MEYVECİLİK ARAŞTIRMA ENSTİTÜSÜ MÜDÜRLÜĞÜ
FRUIT RESEARCH INSTITUTE

e-ISSN: 2148-8770
Yıl /Year**: 2024**
Cilt(Sayı)/Vol.(Issue)**: 11 (2)**
Sayfa/Page**: 79-85**

Araştırma Makalesi/
Research Article

DOI: 10.51532/meyve.1564633

Meyve Bilimi Fruit Science

Geliş Tarihi (date of arrival): 2024-10-11
Kabul Tarihi (date of acceptance): 2024-11-01

In vitro Propagation Techniques for 'Ferragnes' and 'Ferraduel' Almond Cultivars: Concentration Optimization and Rooting Success

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Abstract

In this study, an appropriate *in vitro* propagation technique was determined for 'Ferragnes' and 'Ferraduel' almond varieties, which are used in almond orchards for various reasons. To facilitate shoot and proliferation development in the culture medium, 1 mg L^{-1} and 2 mg L^{-1} concentrations of BAP were added to the MS medium. For the 'Ferragnes' variety, it was found that 1.0 mg L^{-1} BAP concentration was more suitable, resulting in a higher number of leaves. When the BAP concentration was 2 mg L^{-1}, the explants exhibited greater length. Explants started to grow on nutrient medium containing 1.0 and 2.0 mg L^{-1} BAP by the fifth day and 73% of the explants reached a size suitable for subculturing by the end of the third week. The study found that varying the concentrations of IBA did not affect rooting rates. In the rooting medium containing 0.1 mg L^{-1} NAA, rooting success averaged 23.07% with 2.6 roots per plant for 'Ferragnes' and 62.50% rooting success with an average of 2 roots per plant for 'Ferraduel'.

Keywords: Almond, micropropagation, tissue culture, *in vitro*, rooting efficiency.

'Ferragnes' ve 'Ferraduel' Badem Çeşitlerinin *İn Vitro* Çoğaltma Tekniklerinin Geliştirilmesi: Konsantrasyon Optimizasyonu ve Köklenme Başarısı

Özet

Bu çalışmada, badem bahçe tesisinde kullanılan 'Ferragnes' ve 'Ferraduel' badem çeşitleri için uygun bir *in vitro* çoğaltım tekniği belirlenmiştir. Kültür ortamında sürgün gelişimini ve kardeşlenmeyi kolaylaştırmak için MS ortamına 1 mg L^{-1} ve 2 mg L^{-1} konsantrasyonlarında BAP ilave edilmiştir. 'Ferragnes' çeşidi için 1.0 mg L^{-1} BAP konsantrasyonunun daha uygun olduğu ve daha yüksek yaprak sayısına ulaşıldığı görülmüştür. BAP konsantrasyonu 2 mg L^{-1} olduğunda ise eksplantların uzunluğu artmıştır. 'Ferraduel' çeşidi için, 2.0 mg L^{-1} BAP kullanımının sürgün uzunluğu, genişliği, yaprak sayısı ve kardeşlenme oranı için uygun olduğu belirlenmiştir. Eksplantlar, beşinci gün itibariyle 1.0 ve 2.0 mg L^{-1} BAP içeren besin ortamında sürmeye başlamış ve eksplantların %73'ü üçüncü haftanın sonunda alt kültüre alınmaya uygun büyüklüğe ulaşmıştır. Çalışma, farklı IBA konsantrasyonlarının değişmesinin köklenme oranlarını etkilemediğini ortaya koymuştur. 'Ferragnes' için 0.1 mg L^{-1} NAA içeren köklendirme ortamında bitki başına 2.6 kök ile ortalama %23.07 köklenme başarısı ve 'Ferraduel' için bitki başına ortalama 2 kök ile % 62.50 köklenme başarısı elde edilmiştir.

Anahtar Kelimeler: Badem, mikroçoğaltım, doku kültürü, *in vitro*, köklenme etkinliği.

Introduction

Turkiye's geographical location provides an ideal environment for cultivating a wide variety of fruits. Most of the fruit species and varieties grown in Turkiye are temperate climate fruits. One of these fruits, the almond, has successfully adapted to Turkiye's ecological conditions and is recognized as an important species within the *Prunus* genus (Akçay et al., 2005; Ak et al., 2005).

