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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

The Effects of Silver Nitrate and Pre-Cold Treatments on Callus Formation in Strawberry Anther Culture

Gümüş Nitrat ve Ön Soğuk Uygulamalarının Çilek Anter Kültüründe Kallus Oluşumuna Etkileri

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Abstract

Strawberry (*Fragaria* x *ananassa* Duch.) which is one of the widely grown berry species in the world has economic and commercial importance. In commercial strawberry varieties, in order to increase yield and quality, it is necessary to obtain starting materials that are resistant/tolerant to biotic and abiotic stress factors. Biotechnological methods have an important place in strawberry breeding studies due to the long and costly process of classical breeding methods, the genetic expansion of seed production, high ploidy level and strong heterozygosity. Haploid plant production is an efficient breeding method that has been successfully applied to most plant species. However, due to the lack of sufficient haploid studies on strawberry and the fact that a specific protocol for this species has not yet been developed the necessary progress has not been made in this regard. In this study, the effectiveness of some factors determine the success in anther culture which has a significant place in obtaining haploid strawberries was investigated. For this reason, first, different sodium hypochlorite doses (NaOCl; 1%, 2%, 3%) and application durations (10, 15, 20 min) were used to determine the appropriate method for sterilisation, then cold pre-treatments (24, 36, 48, 72 hours at +4 °C) and different silver nitrate doses ($AgNO_3$; 10, 20, 30, 40 mg l-1) were employed for callus induction in Festival strawberry variety. At the conclusion of the study it was observed that the lowest contamination rate (1%) was obtained by soaking in 1% sodium hypochlorite solution for 10 minutes. Cold pre-treatment of flower buds at +4 °C for 36 hours produced the highest callus induction rate (96%). The evaluation of the effect of $AgNO_3$ application at different doses on the callus induction rate revealed that the highest callus induction (82%) was obtained from 20 mg l-1 $AgNO_3$ dosage. This study showed that anther culture practices in strawberry can be improved by using cold pre-treatment, appropriate sterilization method and silver nitrate addition to the medium.

Keywords: Anther, Callogenesis, Silver nitrate, Micropropagation, Haploid

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Öz

Çilek (*Fragaria* x *ananassa* Duch.), dünyada yaygın olarak yetiştiriciliği yapılan, ekonomik ve ticari öneme sahip olan önemli üzümsü meyve türlerinden birisidir. Ticari çilek çeşitlerinde, verim ve kalitenin artırılması için biotik ve abiotik stress faktörlerine karşı dayanıklı veya tolerant başlangıç materyallerinin elde edilmesi gerekmektedir. Çilekte, klasik ıslah çalışmaları süreçlerinin uzun ve masraflı olması, tohumdan üretiminin genetik açılım göstermesi, yüksek ploidi seviyesi ve güçlü heterozigotluk sebebiyle, biyoteknolojik yöntemler ıslah çalışmalarında önemli bir yer tutmaktadır. Haploid bitki üretimi çoğu bitki türünde başarı ile uygulanan etkili bir ıslah yöntemidir. Ancak çilekte haploid bitki üretimi ile ilgili yeterli sayıda çalışmanın olmaması ve bu türe özgü bir protokolün hala geliştirilememesi gibi nedenlerle bu konuda gerekli ilerlemeler sağlanamamıştır. Bu çalışmada, haploid çilek eldesinde önemli bir yer tutan anter kültüründe başarıyı etkileyen bazı faktörlerin etkinliğinin araştırılması amaçlanmıştır. Bu nedenle, Festival çilek çeşidinde kallus indüksiyonu için öncelikle sterilizasyon için uygun yöntemi belirlemek amacıyla farklı sodyum hipoklorit dozları (NaOCl; %1, %2, %3) ve uygulama süreleri (10, 15, 20 dk) ardından farklı soğuk ön uygulamaları (+4 °C'de 24, 36, 48, 72 saat) ve gümüş nitrat dozları ($AgNO_3$; 10, 20, 30, 40 mg l-1) kullanılmıştır. Araştırmadan elde edilen verilerin değerlendirilmesi sonucunda en düşük kontaminasyon oranının (%1) %1'lik sodyum hipoklorit çözeltisinde 10 dakika bekletilerek elde edildiği belirlenmiştir. Çiçek tomurcuklarının 36 saat boyunca +4 °C 'de soğuk ön muameleye tabi tutulması ile en yüksek kallus indüksiyon oranı (%96) sağlanmıştır. Farklı dozlarda $AgNO_3$ uygulamasının kallus indüksiyonu üzerine etkisinin değerlendirilmesi ise en yüksek oranın (%82) 20 mg l-1 $AgNO_3$ dozundan elde edildiğini göstermiştir. Bu çalışma, çilekte anter kültürü çalışmalarının soğuk ön uygulaması, uygun sterilizasyon yöntemi ve besi ortamına gümüş nitrat ilavesi ile geliştirilebileceğini göstermiştir.

