırmalar*. . MSc Thesis, Selçuk University.

Bosela, M. J., & Michler, C. H. (2008). Media effects on black walnut (*Juglans nigra* L.) shoot culture growth *in vitro*: Evaluation of multiple nutrient formulations and cytokinin types. *In Vitro Cellular and Developmental Biology - Plant*, *44*(4), 316-329. doi:10.1007/s11627-008-9114-5

Budak, Y. (2010). *Ceviz Yetiştiriciliği*. Samsun İl Tarım Müdürlüğü, Çiftçi Eğitimi ve Yayım Şubesi Yayınları, 20 p., Samsun. PDF

Caboni, E., & Damiano, C. (2006). *In vitro* propagation of walnut (*Juglans regia* L.): Critical factors for the induction of the rooting response. *Acta Horticulturae*, 705, 329-333. doi:10.17660/ActaHortic.2005.705.44

Caboni, E., & Lauri, P. (1995). Effetto di fattori chimici, fisici e trasformazione localizzata sulla radicazione *in vitro* di noce. *Italus Hortus*, *4*(2), 49-53.

Dong, P., Lichai, Y., Qingming, W., & Ruisheng, G. (2007). Factors affecting rooting of *in vitro* shoots of walnut cultivars. *The Journal of Horticultural Science and Biotechnology*, *82*(2), 223-226. doi:10.1080/14620316.2007.11512223

Driver, J. A., & Kuniyuki, A. H. (1984). In vitro propagation of Paradox Walnut Rootstock. *Hortscience*, 19(4). 507-509.

Er, E., Akyüz, B., Öztürk, A., & Serdar, Ü. (2017). Cevizde Çeşit Değiştirme: Kabukaltı Aşısı. *Bahçe*, *46*(Special Issue-2), 307-312.

Fidancı, A. (2005). Şebin ve KR-2 ceviz çeşitlerinin *in vitro*'da hızlı çoğaltılma tekniklerinin belirlenmesi. *Bahçe*, *34*(1), 239-246.

Fu, Y., Gu, F., & Wu, W. (2003). Sterilization of *Carya illinoensis* Explants in Tissue Culture. *Journal of Anhui Agricultural University*, *31*(2), 169-172.

Gürel, A., Hayta, Ş., Nartop, P., Bayraktar, M., & Fedakar S. O. (2013). *Bitki Hücre, Doku ve Organ Kültürü Uygulamaları*. Ege Üniversitesi Yayınları Yayın No: 58.

Jay-Allemand, C., Capelli, P., & Cornu, D. (1992). Root development of *in vitro* hybrid walnut microcuttings in a vermiculite-containing gelrite medium. *Scientia Horticulturae*, *51*(3-4), 335-342. doi:10.1016/0304-4238(92)90132-V

Kamali, K., Majidi, E., & Zarghami, R. (2001). Micropropagation of GF-677 Rootstocks (*Prunus amygdalus* x P. persica). *Seed and Plant*, *17*, 175-177.

Kepenek, K., & Kolağası, Z. (2016). Micropropagation of Walnut (*Juglans regia* L.). *Acta Physica Polonica A*, *130*(1), 150-156. doi:10.12693/APhysPolA.130.150

Kumar, P. P., & Loh, C. S. (2012). Plant Tissue Culture for Biotechnology. In: A. Altman, & P. M. Hasegawa (Eds.), *Plant Biotechnology and Agriculture-Prospect for the 21st Century* (pp. 131-138) Academic Press. doi:10.1016/B978-0-12-381466-1.00009-2

Kyte, L. (1987). *Plants from Test Tubes. An Introduction to Micropropagation*. Timber.

Leal, D. R., Sánchez-Olate, M., Avilés, F., Materan, M. E., Uribe, M., Hasbún, R., & Rodríguez, R. (2007). Micropropagation of *Juglans regia* L. In: S. M. Jain, & H. Häggman (Eds.), *Protocols for Micropropagation of Woody Trees and Fruits* (pp. 381-390). Springer, Dordrecht. doi:10.1007/978-1-4020-6352-7_35

Leelavathy, S., & Sankar, P. D. (2016). Curbing the menace of contamination in plant tissue culture. *Journal of Pure and Applied Microbiology*, *10*(3), 2145-2152.

Licea-Moreno, R., Fira, A., & Chocov, G. (2020). Micropropagation of valuable walnut genotypes for timber production: new advances and insights. *Annals of Silvicultural Research*, *44*(1), 5-13. doi:10.12899/asr-1932

Lone, I. A. (2017). Effect of different growth regulator combinations on *in vitro* callusing of walnut (*J. regia* L.). *An Asian Journal of Soil Science*, *12*(1), 203-209. doi:10.15740/HAS/AJSS/12.1/203-209

Meier-Dinkel, A., & Wenzlitschke, I. (2015). *Micropropagation of mature Juglans hybrids*. In: M. Beruto, & E. A. Ozudogru (Eds.), Proceedings of the VI International Symposium on Production and Establishment of Micropropagated Plants, ISHS Acta Horticulturae 1155, (pp. 85-92). doi:10.17660/ActaHortic.2017.1155.11

