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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

In vitro Micropropagation of Immortelle Grass (*Helichrysum italicum* (ROTH) G. DON)*

Ölmez Otu (*Helichrysum italicum* (ROTH) G. DON) Bitkisinin *in vitro* Mikroçoğaltımı

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Abstract

Immortelle grass (*Helichrysum italicum* (Roth) G. Don), which spreads in the Southern Marmara and Aegean regions, can be grown in arid and semi-arid regions. In addition, due to its rich essential oil and secondary metabolite content, it has an important place in modern medicine and cosmetics, including traditional treatment methods. Although the propagation of plants by shoot regeneration *in vitro* has been achieved in many plant species, studies on tissue culture in immortelle grass are limited. This study aims to optimize the tissue culture study in immortelle grass and provide a basis for the next *in vitro*, molecular, and secondary metabolite studies. In addition, it promotes the plant by optimizing the healthy and disease-free seedling production method for cultural agriculture in the region. Three different (15%, 25%, and 35%) NaOCl concentrations were tested for 10 and 20 minutes during the sterilization phase of the explants. The most successful result was obtained in the medium containing 35% NaOCl for 10 minutes. Sterilized explants were transferred to MS and Gamborg B5 nutrient media containing BAP, GA, and NAA plant growth regulators for shoot regeneration. The best regeneration in explants was obtained in MS medium containing 0.5 mg L^{-1} BAP, 1 mg L^{-1} GA, and 0.2 mg L^{-1} NAA. No growth was observed in trials containing Gamborg B5, and vitrification and darkening occurred in the explants. After four weeks, the shoots reaching a length of 3 cm were taken into MS and ½MS medium containing 0 MS, 0.5 mg L^{-1} IBA, 1 mg L^{-1} IBA, 1.5 mg L^{-1}, and 2 mg L^{-1} IBA as a rooting medium. 100% rooting was observed in all prepared media within four weeks. As a result of micropropagation studies, the rooted plants were transferred to the acclimatization stage within three months and then moved to the pots in the greenhouse and to the field one month later.

Keywords: *Helichrysum italicum*, Regeneration, Rooting, Acclimatization, MS, Gamborg B5

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Öz

Güney Marmara ve Ege bölgesinde yayılış gösteren ölmez otu bitkisi (*Helichrysum italicum* (Roth) G. Don) kurak ve yarı kurak bölgelerde yetiştirilebilmektedir. Ayrıca zengin uçucu yağ ve sekonder metabolit içeriğinden dolayı geleneksel tedavi yöntemleri dahil modern tıp ve kozmetik alanında önemli bir yere sahiptir. Bitkilerin *in vitro* koşullarda sürgün rejenerasyonu yoluyla çoğaltılması birçok bitki türünde elde edilmiş olmasına rağmen, ölmez otunda doku kültürü üzerine yapılan çalışmalar oldukça sınırlıdır. Bu çalışmanın amacı, ölmez otu bitkisinde doku kültürü çalışmasının optimizasyonunun yapılması ve bir sonraki *in vitro*, moleküler ve sekonder metabolit çalışmalarına zemin sağlamaktır. Bunun yanı sıra bölgede kültür tarımı için sağlıklı ve hastalıksız fide üretimi yönteminin optimize edilerek bitkiyi tanıtmaktır. Eksplantların sterilizasyon aşamasında üç farklı (%15, %25 ve %35) NaOCl konsantrasyonu 10 ve 20 dakika süre ile denenmiştir. En başarılı sonuç 10 dakika %35 NaOCl içeren ortamdan elde edilmiştir. Steril edilen eksplantlar sürgün rejenerasyonu için BAP, GA ve NAA bitki büyüme düzenleyicileri içeren MS ve Gamborg B5 besin ortamlarına aktarılmıştır. Eksplantlarda en iyi rejenerasyon 0.5 mg L^{-1} BAP, 1 mg L^{-1} GA ve 0.2 mg L^{-1} NAA içeren MS ortamında elde edilmiştir. Gamborg B5 içeren denemelerde herhangi gelişim gözlenememiş olup eksplantlarda vitrifikasyon ve kararma oluşmuştur. Dört hafta sonunda 3 cm uzunluğa ulaşan sürgünler köklendirme ortamı olarak 0 MS, 0.5 mg L^{-1} IBA, 1 mg IBA, 1.5 mg L^{-1} ve 2 mg L^{-1} IBA içeren MS ve ½MS ortamına alınmıştır. Hazırlanan tüm ortamlarda dört hafta içerisinde %100 köklenme görülmüştür. Mikroçoğaltım çalışmaları sonucu üç ay içerisinde köklenmiş bitkiler aklimatizasyon aşamasına alındıktan sonra seradaki saksılara ve bir ay sonra tarlaya aktarılmıştır.