Anahtar Kelimeler: Anter, Kallogenesis, Gümüş nitrat, Mikroçoğaltım, Haploid

1. Introduction

Strawberry (*Fragaria* x *ananassa*), which is a commercially important crop, is one of the most produced and consumed berry fruit throughout the world. Its suitability for early harvest and its high export opportunities have increased its importance day by day, and gave rise to the strawberry breeding studies (Lahiri et al., 2022; Simpson, 2018; Bayram et al., 2016). Unlike many agricultural products strawberry can provide high value in fresh and processed markets. In addition to fresh consumption, it can also be consumed by freezing or drying, or as fruit juice, jam, marmalade, dessert, cake, ice cream and liquor (Chandler et al., 2012; Witter et al., 2012). The fact that the strawberry fruit can be consumed in different ways allows it to be grown in different ecologies throughout the year (Davis et al., 2007). It is also a beneficial food source for human health, as it is rich in antioxidants, vitamin C, fiber, polyphenols and potassium (Aaby et al., 2018; Andrianjaka-Camps et al., 2017; Michalska et al., 2017). Turkey ranks 4th in the world with a production value of 486 705 tons in 8.8 million tons of strawberry production (Anonymous, 2021).

Although the yield, fruit size and quality characteristics of strawberry genotypes have been improved with classical breeding methods, the increase in yield and quality and tolerance to stress factors have still not been fully achieved in commercial strawberry cultivars (Hummer et al., 2022; Mezzetti., 2013). The reasons for this situation are the time-consuming and costly process of classical breeding in strawberry, genetic expansion in production from seed, the high ploidy level and the difficulties caused by strong heterozygosity. Haploid technology, which is one of the biotechnological methods and a useful tool for plant breeding, allows the rapid development of productive, high quality, disease and pest resistant varieties (Nguyen et al., 2012). Successful results have been obtained by culturing anthers which have not reached their full maturation stage and contain mononuclear microspores that have reached the first pollen mitosis stage, under in vitro condition in approximately 250 different plant species until today (Irikova et al., 2011). Since strawberry is a herbaceous plant, it is a much more advantageous species in terms of hapliod technique compared to other woody fruit species. Obtaining haploid plants from anthers by callus induction in vitro is a very effective method for strawberry breeding studies. After callus formation, haploid plant production occurs by indirect embryogenesis or indirect organogenesis (Na et al., 2019; Niazian et al., 2017). As with other tissue culture techniques, there are many internal and external factors that affect the embryonic response in androgenesis (Pehlivan et al., 2017; Dunwell, 2010). The success of anther culture is related to factors such as genotype, donor plant physiology, microspore/pollen developmental stage, pre-treatments (temperature shock) and culture conditions (plant growth regulator and carbon source) (Na et al., 2019). The effects of culture medium type, myo-inositol, auxin and cytokinin combination treatment, silver nitrate ($AgNO_3$) and ferric ethylenediaminetetraacetic acid (Fe-EDTA) to callus induction in strawberry were determined from anther culture (Na et al. 2019). In addition to these factors, the effectiveness of heat shock application was investigated in a study by Kim et al 2020. Na et al. (2011) also investigated the effects of cold pre-treatment and medium content on anther culture of strawberry.