Nut trees are among the most important horticultural crops, with both production and consumption rising dramatically due to high economic returns and nutritional benefits. Over the past decade, global tree nut production has increased by 48%, reaching approximately 4.5 million metric tons (Vahdati et al., 2021). The almond (*Prunus dulcis* Mill.) originates from Western and Central Asia, spreading from there to China, Iran, the Middle East, and Anatolia. In Turkiye, almonds are typically grown in arid regions. Almond cultivation is widespread across various regions of Turkiye, especially in areas with a Mediterranean climate. Almonds are one of the most important horticultural crops globally (Kester and Ross, 1996). The traditional breeding of woody fruit species is a slow and time-consuming process due to high levels of heterozygosity and long generation cycles (Ainsley et al., 2000). Almond cultivation holds a significant position in the global nut production industry. According to data from the past decade, the United States ranks first in almond production, contributing 58.8% of the global total, followed by Spain with 10.7%, and Iran with 4.4%. Turkiye ranks fifth, accounting for 3.1% of global production (Ak, 2008; Isgin and Ak, 2011; Eldoğan et al., 2014).

Micropropagation techniques provide significant advantages in terms of sufficient and suitable

material supply in garden establishment. The most important of these advantages is the production of plants free from viruses and diseases that negatively affect the quality of the product and can be transmitted to the plant through vegetative propagation (Eskimez and Polat, 2023). Tissue culture in plants is utilized for propagating plants that are difficult to reproduce through traditional methods, creating somaclonal resistance to plant diseases, conducting breeding research, selecting resistant individuals, preserving genetic resources, cleansing plants of diseases, and producing biochemical products such as secondary metabolites (Rugini and Verma, 1982). Tissue culture is a crucial method for clonal propagation in many horticultural plants. Through tissue culture, a large amount of new propagation material can be obtained, genetic material can be preserved *in vitro*, and clonal cleaning can be performed to produce grafted seedlings with high plant health. One of the disadvantages of micropropagation is its cost, labor-intensive nature, and low survival rate when transferring *in vitro* propagated plants to external conditions. The most important factor limiting the success of the micropropagation method is the acclimatization process of the obtained plants. The plant is negatively affected by stressful conditions during the acclimatization process (Ekinci et al., 2024).

In this study, the regeneration shoot tips and nodal explants of the important almond cultivars 'Ferragnes' and 'Ferraduel' were investigated under *in vitro* conditions. In addition, determining the *in vitro* tolerance of these varieties, which are widely used in semi-arid conditions, will also contribute to micrografting studies. The effects of different phytohormones and concentrations on the adventitious shoot formation from various explants were studied, leading to the development of an effective protocol for *in vitro* conditions. The aim of this study is to develop an *in vitro* micropropagation protocol for the important almond cultivars 'Ferragnes' and 'Ferraduel,' which have been widely used in our region in recent years, and to address the challenges related to adaptation to external conditions. It is anticipated that this study will serve as a reference for future biotechnological research on almonds.

Material and Methods

This research was conducted in the tissue culture laboratory of the Department of Horticulture at Harran University's Faculty of Agriculture and in the tissue culture laboratories of the company Tech 4 Balanced Life (T4bL), which operates within Harran University Technopark. Shoots taken from the 'Ferragnes' and 'Ferraduel' almond varieties were used as the plant material.

Shoots taken from the 'Ferragnes' and 'Ferraduel' varieties after they emerged from the dormancy period were transported to the laboratory in a container containing citric acid and ascorbic acid. The leaves of the newly sprouting cuttings were carefully removed without damaging the shoots, and 20 mm long explants with a single bud were obtained. To minimize water loss, the cuttings were washed under tap water for 60 minutes. They were then immersed in a solution containing fungicide for 30 minutes, followed by rinsing with sterile distilled water. Afterward, they were placed in 70% ethanol for 30 seconds and rinsed again with sterile distilled water. For surface sterilization of the plant material, the explants were immersed in 0.1% HgCl for 7-8 minutes, rinsed with sterile distilled water, and then subjected to surface cleaning in 0.3% CaCl2 for 4 minutes. In the final step, the explants were rinsed with distilled water five times for 5 minutes each, completing the surface sterilization process.

