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Genotypic Responses of Some Besni Pepper (*Capsicum annuum* L.) Genotypes to Anther Culture

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ABSTRACT
In this study, the response of Besni pepper (*Capsicum annuum* L), one of the local varieties of Turkey, to anther culture was determined. A total of 26 Besni pepper genotypes and 3 control cultivars were examined for their response to anther culture. One hundred and fifty anthers from each pepper genotype were cultured under in vitro conditions. A significant difference (0.0%-45.3%) was found among the genotypes in terms of response to anther culture. The highest embryo formation rate was found in genotype B11 with 45.3% (63 embryos) and the highest transformation rate to plant was found in genotype B15 with 30 plants (68%). Compared to control varieties, Besni pepper genotypes produced significantly more embryos. All genotypes except two out of 26 genotypes used produced more or less (1-68) embryos. B4, B10, B11, B12, B15, and G6 genotypes produced more than 25% of embryos and were separated from the control and other genotypes. The average embryo formation rate of the genotypes collected from Besni and Gölbaşı districts was 13% and 7%, respectively, while the embryo formation rate of the control varieties was only 0.2%. It was concluded that the Besni pepper population was highly responsive to androgenetic haploid. The highly responsive genotypes that form high embryos such as B4, B10, B11, B12, B15, and G6 have the potential to be used in developing new breeding lines and in studies investigating the genetics of anther culture.

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Bazı Besni Biberi (*Capsicum annuum* L.) Genotiplerinin Anter Kültürüne Karşı Tepkileri

ÖZET
Bu çalışmada, Türkiye'nin yerel çeşitlerinden biri olan Besni biberinin (*Capsium annuum* L.) anter kültürüne cevabı belirlenmiştir. Toplam 26 Besni biber genotipi ve 3 kontrol çeşidi anter kültürüne cevapları açısından incelenmiştir. Her biber genotipinden 150 anter in vitro koşullarda kültüre alınmıştır. Anter kültürüne cevap açısından genotipler arasında anlamlı bir fark (%0,0-%45,3) bulunmuştur. En yüksek embriyo oluşum oranı %45,3 (63 embriyo) ile B11 genotipinde, en yüksek bitkiye dönüşüm oranı ise 30 bitki (%68) ile B15 genotipinde bulunmuştur. Kontrol çeşitleriyle karşılaştırıldığında, Besni biber genotipleri anlamlı olarak daha fazla embriyo üretmiştir. Kullanılan 26 genotipten ikisi hariç tüm genotipler daha fazla veya daha az (1-68) embriyo üretmiştir. B4, B10, B11, B12, B15 ve G6 genotipleri %25'ten fazla embriyo üretmiş ve kontrol ve diğer genotiplerden ayrılmıştır. Besni ve Gölbaşı ilçelerinden toplanan genotiplerin ortalama embriyo oluşum oranı sırasıyla %13 ve %7 iken, kontrol çeşitlerinin embriyo oluşum oranı sadece %0,2'dir. Besni biber popülasyonunun androgenetik haploide oldukça duyarlı olduğu sonucuna varılmıştır. Yüksek embriyo oluşturan B4, B10, B11, B12, B15 ve G6 gibi oldukça duyarlı genotipler yeni ıslah hatlarının geliştirilmesinde ve

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anter kültürünün genetiğinin araştırıldığı çalışmalarda kullanılma potansiyeline sahiptir.