Anahtar Kelimeler: *Helichrysum italicum*, Rejenerasyon, Köklendirme, Aklimatizasyon, ½ MS, Gamborg B5

1. Introduction

The immortelle grass, belonging to the Asteraceae family, is a shrubby and perennial plant. It has yellow flowers, furry leaves and stems and can grow up to 90 cm in height. Due to the long-lasting appearance of its showy flowers, it is known as the immortal flower, the unfading flower, and the everlasting flower in popular culture. Additionally, it has a sharp scent that can last for a long time. This genus has more than 600 species worldwide, with its native range including Africa (244 species in South Africa), Madagascar, Australia, Asia, and Eurasia. It also grows in the steppes of America, Scandinavia, the Atlantic, Europe, the Balkans, Russia, Siberia, the Caucasus, Central Asia, Mongolia, China, and Türkiye in sandy and semi-hard soils (Umaz and Umaz, 2020). In the Turkish flora, this genus is represented by 27 taxa, 15 of which are endemic and commonly found in Anatolia (Albayrak et al., 2010). *Helichrysum* species have ornamental value due to their unique morphological features, flower structure, and colors. Moreover, essential oils and other secondary metabolites obtained from the flowers and vegetative parts of the plant are known to have anti-inflammatory, anti-allergic, and antimicrobial effects (Dimitrova and Nacheva, 2018).

Medicinal aromatic plants are a group of plants rich in biochemicals called secondary metabolites. One of the most important functions of secondary metabolites is to play a role in the defense systems of the plant against biotic and abiotic stress conditions (Umarusman et al., 2019). The immortelle grass is a plant that is rich in biochemical compounds. The types and amounts of these compounds present in the plant vary depending on the plant part from which they are isolated, the season in which the plant is found, and the conditions under which the plant is grown. The main compounds found in the plant include α-pinene, 2-methylcyclo-hexyl pentanoate, neryl acetate, 1,7-di-epi-αcedrene, γ-curcumene, and thymol (Ninčević et al., 2019). Studies on the compounds found in the plant have shown that α-pinene in particular, has a cytotoxic effect on T leukemia cells, lung cancer cells, and cervical cancer cells, thereby preventing the cancer progression (Staver et al., 2018).

In vitro micropropagation provides the opportunity to produce a large number of clonal plants of a plant part under aseptic and controlled conditions, utilizing the totipotent ability of the plant. One of the most significant advantages of micropropagation is that the resulting plants are genetically identical and have similar forms. Due to its small seeds and low germination performance, *Helichrysum* species are challenging to propagate. Additionally, the limited number of cuttings that can be taken from each Helichrysum seedling makes it challenging to meet the high demand for seedlings. *In vitro*, micropropagation of Helichrysum provides a solution to overcome these challenges. Therefore, this study aims to optimize the *in vitro* micropropagation of *Helichrysum* to contribute to both its commercial production and future *in vitro* studies.

2. Materials and Methods

2.1. Plant Material

The starting material used in the study was obtained from the collection garden of the Tekirdag Namik Kemal University Faculty of Agriculture Research and Production Unit (ZIRAATBIYOTEK). Explants were taken from 1 year old *Helichrysum italicum* plants before flowering in spring.

2.2. In Vitro Culture Conditions

In the conducted study, MS (Murashige and Skoog, 1962) basic nutrient medium and Gamborg B5 (Gamborg et. al., 1976) nutrient medium were used as the nutrient media. 3% sucrose was used as the carbon source in these media. 6.5 g L^{-1} plant agar (Duchefa) was used as the gelling agent in the prepared media. Since shoot cultures released phenolic compounds, 0.06% activated charcoal was added to the media. Plant growth regulators (BAP, GA, NAA, and IBA) used in the experiments were dissolved in appropriate solvents. Then, stock solutions were prepared with distilled water and added to the media in the required amounts and concentrations. The pH of the nutrient media was adjusted to 5.6-5.8 using 1 N NaOH or HCl. The prepared nutrient media were sterilized at 121°C, 1.2 kg cm^2 (Tekbal-ST2) under pressure for 20 minutes. All cultures were incubated at 24±2°C with a 16-hour light 8-hour dark photoperiod under blue-red LED light.