In the climate chamber used, the lights were set to provide 16 hours of light and 8 hours of darkness, with a measured light intensity of 3000 lux. The temperature within the chamber was maintained between 25°C and 27°C, and the humidity level was measured at an average of 59%.

The first culture medium consisted of vitamin-enriched Murashige and Skoog (1962) (MS), 1 mg L^{-1} BAP (6-benzylaminopurine), 1 g L^{-1} activated carbon, 30 g L^{-1} sucrose, and 7 g L^{-1} agar. The pH of the medium was adjusted to 5.8 with 1 N HCl or NaOH before sterilization. The second culture medium was also prepared using the same MS medium but with 0.5 mg L^{-1} BAP, 0.01 mg L^{-1} IBA (indole-3-butyric acid), 30 g L^{-1} sucrose and 7 g L^{-1} agar. The pH of this medium was also adjusted to 5.8, similar to the first medium. The preparation of these media was guided by previous studies on almonds (Gürel and Gülşen, 1998; Ainsley et al., 2000; P´erez-Tornero et al., 1999; Lauri et al., 2001; Silveira, 2000; Addi et al., 2008; Arıcı, 2008; Işıkalan et al., 2010; Shekafandeh, 2010; Jimenez et al., 2012; Ak et al., 2021).

Each shoot, averaging 2.5 cm in length, was separated from the cuttings and placed into tubes. The first rooting medium consisted of 2.0 mg L^{-1} IBA, 30 g L^{-1} sucrose, 5 g L^{-1} agar and half-strength MS. The second rooting medium was similar, containing 2.0 mg L^{-1} IBA, 30 g L^{-1} sucrose, 5 g L^{-1} agar, and half-strength MS. The third rooting medium included 0.1 mg L^{-1} IBA, 30 g L^{-1} sucrose, and 7 g L^{-1} agar in full-strength MS. The fourth medium comprised 0.5 mg L^{-1} IBA, 0.1 mg L^{-1} BAP, 30 g L^{-1} sucrose, and 7 g L^{-1} agar in full-strength MS. The fifth rooting medium was made up of 0.1 mg L^{-1} NAA, 30 g L^{-1} sucrose, and 7 g L^{-1} agar in full-strength MS.

After approximately one month, the plantlets that had sufficiently rooted *in vitro* had their roots washed to remove the agar. They were then

transferred to containers filled with autoclaved peat and perlite in a 1:1 ratio. To prevent moisture loss, the plantlets were covered with transparent containers. These containers, which did not block light, were used to maintain humidity. After three weeks, the covers were gradually opened each week (for 5, 6, 7, 8, 9, and 10 minutes) to help the plantlets acclimate to external conditions.

In the study on the *in vitro* micropropagation of 'Ferragnes' and 'Ferraduel' almond cultivars, the statistical analysis of the findings was conducted using a randomized complete block design. The experiment was repeated three times independently, with 10 explants considered in each repetition. When treatments were found to be statistically significant, differences between the mean values were subjected to LSD (Least Significant Difference) test at the 5% significance level.

Hierarchical clustering analysis (HCA) and principal component analysis (PCA) were conducted via the Software R (Version 4.1.1, R Foundation for Statistical Computing, Vienna, Austria).

Results

Determination of Sterilization Protocol

In vitro studies, the primary procedure is the proper sterilization of plant materials. Therefore, the first and most crucial step is to establish an appropriate sterilization protocol for the specific species or cultivar. Sterilization methods vary depending on the plant's growing environment, the area from which the explants are taken, and the woodiness of the explants. Common disinfectants used for surface sterilization of explants include ethanol, Ca, NaOCl, $AgNO_3$, and $HgCl_2$ (Babaoğlu et al., 2002). Typically, surface sterilization in *in vitro* work is completed by shaking explants in a solution containing different concentrations of NaOCl with a small amount of Tween-20 for about 15 minutes, followed by washing 3 to 5 times with sterile distilled water for 5 minutes each (Ainsley et al., 2001; Ak et al., 2021). However, some researchers have added additional stages to the sterilization process. In similar studies (Ainsley et al., 2000; Pruski et al., 2005; Ak et al., 2021), a preliminary cleaning step was performed by rinsing explant sources under running water for varying periods before surface sterilization. In this study, surface sterilization using an 8% NaOCl solution with 1-2 drops of Tween-20 did not cause any contamination. Therefore, it was determined that applying various sterilization methods used in other studies was unnecessary for the surface sterilization performed in this thesis. An 8% NaOCl solution with 1-2 drops of Tween-20 was found to be the most suitable concentration for surface sterilization of the explants.