Although there are studies on strawberry anther culture around the world, there is no study on this subject in Turkey. Since there are not enough androgenesis studies in strawberry and a specific protocol for this species has not yet been developed, it has become inevitable to identify and develop effective protocols. In order to develop an effective protocol, it is necessary to first determine the factors affecting callus induction in anther culture. In this study, it was aimed to establish an effective protocol for callus induction from anther culture in Festival strawberry cultivar. In this context, the effects of sodium hypochlorite (NaOCl), pre-cold treatment and silver nitrate ($AgNO_3$) applications on callus formation were investigated.

2. Materials and Methods

2.1. Plant material

The 'Festival' strawberry cultivar used as plant material in the research was obtained from a commercial company. This cultivar is a hybrid of Oso Grande and Rosa Linda and is a short-day variety. Measurements and analyses were carried out in Akdeniz University Vocational School of Technical Sciences Laboratories.

2.2. Method

2.2.1. Growing donor plants

The plants of cv. Festival were grown in the greenhouses of Akdeniz University Research and Application Farm in the 2021-2022 growing season. Fresh tube seedlings were planted in the greenhouse soil in the autumn planting period. In order to meet the nutrient requirements, fertilization and cultural treatments were carried out before and after planting.

2.2.2. Removal of buds

Anthers taken from buds of different sizes were stained with acetocarmine and examined under a microscope in order to determine the appropriate bud size. In the cytological examination, buds (1-5 mm) containing anthers with microspores in the middle or late mononuclear period were taken. The development period of the buds (in terms of bud size) containing the anthers at the appropriate period for both cultivars was determined and the anthers in the buds were used as explants. Buds containing microspores at the appropriate stage from donor plants were collected in falcon tubes between 06:30 and 07:30 in the morning and brought to the tissue culture laboratory in an ice box.

2.2.3. Pre-cold application

In order to determine the effectiveness of the pre-cold application and increase the efficiency of callus induction from anther culture in strawberry, flower buds are exposed to +4 °C for different lengths of time. Buds at the appropriate pollen stage were collected from the plant and then were placed in the refrigerator in 90 mm diameter plastic petri dishes with moistened filter papers to be subjected to cold application at +4 °C for 24, 48, 36, 72 hours.

2.2.4. Surface sterilization of buds

Different sodium hypochlorite (NaOCl) doses (1%, 2%, 3%) and application durations (10, 15, 20 min) were tried for surface sterilization. Buds were kept in 70% ethanol solution for 30 seconds and then in different concentrations of NaOCl solutions containing 1-2 drops of Tween-20 for different durations. Sterilization was completed by passing buds through sterile distilled water three times.

2.2.5. Anther culture

After collecting from the buds with scalpel and forceps in a sterile cabinet, anthers were cultured on MS medium containing 0,4 mg l−1 BA+0,1 mg l−1 IAA+2,0 mg l−1 2,4-D and 30 g l−1 sucrose (*Figure 1*). In order to determine the effects of $AgNO_3$ on callus formation, different doses of $AgNO_3$ (10, 20, 30, 40 mg l −1) were also added to the MS medium. Experiments were conducted in petri plates measuring 90 mm in length x 20 mm in height and 10 anthers were placed in each petri dish. Cultures were maintained at 32°C for 48 hours then at 25°C in the dark for 8 weeks. During the study observations were made twice a week and the number of callus forming explants were recorded.

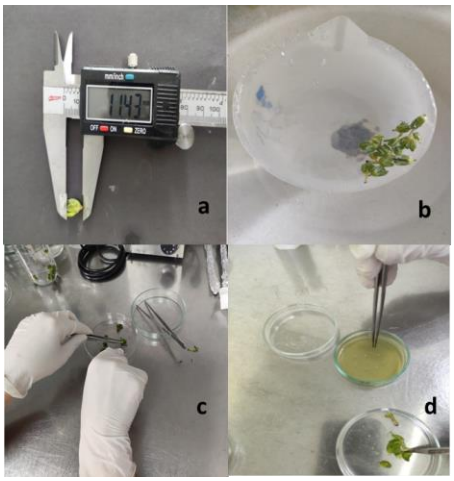

Figure 1. Explant preparation steps for anther culture a. Selection of appropriately sized flower buds b. Selected closed flower buds c. Removal of anthers d. Anther explants placed on petri plates containing MS medium