2.3. Explant Preparation and Surface Sterilization

The fresh shoots of immortelle grass seedlings found in the collection garden were preferred as explants in the study. The collected shoots were washed with 2-3 drops of commercial detergent on a magnetic stirrer for 5

minutes as a pre-sterilization step and then rinsed under running tap water for approximately 1 hour. The pre-sterilized explants were placed in a sterile cabinet and soaked in a 70% ethanol solution for 10 seconds. Then, they were sterilized in a magnetic stirrer in a commercial laundry bleach solution diluted to 15%, 25%, and 35% for 10 and 20 minutes, respectively. Finally, they were rinsed thrice with autoclaved distilled water for 5 minutes each time (Daneshvar Royandazagh and Pehlivan, 2016). The obtained sterile explants were prepared for *in vitro* culture environments in different doses and combinations of plant growth regulators to improve shoot regeneration.

2.4. Regeneration Conditions of Explants

The sterilized explants were transferred to nutrient media containing different plant growth regulators to induce regeneration. In this stage, BAP, GA, and NAA were added to solid MS and Gamborg B5 nutrient media in various combinations. The dosage of plant growth regulators added to the nutrient media varied between 0.2-2 mg L^{-1} (*Table 1*). Control nutrient media without plant growth regulators were prepared for both nutrient media. The experiments were performed with five replicates, and the culture medium that provided the best shoot development was determined.

2.5. Rooting of Shoots

The shoots developed in the special nutrient media prepared for regeneration will be transferred to rooting experiments in MS and ½ MS nutrient media containing different concentrations of IBA (0.5, 1, and 2 mg L^{-1}). The rooting experiments will be carried out in sterilized glass jars, with 4-5 shoots placed in each jar.

2.6. Acclimatization

The rooted plants will be washed with clean water to remove any residue from the nutrient media. They will then be transplanted into vials filled with previously sterilized and moistened peat. To prevent sudden moisture loss of the plants, the vials will be placed in a suitable container and covered entirely with cling film before being transferred to the plant growth room. Over the course of three weeks, holes will gradually be made in the cling film to acclimate the plants to external conditions. After three weeks, the plants will be transferred to field conditions.

2.7. Statistical Assessment of Regeneration Studies

The experiments were conducted with five replications. The statistical analysis of the data obtained from the regeneration studies was performed using the SPSS ver. 22 statistical program and One-Way Anova post hoc tests were conducted with the Duncan test (Snedecor and Cochran, 1967).

3. Results and Discussion

Due to the limited vegetative and generative propagation of the immortelle grass, optimization of the micropropagation method is essential. By using different explants, basic nutrient media, plant growth regulators, and culture conditions in micropropagation studies, a large number of plants can be obtained from a few explants (Gupta et al., 2020). Sterilization is difficult in immortelle grass due to the hairy nature of its leaves (Clasquin and Henry, 2002). In addition, blue and red LED lights were used in growth chamber in this study. According to the study of Silva et al., red and blue LED lights provided better growth and development compared to white light (Silva et al., 2014). In another study, it was seen that blue and red light gave better results in shoot development (Ramírez-Mosqueda et al., 2017). In this study, according to the results obtained from the surface sterilization study, 100% contamination was observed in both trials conducted with 15% commercial bleach. In another trial using 25% commercial bleach, 60% contamination occurred in the 10-minute trial and 40% contamination occurred in the 20-minute trial. Although the best result was obtained in the trial with 35% commercial bleach, where 20% contamination was observed in the trials conducted for both 10 and 20 minutes, it was found that the 10-minute trial achieved the best result because the explant browning rate was high in the explants left for 20 minutes (*Table 1*). Morone-Fortunato et al. reported successful results in their micropropagation study of *H. italicum* using explants treated with a 0.1% (w/v) $HgCl_2$ solution for 15 minutes with magnetic stirring (Morone-Fortunato et al, 2010). In another study by Giovanni et al. on the same genus, the best results were obtained by immersing explants in a solution of 1% NaOCl and two drops of Tween 20 for 20 minutes, after being immersed in a 70% ethanol solution for 30 seconds (Giovanni et al., 2003). In the sterilization study of *H. arenarium* by Figas et al., (2016) explants were immersed in a 70% ethanol solution for 1 minute, then immersed in a solution

