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Araştırma
(Research)

Effect of Genotype, Cold Pre-Treatment, Incubation Conditions and Media on Embryo and Plantlet Formation in Pepper Anther Culture*

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Abstract

Objectives: The aim of this study was to investigate the effects of various culture conditions on embryoid and plantlet formation in pepper anther cultures. To achieve this, the effects of media compositions, vitamin B_{12}, activated charcoal, bud treatments, and incubation temperatures were evaluated.

Materials and Methods: The study was conducted at the screenhouse and tissue culture laboratory of Gaziosmanpaşa University. Three pepper (*Capsicum annuum* L.) genotypes—Belissa F_1, Bafra F_1, and İstek F_1—and their F_2 populations were used to examine the effects of different culture conditions on embryoid formation. Donor plants were grown in a controlled environment using Hoagland's nutrient solution and an integrated pest management approach. Anthers were cultured in DDVX medium, modified with different concentrations of vitamin B_{12} (control, 0.03 mg L^{-1}, 0.05 mg L^{-1}), and in Double Layer (DL) medium with 0.10% and 0.20% activated charcoal. To stimulate embryoid formation, buds were subjected to cold shock at 4°C for 24 or 48 hours, and incubation temperatures ranging from 9°C to 35°C were tested.

Results: In DDVX medium, 702 embryoids and 124 haploid plantlets were obtained. The highest embryoid formation (51) was observed in the Belissa F_2 genotype under conditions of 24-hour pre-treatment at 4°C, no vitamin B_{12} supplementation, and incubation at 35°C. In the Bafra and İstek genotypes, vitamin B_{12} (0.03–0.05 mg·L^{-1}) and 35°C incubation significantly enhanced embryoid formation. A 24-hour pre-treatment at 4°C yielded the best results, while a 48-hour pre-treatment did not provide any additional benefit. In DL medium, 274 embryoids and 49 haploid plantlets were produced. The highest number of embryoids (34) was observed in the Belissa F_2 genotype under control conditions with 0.20% activated charcoal and incubation at 9°C. In the Bafra and İstek genotypes, 0.10–0.20% activated charcoal and 9°C incubation provided the most effective results. Unlike DDVX, bud pre-treatments in DL medium did not show a clear advantage. DDVX medium was 256% more successful in embryoid formation compared to DL medium. F_2 populations produced higher embryoid numbers compared to F_1 populations. In DDVX, 35°C incubation was more effective, while in DL, 9°C incubation yielded better results.

Conclusion: The study demonstrated that genotype, nutrient medium, and incubation conditions significantly influence embryoid formation in pepper. DDVX medium produced significantly more embryoids and plantlets compared to DL medium.

Key words: DDVX medium, double layer medium, activated charcoal, vitamine B_{12}, haploidy

Genotip, Tomurcuk Uygulamaları, İnkübasyon Koşulları ve Besion Ortamlarının Biber Anther Kültüründe Embriyo ve Bitkicik Oluşumuna Etkisi

Öz

Amaç: Bu çalışmanın amacı, biberde anther kültüründe embriyoid ve bitkicik oluşumuna etki

eden çeşitli kültür koşullarını araştırmaktır. Bu amaçla, ortam bileşimleri, vitamin B_{12}, aktif kömür, tomurcuk uygulamaları ve inkübasyon sıcaklıklarının etkileri değerlendirilmiştir.

Materyal ve Yöntem: Çalışma, Gaziosmanpaşa Üniversitesi'nde tül sera ve doku kültürü laboratuvarlarında yürütülmüştür. Üç biber (*Capsicum annuum* L.) genotipi—Belissa F_1, Bafra F_1 ve İstek F_1—ve bunların F_2 popülasyonları kullanılarak, farklı kültür koşullarının embriyoid oluşumuna etkileri araştırılmıştır. Donör bitkiler, Hoagland besin çözeltisi ve entegre zararlı yönetimi yaklaşımıyla kontrollü ortamda yetiştirilmiştir. Antherler, vitamin B_{12}'nin farklı konsantrasyonları (kontrol, 0.03 mg L^{-1}, 0.05 mg L^{-1}) ile modifiye edilen DDVX ve 0.10% ve 0.20% oranında aktif kömür eklenmiş Double Layer (DL) ortamlarında kültüre alınmıştır. Embriyoid oluşumunu teşvik etmek amacıyla tomurcuklara 4°C'de 24 veya 48 saat süreyle soğuk şoku uygulanmış ve inkübasyon sıcaklıkları olarak 9°C ile 35°C arasında değişen koşullar denenmiştir.

Bulgular: DDVX ortamında 702 embriyoid ve 124 haploid bitkicik elde edilmiştir. En yüksek embriyoid oluşumu (51), Belissa F_2 genotipinde, 4°C'de 24 saatlik ön uygulama, vitamin B_{12} eklenmemesi ve 35°C inkübasyon koşullarında elde edilmiştir. Bafra ve İstek genotiplerinde, vitamin B_{12} (0.03–0.05 mg·L^{-1}) ve 35°C inkübasyon, embriyoid oluşumunu anlamlı derecede artırmıştır. 4°C'de 24 saatlik ön işlem en iyi sonuçları verirken, 48 saatlik ön işlem herhangi bir ek fayda sağlamamıştır. DL ortamında ise 274 embriyoid ve 49 haploid bitkicik elde edilmiştir. En yüksek embriyoid sayısı (34), Belissa F_2 genotipinde, kontrol koşullarında %0.20 aktif kömür ve 9°C inkübasyon ile elde edilmiştir. Bafra ve İstek genotiplerinde, %0.10–0.20 aktif kömür ve 9°C inkübasyon en verimli sonuçları sağlamıştır. DDVX ortamının aksine, DL ortamında tomurcuk ön işlemleri belirgin bir avantaj sağlamamıştır. DDVX ortamı, DL ortamına kıyasla embriyoid oluşumunda %256 daha başarılı olmuştur. F_2 popülasyonları F_1 popülasyonlarına kıyasla daha yüksek embriyoid oluşturmuştur. DDVX ortamında 35°C, DL ortamında 9°C inkübasyon daha etkili olmuştur.