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INTRODUCTION

Pepper (*Capsicum annuum* L.), a member of the *Solanaceae* family, is an important vegetable species that originated in South America (Vural et al., 2000). The economically important species of the genus *Capsium*, which includes 43 species (Barboza et al. 2019), are *C. annuum*, *C. frutescens*, *C. pendulum*, *C. baccaum,* and *C. pubescens* (Heiser and Smith, 1953; Samos and Kundt 1984). A diploid and self-fertile species with 2n=24 chromosomes, *C. annuum*, is the most cultivated and economically important species of *Capsicum* in both Türkiye and the world (Gyulai et al., 2000). Approximately 37 million tons of fresh (green and red) and 4.9 million tons of dried pepper are produced in the world and the world's top pepper-producing countries are China (16.81 million tons), Mexico (3.11 million tons), Indonesia (3.02 million tons) and Türkiye (3.01 million tons) (FAO, 2022). As in other plant species, the germplasm of pepper is faced with some threats arising from environmental conditions and cultivation activities. Hence, protecting pepper genetic resources and exploiting them in breeding programs is very important. During the cultivation adventure of pepper in Turkey, pepper genotypes known by the name of the regions have been selected and developed by farmers in different regions such as Demre, Uşak, Karaisalı, Gaziantep, Urfa, and Arapkir pepper. One of them is Besni Pepper, which is known as the Besni district of Adıyaman province (Şahin et al., 2022).

Turkiye is an important plant genetic resource due to its geographical location and different climate and soil conditions (Küçük 2001). Local varieties (landraces) with high variation are very important for breeding studies due to their tolerance to biotic and abiotic stresses and their adaptation to different environmental and cultural conditions (Küçük et al., 2003). Breeding studies carried out to date for certain purposes (high yield etc.) have narrowed the genetic diversity of cultivated vegetable species. Therefore, it is important to include genetic resources that can expand the genetic infrastructure with high adaptability and exotic traits (taste, aroma, phytochemical content, etc.) in breeding programs (Xie et al., 2014).

Long periods are needed to meet the DUS (Distinct, Uniform, Stable) criteria in open-pollinated variety breeding and to produce pure parent lines in hybrid variety breeding with conventional breeding methods. Obtaining homozygous pure lines/varieties by using conventional methods requires a long time: 10-12 years/generation for open-pollinated and 6-7 years/generation for self-pollinated species (Keleş et al., 2015). The introduction of tissue culture and the ability to obtain new plants from different organs of plants with totipotency have provided plant breeders with new opportunities to accelerate new cultivar breeding (Heiser, 1976; Andrews, 1985; Hatipoğlu, 1997; Babaoğlu et al., 2001; Comlekcioglu and Ellialtioglu, 2018; Yılmaz and Güntay, 2023).

The ability to produce haploid plants *in vitro* conditions from cells with haploid chromosome numbers, such as pollen and egg cells, is very important as it shortens the cultivar breeding process. After Guha and Maheshwari (1964) demonstrated that haploid plants could be obtained by culturing anthers containing immature pollen grains of *Datura innoxia*, androgenic plant production techniques were developed very rapidly. Haploid plants can be obtained from anther culture and isolated microspore culture in many plant species. The advantageous method for producing haploid plants is the anther culture technique, which contains thousands of microspores and allows more than one haploid/double haploid plant to be formed from a single anther under suitable conditions (Gönülşen, 1987). The pioneering success of Wang et al. (1973) in China and George and Narayanaswamy (1973) in India in developing haploid embryos from *C. annuum* anthers led to significant advances in this field. Furthermore, the initiation of *in vitro* androgenesis studies on local pepper genotypes by Abak (1983) in Turkiye signifies the global importance and ongoing research of these techniques. Although genotypic selectivity exists, anther culture is the main method of producing haploid/double haploid plants in pepper. However, the complex process of haploid embryo induction and transformation to plant remains a significant challenge and hinders the efficient production of double haploid peppers (Seguí Simarro 2016). The complex interaction of various factors, including the genotype of the donor plant, growth conditions and physiological status of the donor plant, the developmental stage of microspores, anther pretreatments, optimum media combinations, and precise *in vitro* culture conditions play determining roles in achieving successful results (Çiner and Tipirdamaz, 2001; Nowaczyk and Kisiala, 2006). The natural variation of pepper species to androgenesis further emphasizes the importance of identifying and introducing high-frequency responsive genotypes for breeding to ensure the successful production of haploid embryos/plants. Therefore, it is very

important to identify genotypes with the ability to produce highly androgenic haploid plants in the germplasm and to transfer this trait to new lines (Ercan and Şensoy, 2011; Irikova et al., 2011a, b).