containing 9% $Ca(OCl_2)$ and Tween 20 for 12 minutes, and finally rinsed with sterilized distilled water to obtain successful results (Figas et al., 2016). In another study on *H. arenarium*, Clasquin and Henry reported a successful sterilization protocol in which explants were stirred on a magnetic stirrer for 30 minutes in a 0.1 M $KMnO_4$ solution, immersed in a 70% ethanol solution for 1 minute, a 15% H_2O_2 solution for 15 minutes, and a 12% NaOCI solution for 10 minutes (Clasquin and Henry, 2002).

Table 1. *Sterilization study results*

Treatment	Contamination**	Browned Explant**
15% bleach – 10 minutes	10.00 ±0.00a	10.00 ±0.00a
25% bleach – 10 minutes	6.00±1.00b	8.00±0.57ab
35% bleach – 10 minutes	2.00±0.57c	2.00±0.57c
15% bleach – 20 minutes	10.00±0.00a	10.00±0.00a
25% bleach – 20 minutes	4.00±0.57bc	6.00±0.57b
35% bleach – 20 minutes	2.00±0.57c	3.00±0.57c

$p \leq 0.01$

**Averages shown with different lowercase letters in each column, according to Duncans multiple test; It is statistically different at the 0.01 significance level.

In vitro micropropagation method, which is one of the vegetative propagation methods, is important for producing seedlings free from diseases and in high quantities. Two different basic nutrient media (MS and Gamborg B5) were used in the micropropagation study conducted in this study. Regeneration and shoot development were provided by adding different doses of BAP, GA and NAA as plant growth regulators to these nutrient media. According to the results of the Duncan test, the number of shoot formations per explant varies between 2 and 12.40 in MS basic nutrient media. The lowest shoot formation per explant was observed in the MS control, while the highest shoot formation per explant was observed in the MS 0.5 mg L^{-1} BAP + 1 mg L^{-1} GA + 0.2 mg L^{-1} NAA nutrient medium. No development was observed in the Gamborg B5 nutrient media, and vitrification occurred so that no results could be obtained (*Figure 2*). The percentage of shoot formation varied between 19.99% and 66.66%, with the highest percentage of shoot formation observed in the MS 0.5 mg L^{-1} BAP + 1 mg L^{-1} GA + 0.2 mg L^{-1} NAA and MS 1.5 mg L^{-1} BAP + 1 mg L^{-1} GA + 0.2 mg L^{-1} NAA nutrient media. The lowest percentage of shoot formation was observed in the MS control nutrient medium (*Table 2*). The average shoot length varied between 0.42 cm and 2.64 cm in MS basic nutrient media. The highest shoot length was observed in the MS 0.5 mg L^{-1} BAP + 1 mg L^{-1} GA + 0.2 mg L^{-1} NAA nutrient medium, while the lowest shoot length was observed in the MS control nutrient medium. Since no development was observed in the Gamborg B5 nutrient media, the average shoot length could not be calculated (*Table 2*). As the results we obtained in the study of Clasquin and Henry in *H. arenarium*, no shoot development was observed in the experiments with Gamborg B5, however, callus and vitrification formation were observed (Clasquin and Henry, 2002). Giovanni et al. in their study on *H. italicum* and *H. stoechas*, the best shoot growth was obtained in media containing 2.66 µM BAP in both species (Giovanni et al., 2003). In another micropropagation study of Dimitrova and Nacheva in *H. italicum*, trials were established by adding BAP, KIN and ZT to DKW and MS basic nutrient media. As a result of the study, it was reported that the best shoot growth medium was MS containing 5 µM BAP + 0.005 µM IBA and DKW basic nutrient medium containing 5 µM KIN + 0.005 µM IBA (Dimitrova and Nacheva, 2018). Perrinia et al. (2009) aimed to form callus in the leaves of *H. italicum* and then to achieve shoot development by organogenesis. They performed micropropagation study by combining and modifying MS and NN media as nutrient media. In this study, different concentrations of TDZ and NAA were used as plant growth regulators, and the best shoot growth results were obtained in environments without plant growth regulators. According to the data obtained because of this study, it has been reported that the organogenic capacity of *H. italicum* is high (Perrinia et al., 2009). In another study by Figas et al. in *H. arenarium*, KIN and combinations of KIN and IBA were tried. According to the results they obtained, the best shoot growth was achieved in media containing 5 mg L^{-1} KIN and 0.5 mg L^{-1} IAA (Figas et al., 2016). In Tastekin's master's thesis study, the best shoot growth was obtained in the micropropagation stage of St. John's Wort in a medium containing 0.5 mg L^{-1} BAP and 2.5 mg L^{-1} NAA (Tastekin, 2020). In the micropropagation study performed in mint, Khan et al. showed that the best results were obtained from the combination of BAP and NAA in their trials in which they combined BAP with NAA and IBA (Khan et al., 2021). Hirakawa and Tanno experimented with BA and GA plant growth regulators at different concentrations added to ½MS in their micropropagation study in hops. According to the results they obtained, the best shoot