Sonuç: Bu çalışmada, genotip, besin ortamı ve inkübasyon koşullarının biberde embriyoid oluşumunu belirgin şekilde etkilediği anlaşılmıştır. DDVX ortamı, DL ortamına göre önemli düzeyde daha fazla embriyoid ve bitkicik üretmiştir.

Anahtar Kelimeler: DDVX ortamı, double layer ortamı, aktif kömür, vitamin B_{12}, haploid

Introduction

Pepper (Capsicum spp.) is one of the most economically significant vegetable species worldwide, characterized by extensive genetic diversity, including a wide range of types and varieties. Breeding studies play a crucial role in the development and improvement of pepper cultivars. Despite substantial advancements in conventional breeding, anther culture remains a valuable technique in pepper breeding programs. The development of homozygous lines through classical breeding methods is a time-consuming process. However, biotechnological approaches, particularly anther culture, enable the rapid production of homozygous lines, thereby accelerating the breeding process.

Anther culture is an effective in vitro technique that facilitates the generation of haploid plants in pepper, offering significant advantages in terms of genetic purity, homozygosity, and breeding efficiency (Rodeva et al., 2007; Todorova et al., 2013). In this method, anthers are excised from flower buds at an appropriate developmental stage and cultured under sterile conditions, inducing embryo development from microspores within the anthers. Subsequent chromosome doubling in haploid plants leads to the formation of completely homozygous dihaploid lines, while in some cases, androdiploid lines may develop spontaneously (Irikova et al., 2011; Nowaczyk et al., 2015).

Pepper (*Capsicum* spp.) is among the plant species that respond to anther culture. The success and efficiency of anther culture are influenced by numerous factors. Although certain techniques have been widely accepted in previous studies, ongoing research continues to explore alternative approaches to enhance success rates. The first haploid plants in pepper were obtained through anther culture in Capsicum annuum and Capsicum frutescens (Irikova et al., 2016). Initially, anther culture in pepper exhibited a very low success rate; however, extensive research has led to significant improvements in efficiency. These studies have demonstrated that multiple factors influence the success of the process. To date, research has consistently highlighted the critical roles of genotype, the physiological status of the donor plant, and environmental conditions in determining the effectiveness of anther culture.

Genotype is one of the most significant factors influencing success in anther culture. While some genotypes exhibit a high androgenic response, others may show a low or even no response (Jha et al., 2021; İlhan and Kurtar, 2022). In addition to genotype, various studies have highlighted the impact of several factors on the anther culture success. These include the physiological condition and growth environment of the donor plants (Grozeva et al., 2013), the developmental stage of the flower buds (Shrestha et al., 2011), low-temperature treatments applied to flower buds (Popova et al., 2016), the composition of the nutrient medium, and the use of vitamins and hormones (Irikova and Rodeva, 2004; Grozeva and Nankar, 2020). Moreover, incubation conditions for the anthers also play a crucial role in embryoid formation (Grozeva and Nankar, 2020).

This study investigates the effects of low-temperature pretreatment applied to flower buds of selected F_1 pepper genotypes and F_2 genotypes derived from these genotypes, as well as the influence of various anther incubation conditions on embryoid and plantlet formation. The results of this study are intended to contribute to the optimization of anther culture protocols, with the goal of enhancing its efficiency in pepper breeding.

Materials and Methods

This study was conducted in the Gaziosmanpaşa University, Faculty of Agriculture, within a screenhouse and tissue culture laboratory. The plant materials used in this study consisted of three pepper cultivar (*Capsicum annuum* L. cvs 'Belissa F_1', 'Bafra F_1' and 'İstek F_1') along with their respective F_2 populations. Among these genotypes, the Belissa F_1 variety was self-pollinated in Antalya, while the other F_1 genotypes were self-pollinated in Tokat to obtain F_2 populations. Belissa F_1 is characterized by its vigorous and tall growth habit, Kapia-type fruit morphology, high tolerance to cold, and superior fruit-setting ability. Bafra F_1 exhibits a robust plant structure, with a high degree of earliness and productivity. It is resistant to heat and drought stress, producing high-quality, glossy dark green, bitter-tasting, pointed fruits. İstek F_1 also possesses a vigorous plant structure and demonstrates early and high-yielding characteristics. The fruits are elongated, resembling a bell pepper morphology, with a green exterior and thin flesh.

Cultivation of Donor Plants: The seeds of donor plants were sown in a sterile peat medium. Pepper seedlings with 5–6 true leaves were subsequently transplanted into coconut fiber blocks in the screenhouse with eight plants cultivated per genotype. Irrigation and fertilization were carried out using the fertigation method with Hoagland's nutrient solution (Hoagland & Arnon, 1950). To maintain a balance between vegetative and generative growth, regular pruning was performed to promote consistent flower bud formation. To minimize stress in donor plants, chemical pest control was avoided whenever possible. Instead, integrated pest management strategies were employed, including the use of yellow and blue sticky traps and insect nets to mitigate disease and pest infestations.

Determination of Microspore Development Stages: In order to determine the developmental stages of anthers and account for potential differences between genotypes, flower buds were first grouped based on their morphological characteristics. To assess the microspore developmental stages in anthers from these morphologically classified groups, a crushed preparation technique was used in conjunction with the acetocarmine staining method, a rapid and practical staining approach. The staining procedure followed the method described by Elçi (1982). To determine the optimal anther and microspore stage, an integrated evaluation of bud size, anther coloration, and microspore nuclear division stages was conducted. Anthers identified at the most appropriate developmental stage were selected for culture.

Cold Treatments: Flower buds that had reached the appropriate developmental stage for anther culture were collected early in the morning and immediately transferred to a refrigerator set at +4°C. The buds were maintained under dark conditions for 24 and 48 hours before being transferred to a sterile environment for disinfection. Surface sterilization was initiated without delay to minimize potential contamination. In the control treatment, flower buds were not exposed to cold application. Instead, they were collected from donor plants and immediately sterilized without waiting.