Several previous studies have reported that different genotypes responded differently to androgenesis in pepper. Keleş et al. (2015) used seven Charleston, six bell, eight capia, and seven green pepper genotypes and found significant differences in response to anther culture both among different pepper types and within pepper types. The highest embryo formation (20% to 5.7%) was obtained from the anthers of bell peppers, while the androgenic response of green peppers was the lowest (6.7–3.7%). Embryo formation was found to be up to 14% and 10% in capia and Charleston peppers, respectively. Ozsan and Onus (2017) reported that among four varieties, the most positive response was obtained from the capia pepper variety "Belissa" in different growing media. Ata et al. (2019) investigated the effects of different climatic conditions, different cultivation media, and genotypes on haploid embryo induction in pepper. The highest embryo formation was recorded in the İnan3363 variety with 22.14%, while the lowest was recorded in the 421 genotype with 1.40%. Shimira et al. (2019) investigated the responses of the Rwandan-origin Pili-Pili pepper variety (*C. chinense*) and Turkish pepper varieties A111, Kahramanmaraş, İnan3363 and Urfa (*C. annuum*) to anther culture in the Mediterranean Region conditions. It was reported that İnan3363 and A111 pepper varieties produced 19.4% and 4.46% haploid embryos, respectively, while the Rwandan-origin Pili-Pili variety did not produce embryos. In addition, it was determined that the appropriate anther development stage of the Pili-Pili variety, whose flower buds were examined, was not the same as the Turkish varieties, and the phase transition times of the flower buds were very short. In a study using 34 long green, 13 bell pepper, 13 Charleston, 6 California wonder, and 23 capia advanced breeding lines as plant material, it was emphasized that the response to androgenesis was dependent on the genotype and culture in medium containing activated charcoal for up to 35 days was recommended. It was also noted that the use of a medium without activated charcoal after 35 days was important to break the resistance and increase induction efficiency (Pınar et al., 2020).

Characterization of plant genetic resources and their use in plant breeding and production is very important. In species propagated from seeds such as pepper, whether open-pollinated or hybrid cultivar breeding, it is important to produce homozygous individuals. The production of pure lines that require long periods by the classical method (inbreeding) can be reduced to 1.5-2 generations by the haploidization method. For this reason, determining the response of genetic resources to haploidy applications is important. In this study, Besni pepper, one of the landraces that are a part of the genetic richness of Turkey, was used as plant material. For this purpose, a total of 26 genotypes were collected from different villages and different farmers of Besni and Gölbaşı districts. The response of these genotypes, which were morphologically characterized (Şahin et al., 2022), and three control varieties, namely Yalova Çorbacı, Sera Demre, and Cırgalan, to anther culture was investigated.

MATERIAL and METHOD

Experimental site and plant material

This study was carried out in the research greenhouses and laboratories of Erciyes University Faculty of Agriculture located at latitude 38° 42' 33" N and longitude 35° 32' 33". In the study, a total of 26 Besni pepper genotypes were collected from the villages of Besni district (20) and Gölbaşı district (6), and a total of 29 pepper genotypes, including Cırgalan, Yalova Çorbacı, and Sera Demre as control varieties, were used (Table 1). Pepper genotypes were collected by visiting villages and interviewing farmers. In terms of fruit shape, 29 genotypes include three groups: conical, bell, and elongated (Şahin et al. 2022).