3. Results and Discussion

3.1. Surface sterilization of the buds

Microbial contamination is one of the most important threats in in vitro tissue culture (Daud et al., 2012). Virus, bacteria or fungal pathogens cause losses in plant tissue culture resulting in tissue necrosis, reduced shoot growth and reduced rooting. It is desirable that the sterilization measures are included into the process of culturing plant materials, ensuring only the microbial contaminants are destroyed and their biological activities maintain without being damaged. For this reason, it is necessary to carefully select the type and application periods of the disinfectants to be used in the sterilization process. Although ethanol is a powerful sterilizer, it is a highly toxic chemical for the explants. It is therefore recommended to keep the application time short. Among the chemicals used for surface sterilization, sodium hypochlorite is the most commonly preferred because it is easily available and can be diluted to appropriate concentrations (Tyagi et al., 2011). Therefore, in this study, different concentrations of NaOCl and different application times were tried for surface sterilization (*Table 1*). Among the different NaOCl doses (1%, 2%, 3%) and application durations (10, 15, 20 min) tried in the study, the best surface sterilization procedure with 1% infection rate and 95% anther survival rate involved keeping the explants in 70% ethanol for 30 seconds and then in 1% sodium hypochlorite for 15 minutes. Although no infection was observed in 3% NaOCl, the survival rate of anthers decreased to 65%.

Table 1. Infection and anther survival rate in surface sterilization for buds

Applications	Anther survival rate (%)	Infection rate (%)
1% NaOCl 10 minutes	92	2
1% NaOCl 15 minutes	95	1
1% NaOCl 20 minutes	90	1
2% NaOCl 10 minutes	90	1
2% NaOCl 15 minutes	85	1
2% NaOCl 20 minutes	85	0
3% NaOCl 10 minutes	70	0
3% NaOCl 15 minutes	70	0
3% NaOCl 20 minutes	65	0

3.2. Pre-cold application

Temperature shocks induce androgenetic response in different plants (Kiviharju and Pehu, 1998). The available stresses can be heat shock treatment (Kim et al., 2020) as well as cold pre-applications (Na et al., 2011). Cold pre-treatment is widely used to promote androgenesis in many other plants. It has been determined that the cold pre-treatment also provides an effective anther culture of strawberry (Shahvali-Kohshour et al. 2013).

The most effective cold pre-application for callus induction from anther culture of strawberry Seolhyang cv. was obtained by keeping it at 4°C for 72 hours (Na et al., 2011). In Camarosa, Selva and Paros strawberry cultivars at 4°C for two days and in Pajaro cv. at 4°C for three days cold pre-application were the best for promoting anther callogenesis (Shahvali-Kohshour et al., 2013).

In this study, four different cold pre-applications (24, 36, 48 and 72 hours) were performed. From the results it was determined that, among different pre-cold applications (24, 36, 48, 72 hours at +4°C), the highest callus induction rate (85%) was obtained from 36 hours application at +4°C (*Figure 2*). Although this rate is higher than the callus induction rate obtained from the Seolhyang cv. (Na et al., 2011), the effect of the temperature as pre-treatment on callus induction varies depending on the genotype (Kiviharju and Pehu, 1998).

Cold pre-treatment in strawberry protects microspores by preventing the decay of anther tissues and the release of toxic compounds from rotting anthers. The increase in free amino acid content in microspores caused by cold pre-treatment is thought to mediate the induction of embryogenesis. It also leads to greater survival of embryogenic pollen grains (Shahvali-Kohshour et al., 2013; Shariatpanahi et al., 2006).