Murashige, G., & Skoog, F. (1962). A Revised Medium for Rapid Growthand Bioassays With Tobacco Tissue Culture. *Physiologia Plantarum*, *15*(3), 473-497. doi:10.1111/j.1399-3054.1962.tb08052.x

Ray, S. S., & Ali, N. (2016). Biotic Contamination and Possible Ways of Sterilization: A Review with Reference to Bamboo Micropropagation. *Brazilian Archives of Biology and Technology*, *59*(e16160485). doi:10.1590/1678-4324-2016160485

Ripetti, V., Kevers, C. & Gaspar, T. (1994). Two successive media for the rooting of walnut shoots *in vitro*. Changes in peroxidase activity and in ethylene production. *Advances in Horticultural Science*, *8*(1), 29-32.

Revilla, M. A., Majada, J., & Rodriguez, R. (1989). Walnut (*Juglans regia* L.) micropropagation. *Annales des sciences forestières, INRA/EDP Sciences*, *46*(Suppl), 149s-151s. PDF

Rugini, E. (1984). *In Vitro* Propagation of Some Olive (*Olea europaea sativa* L.) Cultivars with Different Root-Ability and Medium Development Using Analytical Data from Developing Shoots and Embryos. *Scientia Hotriculturae*, *24*(2), 123-134. doi:10.1016/0304-4238(84)90143-2

Saadat, Y. A., & Hennerty, M. J. (2002). Factors Affecting the Shoot Multiplication of Persian Walnut (*Juglans regia* L.). *Scientia Horticulturae*, *95*(3), 251-260. doi:10.1016/S0304-4238(02)00003-1

Scaltsoyiannes, A., Tsoulpha, P., Panetsos, K. P., & Moulalis, D. (1997). Effect of Genotype on Micropropagation of Walnut Trees (*Juglans regia*). *Silvae Genetica*, *46*(6), 326-332.

Schader, M. (2019). The Definition of Necrosis in Plants (Accessed: 25/11/2019) URL

Stevens, M. E., & Pijut, P. M. (2018). Rapid *in vitro* shoot multiplication of the recalcitrant species *Juglans nigra* L. *In Vitro Cellular & Developmental Biology-Plant*, *54*(3), 309-317. doi:10.1007/s11627-018-9892-3

Şen, S. M. (2011). *Ceviz Yetiştiriciliği-Besin Değeri-Folklorü*. ÜÇM yayıncılık, Samsun.

Şirin, E. (2014). Kaman 1 ve Kaman 5 ceviz çeşitlerinin (*Juglans regia* L.) mikroçoğaltımı. MSc Thesis, Gaziosmanpaşa University.

Tarinejad, A. (2013). Effects of disinfectants and antibiotics on contamination during propagation of walnut (*Juglans regia* L.). *Research on Crops*, *14*(1), 219-225.

Tekielska, D., Peňázová, E., Kovács, T., Křižan, B., Čechová, J., & Eichmeier, A. (2019). Bacterial contamination of plant *in vitro* cultures in commercial production detected by high-throughput amplicon sequencing. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, *67*(4), 1005-1014. doi:10.11118/actaun201967041005

Tetsumura, T., Tsukuda, K., & Kawase, K. (2002). Micropropagation of Shinano Walnut (*Juglans regia* L.). *Journal of the Japanese Society for Horticultural Science*, *71*(5), 661-663. doi:10.2503/jjshs.71.661

Tuan, P. N., Meier-Dinkel, A., Höltken, A. M., Wenzlitschke, I. & Winkelmann, T. (2017). *Factors affecting shoot multiplication and rooting of walnut (Juglans regia L.) in vitro*. In: M. Beruto, & E. A. Ozudogru (Eds.), Proceedings of the VI International Symposium on Production and Establishment of Micropropagated Plants, ISHS Acta Horticulturae 1155, (pp. 525-530). doi:10.17660/ActaHortic.2017.1155.77

Vahdati, K., Ashrafi, E. N., Ebrahimzadeh, H., & Mirmasoumi, M. (2009). *Improved micropropagation of walnut (Juglans regia L.) on media optimized for growth based upon mineral content of walnut seed*. In: M.-V. Hanke, F. Dunemann, & H. Flachowsky (Eds.), Proceedings of the I International Symposium on Biotechnology of Fruit Species: BIOTECHFRUIT2008, ISHS Acta Horticulturae 839, (pp. 117-124). doi:10.17660/ActaHortic.2009.839.13

Varghese, N., & Joy, P. P. (2016). Plant tissue culture contaminants identification and its response to fumigation. *Technical Report*, 1-10.

Yiğit, D., Yiğit, N., Aktaş, E., & Özgen, U. (2009). Ceviz (*Juglans regia* L.)'in antimikrobiyal aktivitesi. *Türk Mikrobiyoloji Cemiyeti Dergisi*, *39*(1-2), 7-11.