Shoot Development of Explants and Poliferation

For the development and branching of explants from different cultivars, an MS medium was prepared containing 30 g L^{-1} sucrose, 7 g L^{-1} agar, and either 1 or 2 mg L^{-1} BAP. The effects of these different BAP concentrations on shoot length, width, leaf number, and number of branches were assessed (Table 1).

Different results were observed with varying BAP concentrations for the 'Ferragnes' variety. It was determined that 2.0 mg L^{-1} BAP was suitable for optimizing shoot length, width, leaf number, and branching rate. Both shoot and nodal explants began to sprout within five days on media containing 1.0 and 2.0 mg L^{-1} BAP and by the end of the third week, 73% of the explants had grown to a size suitable for subculturing.

When no activated carbon was used in the medium, the contamination rate increased. Increasing the concentration to 2 g L^{-1} led to issues with plant development and leaf number. This section of the thesis found that 1 g L^{-1} activated carbon in the medium improved leaf number and quality while reducing contamination (Table 2). When comparing the two varieties, it can also be concluded that the 'Ferraduel' variety is more tolerant to higher concentrations of activated charcoal.

In the rooting phase, the effect of different concentrations of IBA was investigated, and explants were placed in a 2.2 g L^{-1} MS medium. The study found that the use of IBA did not affect the rooting rates, and the cultured plantlets did not root. In contrast, in the fifth rooting medium containing 0.1 mg L^{-1} NAA, a rooting success of 23.07% with an average of 2.6 roots was achieved for 'Ferragnes', while 'Ferraduel' showed a rooting success of 62.50% with an average of 2 roots (Table 3).

Acclimatization

Rooted plantlets were washed to remove them from the medium and then transferred to an adaptation environment consisting of a 1:1 mixture of perlite and peat. In this environment, 65% of the plantlets successfully adapted.

In the adaptation phase of plantlets to external conditions, minimizing water loss is crucial. The low adaptation rates observed in plantlets transferred to substrates with excessive perlite are primarily attributed to nutrient and water deficiency. For woody plants, it is essential to maintain high humidity levels in climate-controlled cabinets during *in vitro* propagation and to gradually reduce the humidity to acclimate the plantlets effectively. The process from the very beginning of the studies to this stage is given in Figure 1.

Table 1. Explant growth chart at different BAP concentrations
Çizlge 1. Farklı BAP konsantrasyonlarında eksplant büyüme tablosu

		1st Medium (0 mg L^{-1} BAP)	2nd Medium (1 mg L^{-1} BAP)	3rd Medium (2 mg L^{-1} BAP)	LSD (%5)
Ferragnes	Explant length (cm)	0.00±0.00	2.10±0.65b	4.20±0.52a	0.227*
	Explant width (cm)	0.00±0.00	3.80±0.57a	2.50±0,57b	0237*
	Number of leaves (pcs)	0.00±0.00	19.30±0.15a	16.40±0.45b	0.226*
	Proliferation rate (%)	0.00±0.00	14.60±0.55a	11.60±0.40b	0.453*
Ferraduel	Explant length (cm)	0.00±0.00	2.70±1.53b	4.20±1.15a	0.223*
	Explant width (cm)	0.00±0.00	2.20±0.84b	2.50±0.57a	0.227*
	Number of leaves (pcs)	0.00±0.00	10.20±0.72b	16.40±0.37a	0.320*
	Proliferation rate (%)	0.00±0.00	6.80±0.15b	11.60±0.35a	0.216*

*The values were found to be statistically significant at 5% level.