growth was obtained in the medium containing BA (0.01 mg L^{-1}) at a lower concentration (Hirakawa and Tanno, 2022). In a study conducted by Yesmin (2019) on sugar grass, media were prepared with the combinations of BAP, KIN and NAA added to MS. According to the results obtained in the study, the best shoot growth and shoot length were obtained in media containing 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA (Yesmin., 2019). In the micropropagation study of Petrova et al. in lemon balm, media were prepared with IBA and NAA combinations of BAP, KIN, ZT and 2-iP added to MS. As the results obtained in sugar grass, the best shoot development and shoot length were obtained in the medium containing 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA (Petrova et al., 2021). In the micropropagation study of Morone-Fortunato et al. on *H. italicum*, explants were taken from 20 different genotypes and the results obtained from these genotypes were examined. In this study, which used 1 mg L^{-1} BAP and 0.2 mg L^{-1} IBA for shoot development, it was revealed that genotypes gave different responses under the same conditions. According to the results obtained, more than 50% shoot growth was obtained in 16 genotypes, while the result was between 33-47% in the remaining 4 genotypes (Morone-Fortunato et al., 2010).

Table 2. Micropropagation results

Treatment	Average number of shoots per explant**	Percent shoot formation	Average shoot length** (cm)
MS control	2.00±0.44c	19.99 ±2.4b	0.42±0.37c
MS 0.5 mg L^{-1} BAP + 1 mg L^{-1} GA + 0.2 mg L^{-1} NAA	12.40±1.80a	66.66±10.2a	2.64±0.19a
MS 1 mg L^{-1} BAP + 1 mg L^{-1} GA + 0.2 mg L^{-1} NAA	7.20±1.39b	46.66±12.2ab	1.70±0.25b
MS 1.5 mg L^{-1} BAP + 1 mg L^{-1} GA + 0.2 mg L^{-1} NAA	7.00±1.04b	53.33±8.2a	1.88±0.17b
MS 2 mg L^{-1} BAP + 1 mg L^{-1} GA + 0.2 mg L^{-1} NAA	5.40±0.67bc	46.66±12.2ab	2.0±0.17b

$p \leq 0.01$

**According to Duncans multiple tests, the means shown with different lowercase letters in each column; It is statistically different at the 0.01 significance level.