Sterilization of Flower Buds: The collected flower buds were first rinsed under running tap water for approximately one minute. Subsequently, they were immersed in 70% ethanol for one minute, followed by treatment with a 20% sodium hypochlorite solution containing a few drops of Tween-20 for 15 minutes. During this disinfection process, the solution was agitated frequently to enhance effectiveness.

The flower buds were then transferred to a sterile cabinet, where they were rinsed with sterile distilled water for five minutes. This washing step was repeated three times to ensure thorough removal of disinfectants, completing the sterilization process.

Preparation of the Nutrient Medium: In this study, the DDVX nutrient medium, as proposed by Dumas de Vaulx et al. (1981), and the Double Layer (DL) nutrient medium, developed by Dolcet-Sanjuan et al. (1997), were utilized. For the DDVX medium, three different concentrations of vitamin B_{12} (control, 0.03 mg L^{-1}, and 0.05 mg L^{-1}) were incorporated into the C medium. Similarly, for the DL medium, three different concentrations of activated charcoal (control, 0.10%, and 0.20%) were added to the main medium. The pH of each nutrient medium was adjusted prior to sterilization. The prepared media were then autoclaved at 121°C and 1.1 atm pressure for 15 minutes to ensure sterility. Following autoclaving, the media were transferred to Erlenmeyer flasks, which were sealed with aluminum foil. Under aseptic conditions in a laminar flow cabinet, 10 mL of the sterilized nutrient medium was pipetted into each Petri dish to facilitate culture preparation.

Harvest of Anthers and Planting in Nutrient Media: Anthers were carefully excised from flower buds using a sterile scalpel and a pair of curved forceps. Once removed, the anthers were immediately placed onto the nutrient medium, ensuring that their dorsal surfaces were in contact with the medium without submerging them. Sterile, disposable 6 cm plastic petri dishes were used for planting the anthers, with six anthers placed in each dish. A total of five petri dishes, containing 30 anthers, were prepared for each treatment. Following the planting process, the edges of the petri dishes were sealed with thin, sterile plastic film strips to prevent contact with the external environment.

Incubation Treatments: Anthers placed on C medium were incubated in the dark at 9°C and 35°C for 8 days. After this period, the Petri dishes were transferred to the acclimatization room, where the anthers were allowed to develop at 25 ± 2°C under 3000 lux light intensity for a 16-hour photoperiod, followed by 8 hours of darkness. After 4 days in the acclimatization room, the anthers were transferred to 9 cm sterile Petri dishes containing R medium. The anthers were subsequently subcultured onto fresh R medium every 4 weeks. Embryoids appeared on the surface of the anthers approximately 5-6 weeks after being placed in R medium. Once embryoids were visible, they were isolated under sterile conditions using a scalpel and transferred to V medium. To facilitate germination, 10 mL of germination medium was added to sterile Petri dishes, and the isolated embryoids were transferred to the medium. The Petri dishes containing the embryoids were incubated under 3000 lux light intensity with a 16-hour photoperiod at 25 ± 2°C. After 8-10 days in the germination medium, plantlets that began to form leaves were transferred to glass tubes (25 mm diameter, 100 mm length), each containing 15 mL of V medium. The plantlets were maintained under acclimatization room conditions until their secondary roots developed and they reached the 3-4 leaf stage (acclimatization size).

Results

In this study, embryoids were obtained from 22 applications in the Belissa F_1 and F_2 genotypes on DDVX medium, while 14 applications yielded no results. A total of 352 embryoids and 62 haploid plantlets were successfully produced. The highest number of embryoids (51) was obtained from the Belissa F_2 genotype under specific conditions, including a 24-hour pre-treatment at 4 °C, a control medium without vitamin B_{12}, and a 35 °C incubation. However, no single application demonstrated a clear advantage in promoting embryoid formation in the Belissa F_1 and F_2 genotypes on DDVX medium. Various factors, including bud pre-treatment, vitamin B_{12} supplementation, and incubation conditions, influenced embryoid formation to varying extents (Figure 1).

In the double-layer medium, embryoids were obtained from 15 applications, whereas 21 applications did not yield any embryoids. A total of 132 embryoids and 24 haploid plantlets were successfully produced under these conditions. The highest number of embryoids (34) and the highest success rate (113.33%) were recorded in the Belissa F_2 genotype under conditions without pre-treatment, with the addition of 0.20% activated charcoal, and incubation at 9 °C. In the double-layer medium, treatments involving activated charcoal and 9 °C incubation generally led to more favorable results in the Belissa F_1 and F_2 genotypes. However, the effect of bud pre-treatment remained inconclusive, as no definitive pattern was observed (Figure 2).