Table 2. Effects of activated carbon on shoot development
Çizelge 2. Aktif karbonun sürgün gelişimine etkileri

		1st Medium (1 g L^{-1} AC)	2nd Medium (2 g L^{-1} AC)	LSD (%5)
Ferragnes	Number of Leaves	10.44±0.51a	4.35±1.33b	3.816*
	Length of Shoots	1.80±0.30a	0.90±0.62b	0.581*
Ferraduel	Number of Leaves	15.26±2.05a	7.11±2.25b	4.657*
	Length of Shoots	2.12±0.43a	1.81±0.63a	0.659ns

*The values were found to be statistically significant at 5% level.

Table 3. Effects of auxins on root development
Çizelge 3. Oksinlerin kök gelişimine etkileri

		0.1 mg L^{-1} IBA	0.5 mg L^{-1} IBA	1.0 mg L^{-1} IBA	2.0 mg L^{-1} IBA	0.1 mg L^{-1} NAA
Ferragnes	Rooting Success(%)	0.00	0.00	0.00	0.00	23.07
	Number of Roots	0.00	0.00	0.00	0.00	2.60
Ferraduel	Rooting Success(%)	0.00	0.00	0.00	0.00	62.50
	Number of Roots	0.00	0.00	0.00	0.00	2.00

Figure 1. The process from shooting media to acclimatization of plants.
Şekil 1. Bitkilerin sürgün ortamından iklimlendirme odasına kadar olan süreç.

Hierarchical Clustering Analysis (HCA)

Hierarchical clustering analysis (HCA) is a method used to group data based on their similarities. The results of HCA are typically presented in a graph called a dendrogram, which illustrates how the data is clustered hierarchically, visualizing the similarities or distances between the samples (Ekinci et al., 2024). In Figure 2, when examining HCA, groups I, II, and III represent the varieties treated with different BAP concentrations. Groups A, B, and C represent the clusters of the evaluated parameters (explants length, explants width, number of leaves, and proliferation rate).

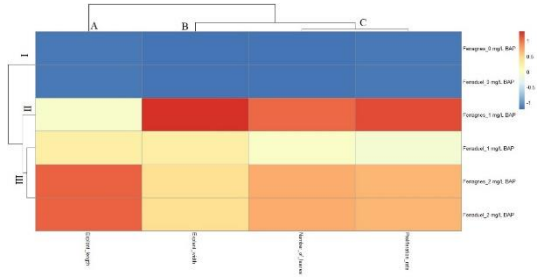

Figure 2. Classification of morphological characteristics evaluated under different BAP concentrations in Ferragnes and Ferraduel varieties using a heat map.
Şekil 2. Ferragnes ve Ferraduel çeşitlerinde farklı BAP konsantrasyonları altında değerlendirilen morfolojik özelliklerin ısı haritası kullanılarak sınıflandırılması.

It has been shown that the Ferraduel variety performed best at a 2 mg L^{-1} BAP concentration, having the highest explant length and number of leaves compared to the other groups. Additionally, it was determined for the Ferragnes variety at 1 mg/l. In groups treated with low BAP concentrations, explant development and proliferation rates were observed to be at lower levels. In the HCA presented in Figure 3, the parameters of number of leaves and shoot length evaluated under activated carbon (AC) applications in Ferragnes and Ferraduel varieties were classified. Groups I, II, and III represent the AC concentrations examined on a variety basis. Groups A and B classified the parameters evaluated in the treatments. The highest number of leaves was found in the Ferraduel-1 g L^{-1} AC application. The Ferragnes-2 g L^{-1} AC application showed a negative effect on the number of leaves and shoot length. It has been reported that BAP and AC concentrations that improve shoot regeneration vary based on species, variety, and even genotype (Balilashaki et al., 2015; Mittal et al., 2016). These results support our study.

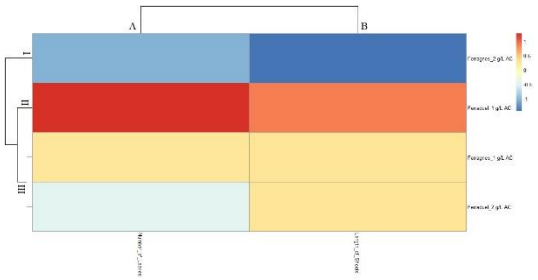

Figure 3. Classification of number of leaves and shoot length parameters evaluated under activated carbon (AC) applications in Ferragnes and Ferraduel varieties using a heat map.
Şekil 3. Ferragnes ve Ferraduel çeşitlerinde aktif karbon (AC) uygulamaları altında değerlendirilen yaprak sayısı ve sürgün uzunluğu parametrelerinin ısı haritası kullanılarak sınıflandırılması.