Cultivation of donor plants

Seeds of the genotypes were sown in a 3:1 ratio peat-perlite mixture under unheated greenhouse conditions on 16.04.2021. The seedlings were fertilized twice with 15:15:15 (N:P: K) + microelement fertilizer until they reached planting size (3-4 true leaves). The electrical conductivity of the fertigation water was adjusted to 2 dS/m. When the seedlings reached the 3-4 true leaf stage, 3 plants from each genotype were planted in an unheated greenhouse at 80x30 cm distances on 25.05.2021. Drip irrigation was used as the irrigation system. Irrigation was done based on plant and soil observations. Fertilization was done depending on the plant development period by fertigation (Vural et al., 2000; Ifas, 2021). Black plastic mulch was used for weed control. Certified pesticides and fungicides for pepper were applied according to the disease and pest occurrence. Weeds between the rows were manually controlled.

Table 1. Pepper genotypes and sources used in the study
Çizelge 1. Çalışmada kullanılan biber genotipleri ve kaynakları

Genotypes	Sources	Fruit shape	Genotypes	Sources	Fruit shape
B1	Oyratlı Village/Besni	Cn	B16	Oyratlı Village/Besni	Cn
B2	Oyratlı Village/Besni	Cn	B17	Oyratlı Village/Besni	Cn
B3	Oyratlı Village/Besni	Cn	B18	Oyratlı Village/Besni	Cn
B4	Oyratlı Village/Besni	Cn	B19	Oyratlı Village/Besni	Cn
B5	Oyratlı Village/Besni	B	B20	Toklu Village/Besni	E
B6	Oyratlı Village/Besni	Cn	G1	Gölbaşı/City Center	E
B7	Oyratlı Village/Besni	E	G2	Gölbaşı/City Center	Cn
B8	Oyratlı Village/Besni	Cn	G3	Gölbaşı/City Center	Cn
B9	Oyratlı Village/Besni	Cn	G4	Maltepe Village/Gölbaşı	Cn
B10	Besni/City Center	Cn	G5	Maltepe Village/Gölbaşı	E
B11	Oyratlı Village/Besni	B	G6	Maltepe Village/Gölbaşı	Cn
B12	Oyratlı Village/Besni	Cn	C1 (Cırgalan)	ERÜ Agricultural Faculty	E
B13	Çamurcu Village/Besni	B	C2 (Yalova Çorbacı)	ERÜ Agricultural Faculty	E
B14	Çamurcu Village/Besni	Cn	C3 (Sera Demre)	ERÜ Agricultural Faculty	E
B15	Oyratlı Village/Besni	Cn			

B: Besni; G: Gölbaşı; C: Control; Cn: Conical; B: Bell; E: Elongate.

Anther culture

Plants were grown under the greenhouse conditions mentioned above, and flower buds on the 30th-40th day of flowering were used. The most suitable anther developmental stage for anther culture is the late-uninucleate or early-binucleate phase (beginning of the first mitotic division) in pepper. The length of the corolla should be equal to or slightly longer than the length of the calyx, and almost half of the anthers contain anthocyanin in this stage (shown with arrowheads in Figure 1) (Dumas de Vaulx et al., 1982; Bal et al., 2003; Büyükalaca et al., 2004; Mangal and Srivasatava, 2019). Flower buds used in anther culture were collected one day in advance at 17:00-18:00 and immediately transported to the laboratory in a cool and humid environment (ice box with ice pack) to preserve their viability. After the buds were washed with tap water to remove external contaminants and rinsed three times with pure water, they were subjected to low-temperature pre-treatment at 4 °C for 24 hours.

Figure 1. Pepper flower buds at the optimum stage for anther isolation (buds at the late-uninucleate or early-binucleate phase, as indicated by arrows in rows 1 and 5) (Büyükalaca et al., 2004).
Şekil 1. Anter izolasyonu için optimum aşamadaki biber çiçekleri (1. ve 5. sıralarda oklarla gösterildiği gibi, geç tek çekirdekli veya erken çift çekirdekli fazdaki tomurcuklar) (Büyükalaca ve ark., 2004).