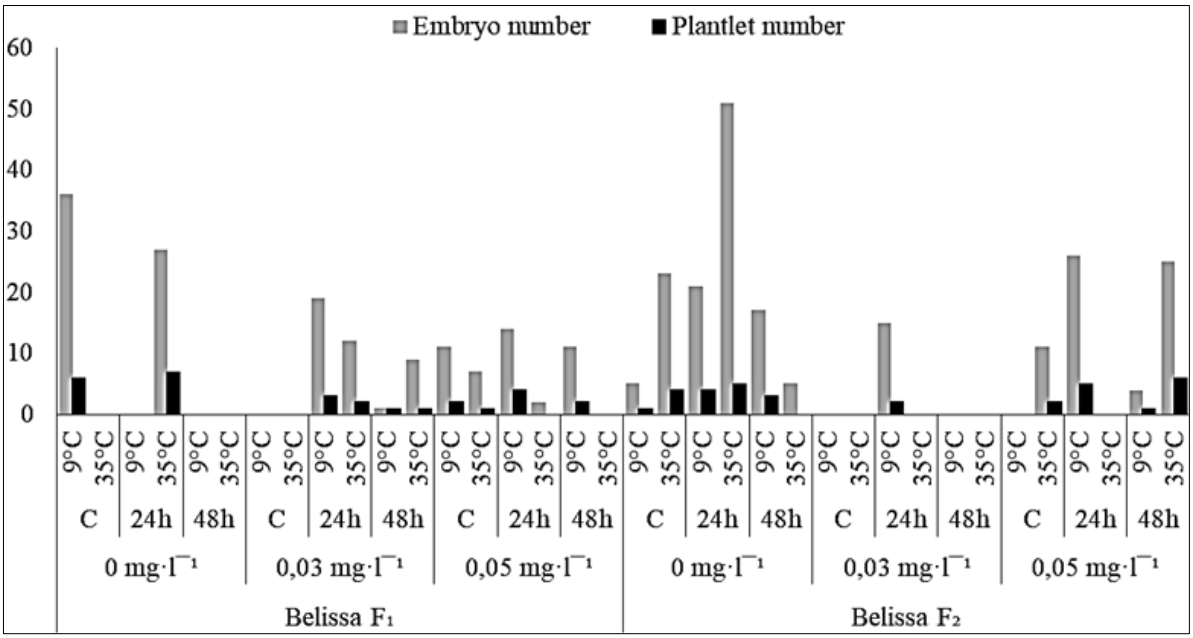

Figure 1. Effect of Vitamin B_{12}, pretreatment and incubation temperature on embryo and plantlet formation in Belissa F_1 and Belissa F_2 genotypes cultured in DDVX medium

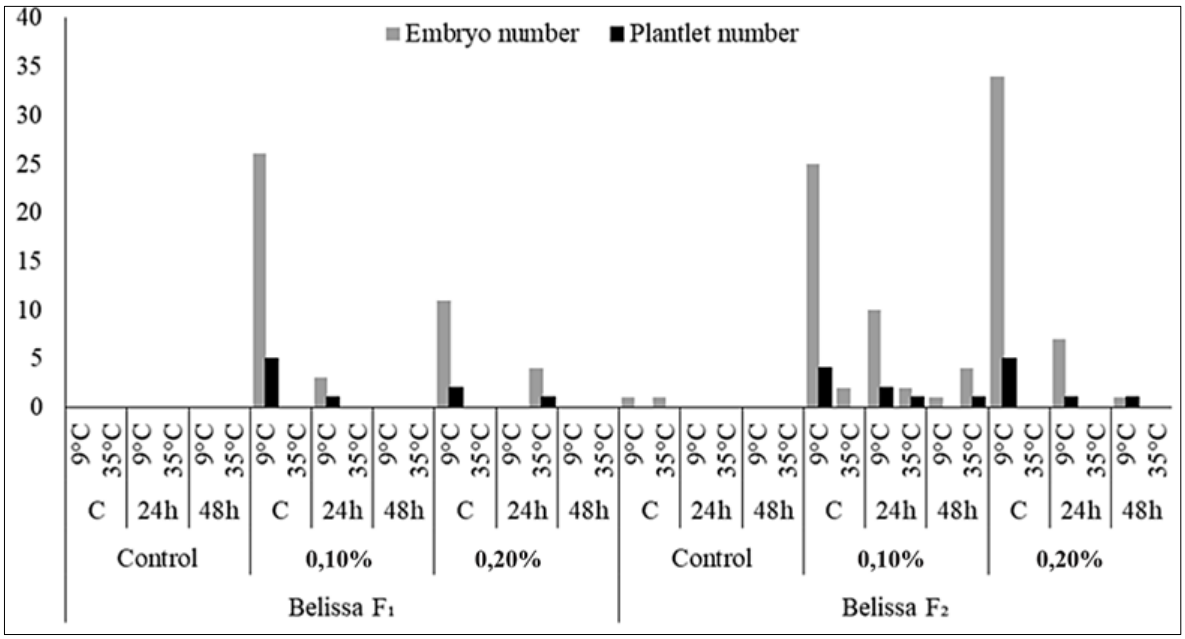

Figure 2. Effect of activated charcoal, pretreatment and incubation temperature on embryo and plantlet formation in Belissa F_1 and Belissa F_2 genotypes cultured in Double Layer medium

In the DDVX medium, embryoids were obtained from 21 applications in the Bafra F_1 and F_2 genotypes, while 15 applications yielded no results. A total of 163 embryoids and 28 haploid plantlets were produced; however, no dihaploid plants were obtained. The highest number of embryoids (19) and the highest success rate (63.33%) were observed in the Bafra F_1 genotype under different applications: (i) a 24-hour pre-treatment at 4 °C, 0.05 mg·L^{-1} vitamin B_{12} supplementation, and incubation at 9 °C; (ii) a 48-hour pre-treatment at 4 °C, incubation at 35 °C, and a vitamin-free control medium. In the DDVX medium, bud pre-treatments and vitamin B_{12} applications at 0.03 and 0.05 mg·L^{-1} were found to enhance embryoid formation in the Bafra F_1 and F_2 genotypes (Figure 3).