This study extensively examines the development of suitable *in vitro* micropropagation protocols for the 'Ferragnes' and 'Ferraduel' almond varieties, which are significant in Turkiye and the Şanlıurfa region. The selection of explants and sterilization protocols is crucial in *in vitro* work. The use of new shoots has yielded positive results. One-year-old shoots were used as plant material, and the performance of shoot tip versus node explants was compared. Shoot tip explants provided better results, with ideal sterilization procedures effectively reducing contamination rates. The effects of activated carbon were also investigated, revealing that a concentration of 1 g/l improved shoot proliferation and quality while reducing contamination. However, the impact of activated carbon varies across different plant species and conditions, indicating a need for further research. Culture media adjusted to a pH of 5.8 and repeated subculturing resulted in a high yield of clonal shoots. NAA was found to be successful in the rooting phase, while IBA inhibited rooting. In this study, it was found that 1 mg L^{-1} and 2 mg L^{-1} IBA did not have an effect on the *in vitro* rooting of 'Ferragnes' and 'Ferraduel' almond varieties. However, the addition of 0.1 mg L^{-1} NAA to the medium had a better effect on root length and number of roots in microshoots. Previous studies recommended various concentrations of NAA for rooting media, including Rugini and Verma (1982) with 0.01 mg L^{-1} NAA for 'Ferragnes'; Lauri et al. (2001) with 0.2 mg L^{-1} NAA for different varieties; Arıcı (2008) with 0.2 mg L^{-1} NAA for 'Myrobolan', 0.02 mg L^{-1} NAA for 'MaxMa-60', 0.2 mg L^{-1} NAA for 'MaxMa-14', 0.02 mg L^{-1} NAA for 'GF-677', and 0.02 mg L^{-1} NAA for 'Garnem'; and Yıldırım et al. (2010) with 0.01 mg/l NAA for 'Ferragnes' and 'Ferraduel'. The findings of this study are consistent with these references. While IBA was reported to be effective in different almond varieties, such as the more intense and compact callus formation with IBA in 'Shahrood-8' and 'Shahrood-10', and less vigorous callus formation with NAA (Shekafandeh, 2010), this study did not find positive effects of the tested IBA concentrations. Instead, NAA was found to yield better results during the rooting stage.
The use of activated carbon alone or in combination with an auxin for the rooting of micropropagated shoots has been studied in several research works. For instance, 0.5 g L^{-1} of activated carbon was found to significantly improve rooting success in pineapple (Firoozabady et al., 2006). In another study, 0.5 g L^{-1} of activated carbon used in combination with NAA in a 1/2 MS medium yielded successful results for micropropagation of *Swertia chirayita* (Joshi and Dhawan, 2007). Similarly, shoots of Fortunella crassifolia, cultured from epicotyl explants in a 1/2 MS medium supplemented with NAA, Kn, and 0.5 g L^{-1} activated carbon, demonstrated maximum rooting success (75%) (Yang et al., 2006). However, excessive amounts of activated carbon have been reported to have negative effects in *in vitro* studies on *Pyrus pyrifolia* (Kadota and Nimmi, 2004) and *Capsicum annuum* (Supena et al., 2006).

Conclusion

In the context of this study, an increase in the amount of activated carbon was found to cause problems in plant development. It was concluded that a concentration of 1 mg L^{-1} of activated carbon was sufficient for the micropropagation of the two almond varieties under investigation.
The study identified that rooting media require relatively low levels of cytokinin and trace amounts of NAA. In micropropagation, the suitability of

laboratory conditions, explant selection, and growth regulator concentrations is of utmost importance. The study emphasized that each variety responds differently under *in vitro* conditions, and factors such as shoot age and explant physiological state affect success rates. In conclusion, the study developed an effective protocol for the micropropagation of 'Ferragnes' and 'Ferraduel' almonds. The technique shows high potential for rapid and efficient clonal propagation and can serve as a model system for other almond varieties.

Funding
This study was supported financially by the Harran University Scientific Research Projects Coordination Unit (HUBAK) with project number 21095.

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