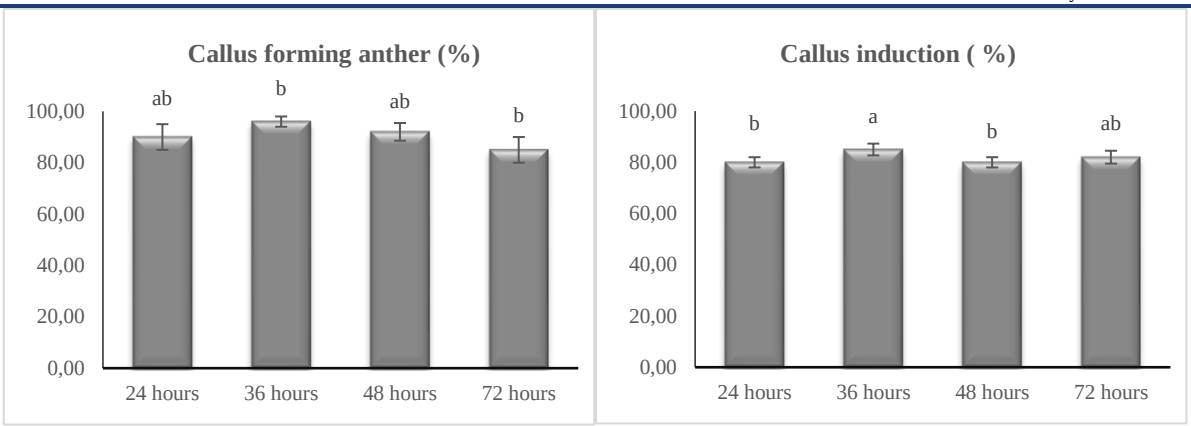

Figure 2. The effects of pre-cold applications on anther development and callus formation of Festival cv.

3.3. The effects of different silver nitrate concentrations on callus formation

Determining the medium and optimizing the most appropriate dose of each chemical component added to the medium is a prerequisite for success in haploid plant production in vitro (Hassan and Islam, 2021). Silver nitrate is one of the components added to the culture medium before the anthers are cultured or during the culture to increase the success of anther culture (Shahvali-Kohshour et al., 2013). Murashige and Skoog (MS) medium, Gamborg B5 medium and Lichter medium were used for callus formation from the anther for haploid plant production in Seolhyang cultivar, and the best results were obtained in MS medium. The effects of $AgNO_3$ on callus formation were also investigated in the same study, and the highest callus formation rate was determined as 41.4% in the MS medium with 25% silver nitrate (Na et al., 2019). Therefore, MS medium was used for the formation of callus from anther culture in the Festival cv. In terms of promoting anther development (anther response rate) and callus induction in different concentrations of $AgNO_3$, the best dose was determined as MS medium containing 20 mg l-1 $AgNO_3$ (*Figure 3*). In the presence of 20 mg l-1 silver nitrate in the MS medium, the highest callus formation rate was 82% (*Figure 4*), which is twice that of the callus formation rate obtained in Seolhyang cultivar (Na et al., 2019). In Camorasa strawberry cultivar, the highest callus formation was obtained in the presence of 15 mg l−1 $AgNO_3$ added to the culture medium (Shahvali-Kohshour et al., 2013).

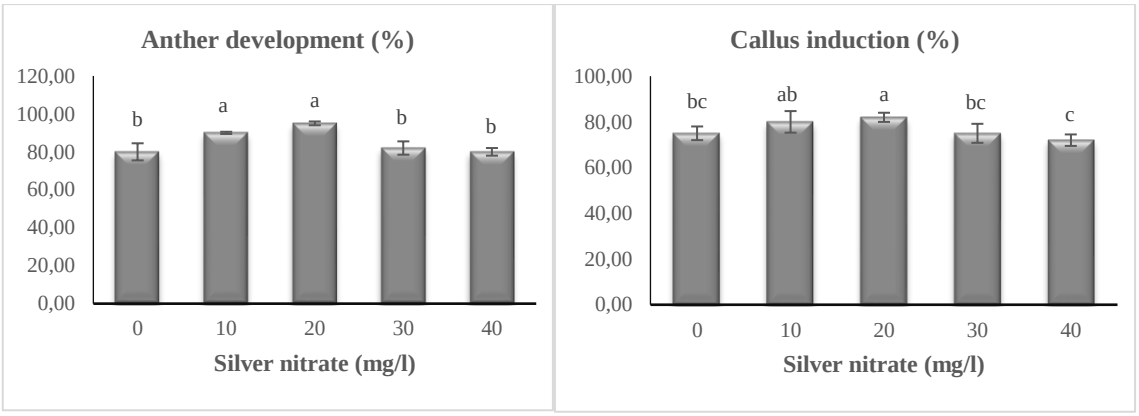

Figure 3. The effects of different concentrations of $AgNO_3$ applications on anther development and callus formation of Festival cv.