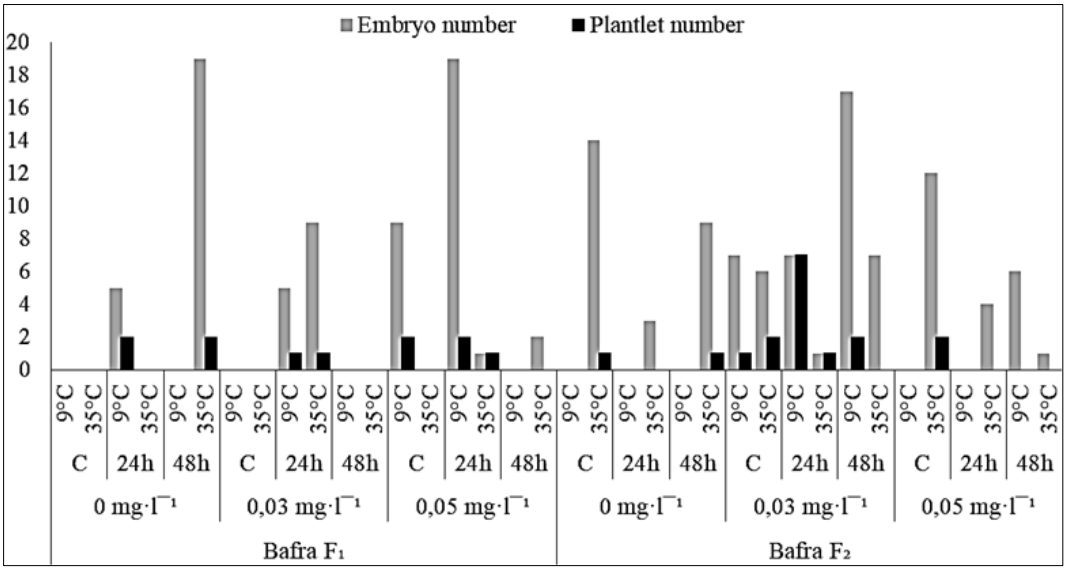

Figure 3. Effect of Vitamin B_{12}, pretreatment and incubation temperature on embryo and plantlet formation in Bafra F_1 and Bafra F_2 genotypes cultured in DDVX medium

In the double-layer medium, embryoids were obtained from 10 applications, while 26 applications yielded no results. A total of 123 embryoids and 19 haploid plantlets were produced, however no dihaploid plants were obtained. The highest embryoid number (32) and the highest success rate (106.67%) were achieved under two conditions: (i) in the Bafra F_2 genotype with a 48-hour pre-treatment at 4 °C and incubation at 9 °C, and (ii) in the Bafra F_1 genotype with 0.10% activated charcoal and incubation at 9 °C (Figure 4).

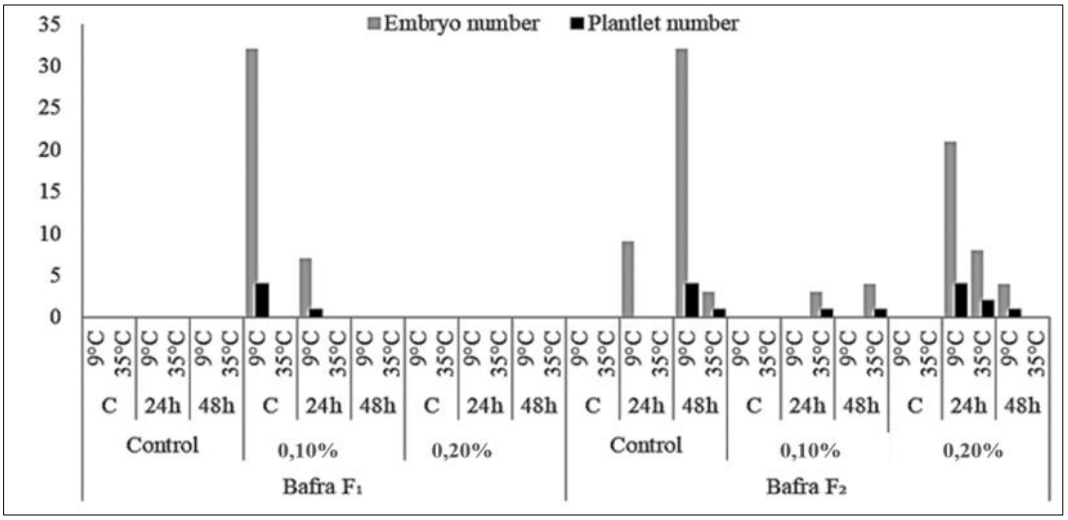

Figure 4. Effect of activated charcoal, pretreatment and incubation temperature on embryo and plantlet formation in Bafra F_1 and Bafra F_2 genotypes cultured in Double Layer medium

In the İstek genotype, embryoids were successfully obtained from 21 applications in the DDVX medium, while 15 applications yielded no response. A total of 187 embryoids and 34 haploid plantlets were produced. The highest number of embryoids (35) and the highest success rate (116.67%) were recorded in the İstek F_1 genotype under the conditions of a 24-hour pre-treatment at 4 °C, incubation at 35 °C, and a vitamin B_{12}-free medium. The findings suggest that in the DDVX medium, bud pre-treatment at 4 °C for 24 and 48 hours, vitamin B_{12} supplementation at concentrations of 0.03 and 0.05 mg·L^{-1}, and incubation at 35 °C had a significant impact on embryoid formation in the İstek F_1 and F_2 genotypes (Figure 5).

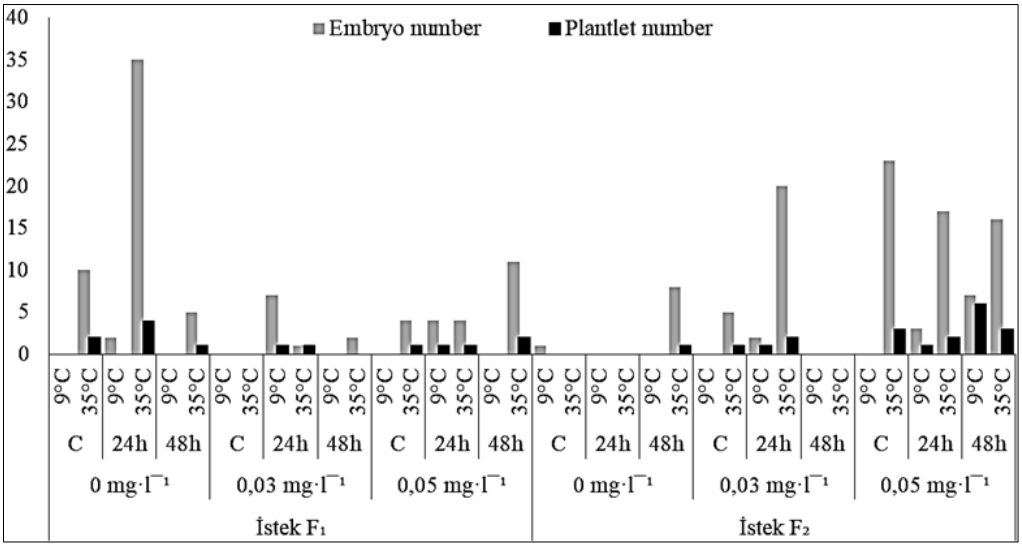

Figure 5. Effect of Vitamin B_{12}, pretreatment and incubation temperature on embryo and plantlet formation in İstek F_1 and İstek F_2 genotypes cultured in DDVX medium