For surface sterilization, the buds were kept in 80% ethyl alcohol for 60 seconds, then in 10% commercial sodium hypochlorite (4.5% sodium hypochlorite) for 13 minutes, and then washed with sterile distilled water three times and placed on sterile paper towels to remove excessive water in laminar air flow cabinet. After sterilization, the calyx, corolla, and filaments were removed from the buds without damaging the anthers, and the anthers were placed on a nutrient medium in 6 cm diameter glass Petri dishes using sterile forceps and scalpels (Figure 2 a, b). Isolated anthers were cultured in the induction medium whose content is given in Table 2. The culture medium was sterilized by autoclaving at 15 psi pressure at 121 °C for 30 min. The pH of the medium was adjusted to 5.8 using 1N HCl and NaOH solution before autoclaving. To prevent denaturation of the hormones, thermolabile hormones were first filter sterilized using 0.20 µm syringe filters and then added to warm (35–40 °C) autoclaved media before solidification. Anthers were planted in 6 cm diameter glass Petri dishes with their dorsal surfaces in contact with the medium (Table 2) using sterile forceps and scalpel. The planted petri dishes were labeled, wrapped with stretch film, and incubated in the dark at 35 °C for eight days for high-temperature pre-treatment. Then, the cultured anthers were incubated at 25±2 ℃ under 16 h light and 8 h dark conditions from the beginning of the culture in a climate cabinet. A total of 150 anthers were cultured from each genotype. The cultured anters and embryo formation were checked regularly and after 45 days, the anthers were transferred to the second hormone-free medium whose content is given in Table 3 (Figure 2 c. d). After the embryos observed in the cultured anthers germinated and reached a length of 0.3-0.5 cm, they were transferred to test tubes containing hormone-free medium (Table 3) (Figure 3 a, b). The plants that reached a certain size (2-3 true leaves) in the tubes were transferred from growth tubes to pots of 5 cm filled with sterile peat: perlite (2:1 v:v) mixture (Figure 3 c). To maintain the humidity around the plants, each pot was covered with polyethylene stretch film and 2-3 holes were opened in the stretch film for sufficient ventilation. These pots were placed in 50-liter transparent containers with lids and acclimatized at 25±2 °C and 16 hours/8 hours (light/dark) photoperiod for 5-7 days. As the plants developed, the lid and stretch film were gradually opened, and the plants were acclimated to external conditions (Figure 3 c; Figure 4). Embryo formation and transformation rates to plants were calculated according to the formulas below.

Embryo formation rate = (Number of embryos/Number of anthers cultured) x 100

Transformation rate to plant = (Number of plants/ Number of embryos) x 100

Tablo 2. Nutrient components used in anther induction medium
Çizelge 2. Anter uyartım ortamının bileşenleri

Chemicals	Concentration
MS	4.3 g
Sucrose	30 g L^{-1}
Activated charcoal	2.5 g L^{-1}
$AgNO_3$ (5 mg L^{-1})	10 mg L^{-1}
Agar	7 g L^{-1}
NAA (1 mg L^{-1})	4 mg L^{-1}
BAP	500 µl L^{-1}

Table 3. Components of hormone-free nutrient media used in anther culture
Çizelge 3. Anter kültüründe kullanılan hormonsuz besin ortamının bileşenleri

Chemicals	Concentration
MS	4.3 g L^{-1}
Sucrose	30 g L^{-1}
Silver nitrate (5 mg/L)	10 mg L^{-1}
Agar	7 g L^{-1}
pH	5.8

Correlation matrix heatmap

A heat map was created using GraphPad Software, version 10.3.1 (GraphPad Software Inc. La Jolla, CA) to visualize the correlation between morphological characters and anther culture results based on Pearson correlation. The correlation matrix heatmap displays values of the Pearson correlation coefficient, which is a measure of the strength of the linear relationship (positive/negative) between two variables. The correlation matrix heatmap shows the values of the Pearson correlation coefficient (Schober et al., 2018). A correlation matrix heat map was created between the morphological traits reported in Şahin et al. (2022) and the response parameters of genotypes to anther culture. The features with $r>0.5$ as a result of correlation analysis were evaluated in detail with one-way ANOVA and Cohen's (1988) eta squared and confidence intervals in the SPSS 22.0 statistical program.