Another factor affecting anther culture is plant growth regulators added to the medium. In a study carried out by the Kim et al. (2019) investigating the effectiveness of different hormones for the propagation of Goha and Seolhyang cultivars in in vitro culture medium, it was determined that BA application was the most effective plant growth regulator for proliferation. Kim et al. (2020) used BAP, IAA, and 2.4-D as plant growth regulators in their study investigating the effects of various factors, including $AgNO_3$, on callus formation and plant regeneration from anther culture in strawberry. The highest callus formation rate of 71.4% was obtained at 25 mg l-1 $AgNO_3$ concentration.

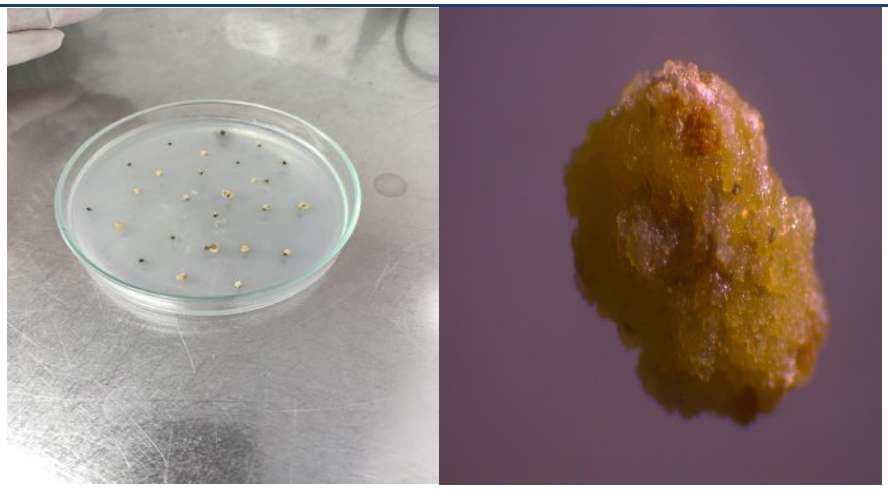

Figure 4. Callus formation from anther explant of Festival cv. on MS medium containing 20 mg l^{-1} $AgNO_3$

Indolacetic acid (IAA), benzyl amino purine (BAP), 2,4-Dichlorophenoxyacetic acid (2,4 D), of which the efficacy has been investigated previously for anther culture (Kim et al., 2020) were added in MS medium for all trials. Since the effects of cold pre-treatment and $AgNO_3$ on callus formation from the anther culture of the Festival cv. were investigated in this study, the hormones added to the nutrient medium were kept at same doses for all applications.

4. Conclusions

Haploid plant production is a very effective method for strawberry breeding. Callus formation is required for haploid plant regeneration through anther. Therefore, in this study we aimed to investigate the cold pre-treatment period, sterilization method and $AgNO_3$ concentrations on callus formation from anther explant using cv. Festival. Our findings suggest that cold pre-treatment of anthers for a period of 36 hours at +4ºC and adding of 20 mg l-1 $AgNO_3$ to the medium were best practices for promoting callus formation. Also, this study showed that strawberry anther culture can be improved by using cold pre-treatment, sterilization method and silver nitrate addition to the medium. We can speculate that our results provide the basis for further research to develop effective protocols for anther culture, a method used in the production of haploid strawberries.

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: Sevinç Şener and Ayşe Gül Nasırcılar; Design: Sevinç Şener, Ayşe Gül Nasırcılar and Ahmet Karaçan; Data Collection or Processing: Sevinç Şener, Ayşe Gül Nasırcılar and Ahmet Karaçan; Statistical Analyses: Sevinç Şener; Literature Search: Sevinç Şener and Ayşe Gül Nasırcılar; Writing Sevinç Şener and Ayşe Gül Nasırcılar.

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