In the double-phase medium, embryoids were obtained from only 5 applications, while 31 applications did not result in embryoid formation. A total of 19 embryoids and 6 haploid plantlets were successfully produced. The highest number of embryoids (8) and the highest success rate (26.67%) were observed in the İstek F_2 genotype under conditions involving a 24-hour pre-treatment at 4 °C, supplementation with 0.20% activated charcoal, and incubation at 9 °C. These results indicate that, in the İstek genotype, bud pre-treatment in the double-phase medium and the inclusion of activated charcoal played a crucial role in promoting embryoid formation (Figure 6).

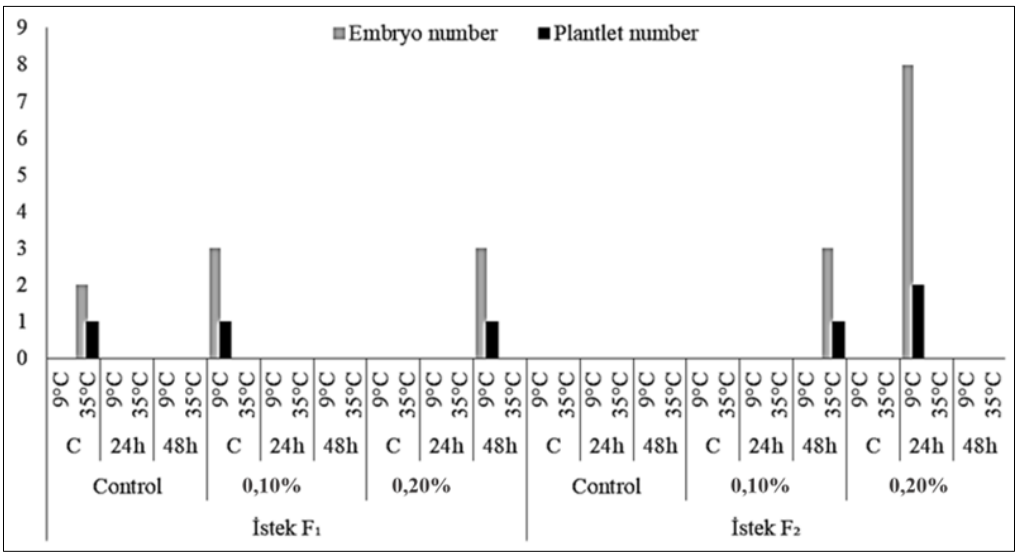

Figure 6. Effect of activated charcoal, pretreatment and incubation temperature on embryo and plantlet formation in İstek F_1 and İstek F_2 genotypes cultured in Double Layer medium

An analysis of the average embryoid productivity of the three genotypes used in the experiment revealed that embryo formation in the DDVX medium was significantly influenced by vitamin B_{12} supplementation. The highest embryoid yield was obtained from the DDVX medium without vitamin B_{12}, followed by the 0.05 mg·L^{-1} and 0.03 mg·L^{-1} vitamin B_{12} treatments, respectively. Among the pre-treatments applied to flower buds, a 24-hour treatment at 4 °C resulted in the highest embryoid formation, while the difference between the control and the 48-hour pre-treatment was not statistically significant. Incubation at 35 °C was more effective than 9 °C in the DDVX medium, and the embryoid

productivity ranking among the genotypes was as follows: Belissa, İstek, and Bafra. Additionally, embryoid productivity in the DDVX medium was higher in the F_2 generation compared to the F_1 generation (Figure 7).

In the Double Layer medium, the addition of activated charcoal enhanced embryoid productivity, with the highest embryoid formation observed at a concentration of 0.10%, followed by 0.20% activated charcoal and the control. Unlike in the DDVX medium, pre-treatments in the Double Layer medium did not promote embryoid formation, as the highest embryoid yield was obtained from the control treatment. Similarly, while 35 °C incubation was more effective in the DDVX medium, embryoid formation in the Double Layer medium was more favorable at 9 °C. The highest embryoid productivity in the Double Layer medium was recorded in the Belissa genotype, followed by the Bafra genotype, while the İstek genotype exhibited very low embryoid production. Consistent with DDVX medium, the F_2 generation showed higher embryoid productivity than F_1 in the Double Layer medium (Figure 8).