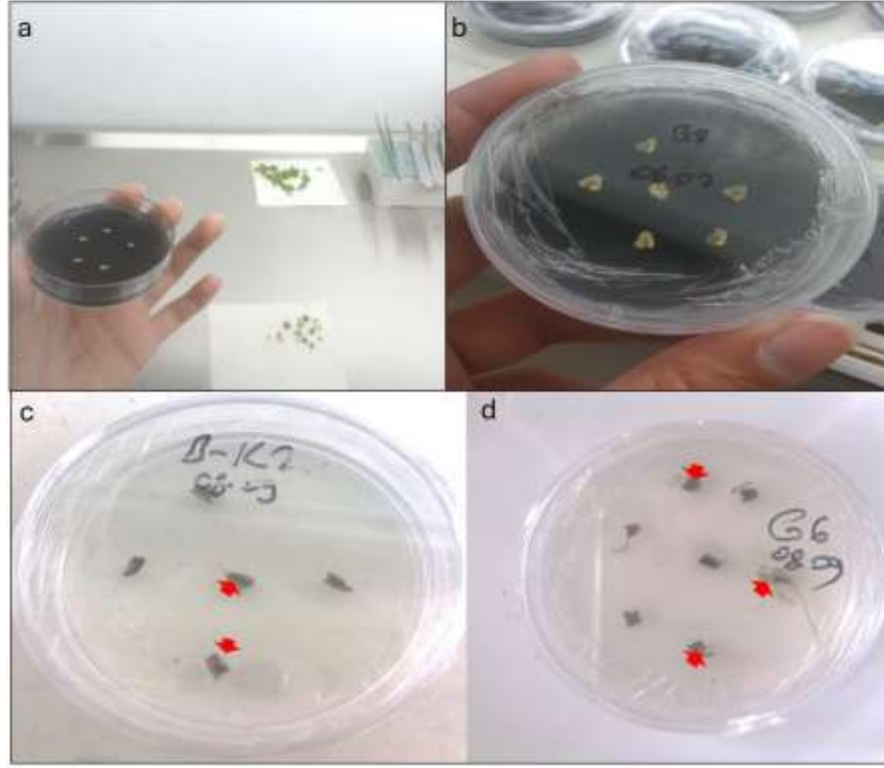

Figure 2. Stages of culturing anthers. a and b: Planting of anthers in the medium, callus (c), embryo, and root formation (d) from cultured anthers (shown with arrowheads).

Şekil 2. Anterlerin kültüre alınma aşamaları. a ve b: Anterlerin ortama ekilmesi, kültüre alınan anterlerden kallus (c), embriyo ve kök oluşumu (d) (ok uçlarıyla gösterilmiştir).