The rooting stage is one of the most important stages for tissue culture studies to achieve results. While the rooting stage can be quite difficult especially in woody plants, it can be achieved relatively easily in herbaceous plants. MS basal nutrient medium was used at ratios of 1/1 and 1/2 for rooting stage of shoots obtained under *in vitro* conditions. Different rates of IBA, which is a plant growth regulator belonging to the auxin group, between 0.5-2 mg L^{-1} were used in these nutrient media. According to the data obtained, all explants were rooted at the end of 30 days. The highest average root length was obtained in the nutrient medium containing 1/1 MS and 1 mg L^{-1} IBA. The lowest root length was obtained in the nutrient medium containing 1/1 MS without plant growth regulator. When the average root number was examined, the highest average root number was observed in the nutrient medium containing 1/1 MS and 1 mg L^{-1} IBA (*Figure 1*). The lowest average root number was observed in the nutrient medium containing 1/2 MS without plant growth regulator (*Figure 1*). Perrinia et al. (2009) reported that they achieved nearly 100% rooting in the MS medium without plant growth regulators in immortelle grass (Perrinia et al., 2009). Figas et al. (2016), on the other hand, set up trials with media containing 0 MS, 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} IAA for rooting in *H. arenarium*. According to the results they obtained, rooting was observed between 85-100% in all media, and when the root length and number of roots were examined, the best result was achieved in the medium containing 0.5 mg L^{-1} IBA (Figas et al., 2016). Again, similar to the results obtained in this study, 100% rooting was obtained in the experiments performed at different concentrations of 0 MS, IAA and NAA in the study of Giovanni et al. on *H. italicum* and *H. stoechas* (Giovanni et al., 2003). In another study, 60-80% successful results were obtained in the trials established with NAA in Dimitrova and Nacheva *H. italicum*, while successful results were between 90-100% in the trials established with IBA (Dimitrova and Nacheva, 2018). In the study of Anrade et al. on *Lavandula vera*, it was tried to achieve rooting by adding different concentrations of IBA and NAA to normal MS and ¼MS nutrient media. According to the results obtained, the best rooting rate was achieved in the medium containing ¼ MS 0.2 mg L^{-1} NAA (Anrade et al., 1999). In the study of El-Banna on thyme, rooting experiments were established by using 0 MS, IBA and NAA at different concentrations. According to the results, while 75% rooting occurred in the control medium, 100% rooting was observed in the medium

containing 1.5 mg L^{-1} IBA, 1 mg L^{-1} NAA and 1.5 mg L^{-1} NAA (El-Banna, 2017). Hirakawa and Tanno reported that they reached the best rooting rate within 2 weeks in ½MS nutrient medium containing 0.05 mg L^{-1} NAA in their study on hops (Hirakawa and Tanno, 2022). In the study of Petrova et al. on lemon balm, 100% rooting was obtained in the control medium without plant growth regulator (Petrova et al., 2021). In a study on sugar grass, Yesmin used 0.2-0.5 mg L^{-1} ratios of IBA, IAA and NAA in MS and ½MS nutrient media for the rooting stage. According to the results obtained, the highest rooting rate was obtained in MS nutrient medium containing 0.2 mg L^{-1} IBA (Yesmin., 2019).