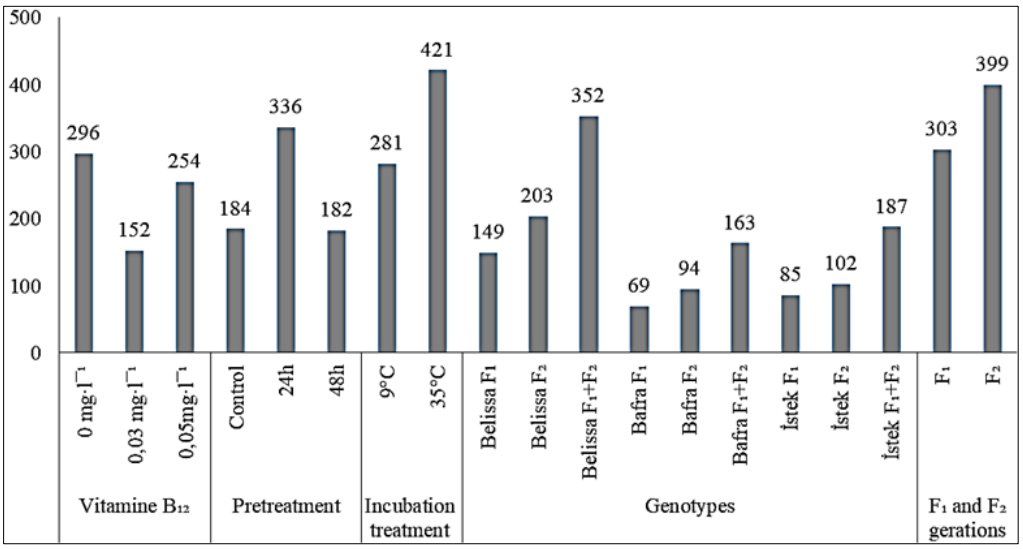

Figure 7. Embryo numbers in response to different treatments in DDVX medium

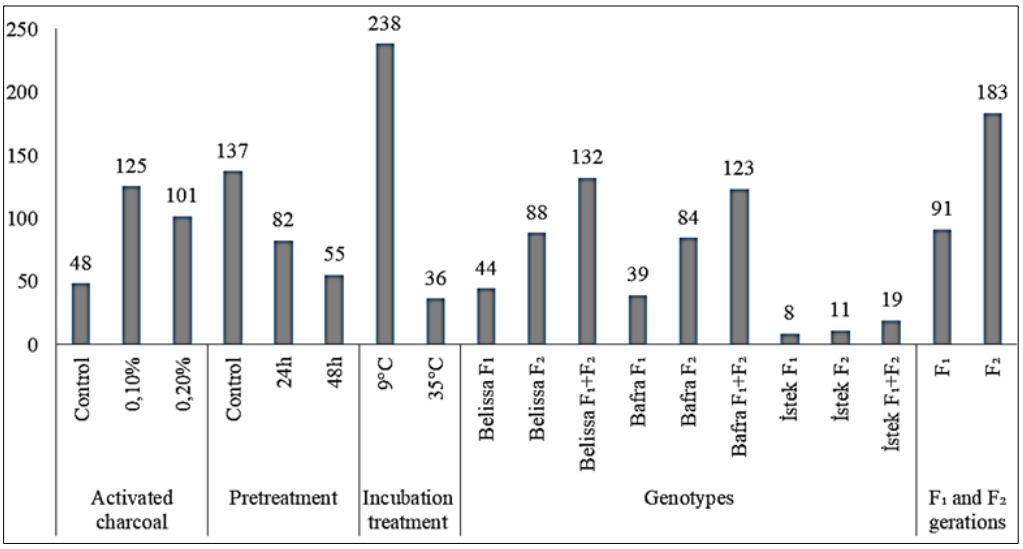

Figure 8. Embryo numbers in response to different treatments in Double Layer medium

A comparison of the two nutrient media showed that a total of 702 embryoids were obtained in the DDVX medium, whereas 274 embryoids were produced in the Double Layer medium. These results indicate that the DDVX medium was 256% more effective in promoting embryoid formation than the Double Layer medium (Figure 9).

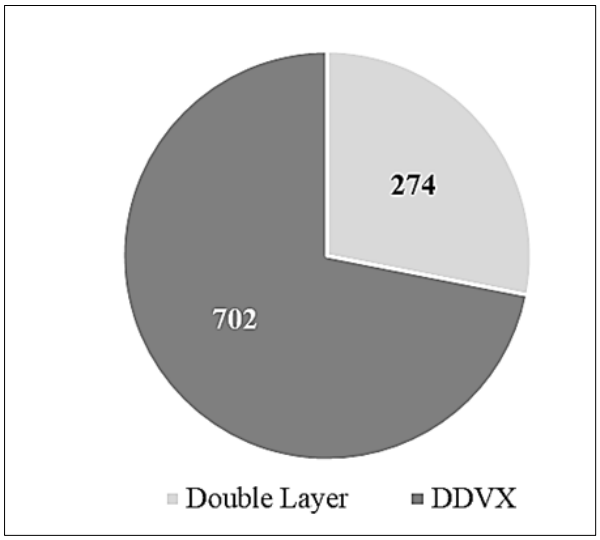

Figure 9. Total number of embryos formed in Double Layer and DDVX media

Discussion

Embryoid and plantlet formation rates varied depending on the specific applications used in this study. In particular, some genotype-dependent applications resulted in high embryoid formation but low plant conversion rates. Similar findings have been reported in the literature, where variations in embryoid formation rates and plant conversion efficiency in anther culture studies have been attributed to factors such as genotype, nutrient medium, and other experimental conditions (Dolcet-Sanjuan et al., 1997; Parra-Vega et al., 2013).

Özkum and Tıpırdamaz (2002), in their studies on pepper anther culture, observed that the embryoid formation rate ranged between 0.5% and 12.5%. However, they also noted that embryoids often failed to convert into plants, with a plant conversion rate of only 0.5% even in the most successful application, which achieved 12.5% embryoid formation. Similarly, Nervo et al. (1995) reported obtaining a total embryoid formation rate of 40.50% and a plantlet conversion rate of 6.3% in their anther culture studies on various pepper genotypes. In another study investigating the effects of season and bud developmental stage on pepper anther culture, Ercan et al. (2006) found that embryo formation frequency ranged from 0.77% to 7.70%, while plant formation from embryos varied between 16.67% and 100.00%. Irikova et al. (2011) also emphasized that success rates in pepper anther culture studies fluctuate based on the applied methodologies.

Anther culture success is influenced by multiple factors, including donor plant growth stage, cultivation conditions, nutrient medium composition, stress treatments, and, most notably, genotype. Many researchers have identified genotype as the primary determinant of success in anther culture (Rodeva et al., 2004; Nowaczyk et al., 2009; Seguí-Simarro et al., 2011; Arı et al., 2016). Shrestha and Kang (2009), in their study on nine different pepper genotypes, demonstrated that each genotype exhibited distinct outcomes in terms of direct and indirect embryoid formation. Taşkın et al. (2011) observed variations in embryoid formation capacities among five pepper genotypes and further noted that genotype performance differed according to the culture medium used. Similarly, Keleş et al. (2015) reported that anther culture success in pepper was highly genotype-dependent. These findings align with the genotype-related variations observed in the present study.

In addition to genotype differences, heterozygosity levels also play a crucial role in determining androgenic success. Irikova et al. (2011) evaluated androgenic success in anther culture studies involving eight parental lines and four hybrids derived from their crosses, revealing that hybrids exhibited lower success rates compared to their parental lines. Similar patterns were observed in this study, where a decline in heterozygosity was associated with increased embryo formation. Specifically, the higher embryoid formation rate observed in F_2 genotypes compared to F_1 genotypes can be attributed to reduced heterozygosity in the F_2 generation due to inbreeding. Başay and Ellialtıoğlu (2013) reported similar findings in eggplant, where standard genotypes produced a higher number of

embryoids than F_1 hybrids. Likewise, Mityko et al. (1995) demonstrated that standard pepper varieties had greater embryo formation rates in anther culture compared to hybrid varieties. Rodeva et al. (2006), in their study on Bulgarian pepper genotypes, reported comparable results. Additionally, Nowaczyk and Kisiala (2006) found that hybrid peppers had lower embryo formation rates than their parental genotypes. The findings of these researchers provide further support for the results obtained in the present study.

In this study, various concentrations of vitamin B_{12} were incorporated exclusively into medium C; however, no significant increase in embryoid formation was observed compared to the control. The effect of vitamin B_{12} also varied depending on genotype. Irikova et al. (2011) emphasized the crucial role of vitamins in improving the success of pepper anther culture and recommended the inclusion of essential vitamins, including vitamin B_{12}, in both media C and R. In contrast to their study, vitamin B_{12} was not used in medium R in the present research. Similarly, Özsan and Onus (2017) reported that in their study, where MS medium supplemented with 0.25% activated charcoal was used as the control, no embryoids were obtained from the control medium. They observed a 3.03% embryoid formation rate in the Belissa genotype when 0.05 $mg \cdot l^{-1}$ of vitamin B_{12} was added, whereas in media without vitamin B_{12}, embryoid formation reached up to 7.86%. Additionally, no embryoids were formed in the Benino and Kanyon genotypes when vitamin B_{12} was applied. These findings suggest that the effect of vitamin B_{12} on embryoid formation is genotype-dependent. In our study as well, embryoid formation was higher in the control medium without vitamin B_{12} for the İstek F_1 and Bafra F_1 genotypes, whereas the İstek F_2 and Bafra F_2 genotypes showed higher embryoid formation rates at vitamin B_{12} concentrations of 0.03 and 0.05 $mg \cdot l^{-1}$. Supporting this observation, Başay and Ellialtıoğlu (2013) reported positive results in specific genotypes in their eggplant anther culture study, where vitamin B_{12} was applied at a concentration of 0.2 $mg \cdot l^{-1}$ in DDV culture medium.

Activated charcoal is a critical factor influencing success in in vitro studies, particularly in anther culture. Its primary mechanism of action is the adsorption of inhibitory substances and harmful gases released from the culture medium or the explant itself (Thomas, 2008). In this study, the highest embryoid formation was achieved with the application of 0.10% activated charcoal. Yang et al. (2009) examined various culture media compositions in pepper anther culture and found that the highest embryoid formation was achieved using a medium containing 1.5 $g \cdot l^{1-}$ activated charcoal. Similarly, Nowaczyk and Kisiala (2006) reported that activated charcoal at a concentration of 0.5 $g \cdot l^{1-}$ enhanced embryoid formation in pepper anther culture; however, they noted that this effect may vary depending on the composition of other medium components.

In anther culture studies, both high and low-temperature shocks have been reported to positively influence androgenic success or callogenesis (Sangwan and Sangwan-Norreel, 1990). Although the precise mechanism underlying the effects of incubation or shock treatments remains unclear, it has been suggested that a reduction in abscisic acid (ABA) levels in cultured anthers may play a critical role (Dolcet-Sanjuan et al., 1997). Numerous studies have demonstrated that incubation treatments in different nutrient media promote either callus or embryoid formation in various plant species (Dolcet-Sanjuan et al., 1997; Matsubara et al., 1998; Xynias et al., 2001; Dias and Correia, 2002). According to the literature, high-temperature stress (35 °C) has been found to be more effective in promoting androgenic success than low-temperature stress (7–9 °C) in pepper (Kim et al., 2004; Barany et al., 2005; Koleva Gudeva et al., 2007). These findings are consistent with the results obtained in this study using DDV medium.

The effectiveness of incubation treatments in anther culture studies may also depend on interactions with other experimental factors. Supena et al. (2006) investigated the effects of incubation temperatures ranging from 35 °C to 9 °C in a double-phase anther culture system and reported that the highest embryoid development was achieved after an 8-day incubation at 9 °C. Similarly, in present study, the 9 °C incubation treatment resulted in the highest embryoid formation in the double-phase medium.

Conclusion

This study demonstrated that anther culture efficiency in *Capsicum annuum L.* is significantly influenced by genotype, nutrient medium composition, incubation temperature, and pre-treatment conditions. The DDVX medium produced a higher number of embryoids than the Double Layer medium, with the highest embryoid formation

observed in the Belissa genotype, followed by İstek and Bafra. Vitamin B_{12} supplementation in the DDVX medium did not consistently enhance embryoid development, whereas the addition of activated charcoal in the Double Layer medium positively affected androgenesis. A 24-hour pre-treatment at 4 °C and incubation at 35 °C promoted embryoid formation in the DDVX medium, while incubation 9°C proved more effective in the Double Layer medium. Overall, these findings emphasize the importance of optimizing culture conditions to enhance haploid and embryoid production in pepper breeding programs, and offer valuable insights for future research on androgenesis in *Capsicum annuum*.

Conflict of interest

The authors declare no conflicts of interest.

Authorship contribution statement

PD: The data were obtained from the doctoral thesis, and she contributed to all stages of the study.

NG: The doctoral thesis advisor, who contributed to every stage of the study.

EP: Contributed to the manuscript preparation, interpretation of results, and discussion.

ESK: Served as the second advisor for the doctoral thesis, providing contributions regarding the techniques and applications for anther culture and assisting in the evaluation of the results

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