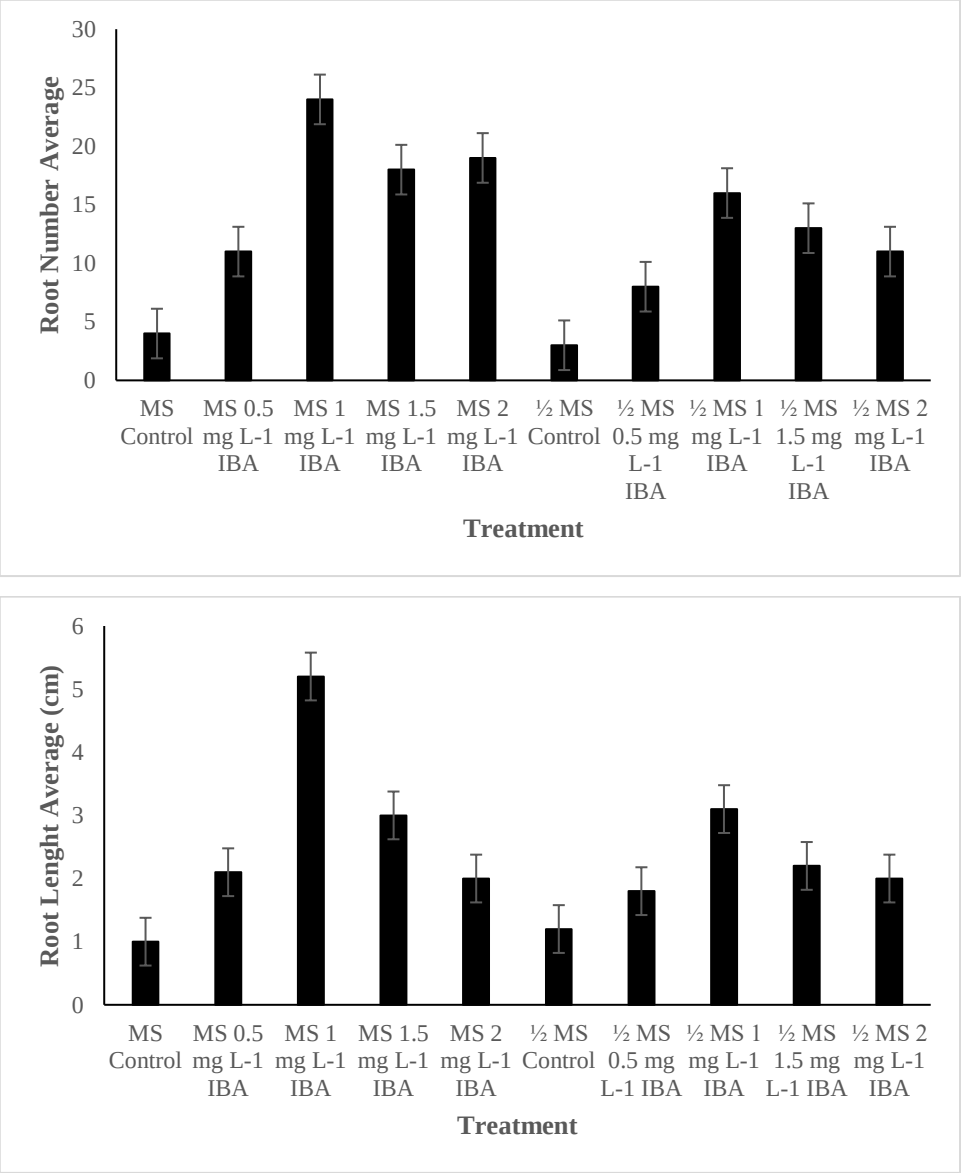

Figure 1. *Rooting study results.*

Rooted plants must go through a gradual acclimation process in order to be transferred to external conditions. This stage, in which plants kept at high humidity and constant temperature *in vitro* conditions are controlled and acclimatized to external conditions, is very important. Especially in herbaceous plants, it is very important to control and monitor the conditions in order to avoid plant losses at this stage. In this study, plants were successfully transferred to external conditions without loss in the acclimatization stage. After the rooted plants were transferred to sterilized peat, after irrigation and leaf moistening, they were covered with airtight cling film and transferred to fully controlled plant growth rooms. After the stretch was kept completely closed during the first week, holes were opened on the stretch and the amount of moisture inside was gradually reduced. The acclimatization process, which continued by increasing the number of holes in the second week, was completed by completely removing the stretch at the end of the third week. Afterwards, the plants were transferred to greenhouse and field conditions and

the acclimatization process was successfully completed and later to field conditions (*Figure 2*). During the acclimatization phase, different soil mixtures and irrigation waters are used according to the plant's demands. In this study, this step was carried out by irrigating sterile peat with distilled water. Hirakawa and Tanno performed the acclimatization process of hops by irrigation with distilled water in a soil mixture containing 3:1:1 peat, vermiculite and akadama (Hirakawa and Tanno, 2022). Petrova et al., on the other hand, carried out the acclimatization stage of lemon balm by irrigating a soil mixture containing soil, peat, perlite, and sand at a ratio of 2:1:1:1 with pure water (Petrova et al., 2021). In Yesmin's study on sugar grass, acclimatization was carried out by irrigating a 1:1 mixture of garden soil and compost with pure water (Yesmin., 2019). Figas et al. watered the *H. arenarium* with water and water containing 25% MS during the acclimatization phase. While 56% acclimatized plants were obtained in irrigation with water, 75% acclimatized plants were obtained in irrigation with 25% MS-containing water (Figas et al., 2016).

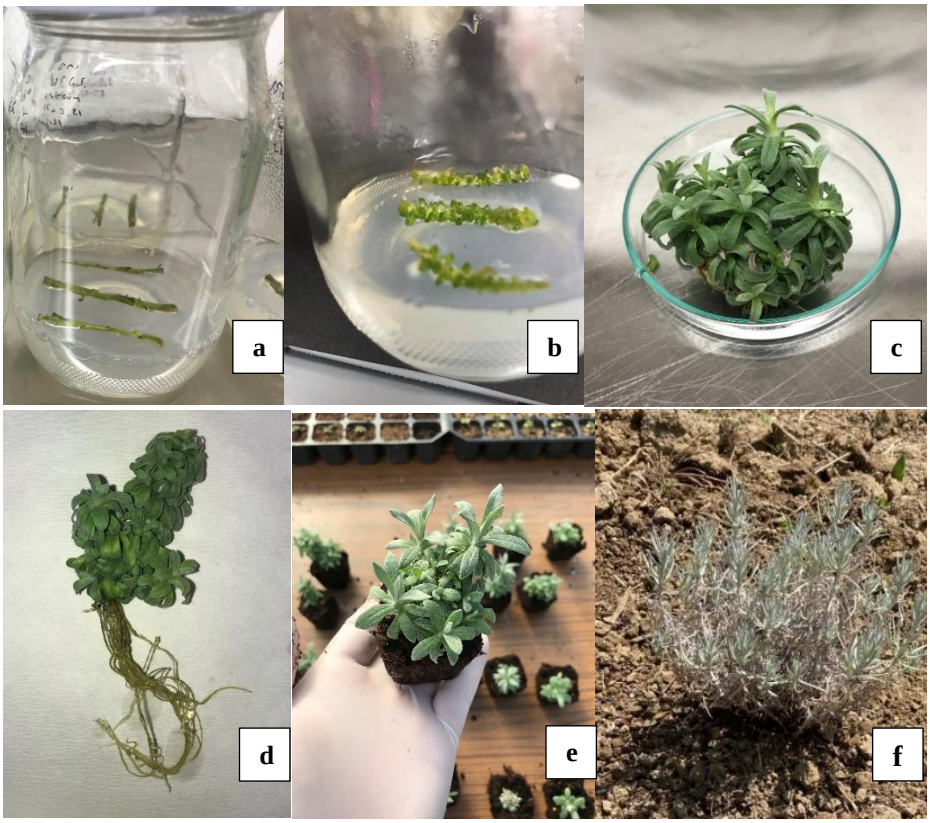

Figure 2. *(a) explants transferred to nutrient media, (b) vitrification in nutrient media containing Gamborg B5, (c) shoot development in 30 days, (d) root development in rooting medium, (e) acclimatized seedlings, (f) 2 months of development in field conditions.*

4. Conclusions

Immortelle grass is a plant that has a very important place among medicinal and aromatic plants that spread naturally in Türkiye. This plant has essential oil and biochemical components used in many sectors, especially in the pharmaceutical, cosmetic and paint sectors. Agriculture is of great importance in our country's economy. For this reason, it is vital to make good use of the agricultural lands owned. Since immortelle grass is not selective in terms of growing conditions, it has the potential to contribute to the country's economy, even in barren lands. For these reasons, it is necessary to encourage the production of healthy and high-quality seedlings of immortelle grass under *in vitro* conditions, their promotion to farmers and cultural agriculture.

According to the information obtained in the literature review for the sterilization of immortelle grass, which is very difficult to sterilize due to its hairy leaves, NaOCl (bleach), which has the lowest cost among the chemicals used and is known to be the least harmful to nature, was preferred. According to the results obtained, it has been observed that the concentration of the commercial bleach solution is as important as the time in order to obtain sterile and healthy plants. Successful results were obtained in the trial, which was kept for a shorter time due to the burning of the plants

kept in high concentration for a long time.

Successful results were obtained by using a lower concentration of plant growth regulator (0.5 mg L^{-1} BAP, 1 mg L^{-1} GA and 0.2 mg L^{-1} NAA) in the micropropagation stage compared to previous studies.

At the rooting stage, as a result of the experiments, 100% rooting was obtained in ½MS basic nutrient medium without plant growth regulator.

The acclimatization stage is crucial for this plant. To prevent plant loss, humidity and temperature should be gradually balanced with external conditions during the controlled acclimation stage. Due to the very high humidity and optimum temperature *in vitro* conditions, sudden moisture loss or heat shock at this stage causes the plant to die.

As a result of the studies carried out, the micropropagation of immortelle grass has been optimized by using lesser amounts of chemicals that are less harmful to nature. In this way, a fast and economical method that can be used to produce of high amounts of seedlings for cultural agriculture has been created. In addition, this study lays the groundwork for future *in vitro*, molecular and secondary metabolite studies in immortelle grass.

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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: Uz, I., Savalan, S.; Design: Uz, I., Savalan, S.; Data Collection or Processing: Uz, I., Savalan, S.; Statistical Analyses: Uz, I., Savalan, S.; Literature Search: Uz, I., Savalan, S.; Writing, Review and Editing: Uz, I., Savalan, S.

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