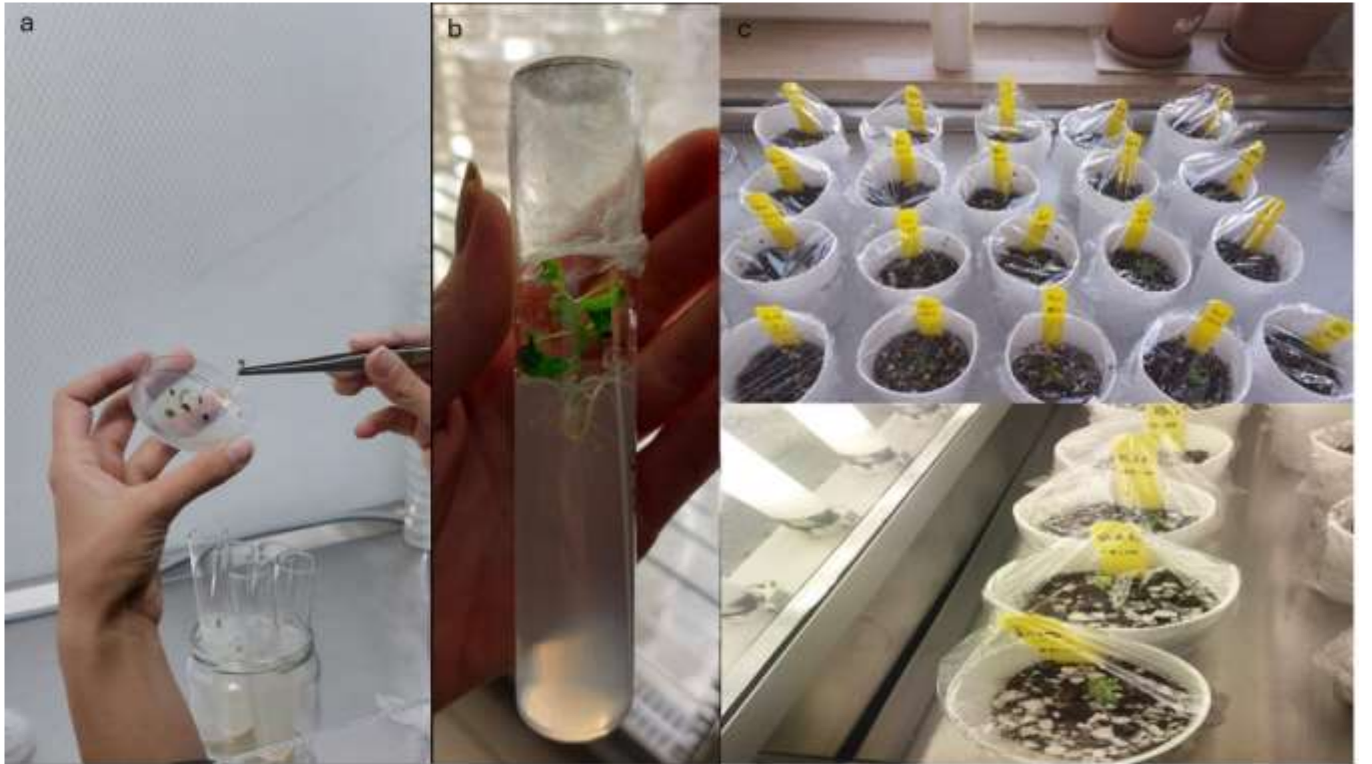

Figure 3. Transfer of developing plantlets to tubes (a and b) and plantlets transferred to sterile peat: perlite mixture and acclimatized to the outside environment (c).

Şekil 3. Gelişen bitkilerin tüplere transferi (a ve b) ve steril torf:perlit karışımına transfer edilen ve dış ortama alıştırılan bitkilerin (c).

RESULTS and DISCUSSION

The responses of pepper genotypes used in the study to anther culture are presented in Table 4. One hundred and fifty anthers were cultured from each of the 29 pepper genotypes used. It was observed that there was a significant variation (0.0%-45.3%) in terms of embryo formation rate (Figure 2) among the genotypes used in the study. While the highest embryo formation rate was determined as 45.3% in genotype B11, no embryo formation was observed in genotypes B7, G5, C1, and C3. The B11 genotype had the highest embryo formation rate, followed by G6 with 31.3% and B15 with 29.3%. While the average embryo formation rate of the genotypes collected from Besni district was 13.1%, the embryo formation rate of the genotypes taken from Gölbaşı district was calculated as 7.08%. The number of haploid plants produced varied from 30 to 0 and haploid plants could not be obtained in 12 genotypes. The highest number of haploid plants was recorded in the B12 genotype with 30, while the lowest number of plants was determined in the B5, B8, and G2 genotypes with 1 plant. While the average haploid plant numbers formed by the genotypes collected from Besni and Gölbaşı were 7.8 and 1.3, respectively, haploid plant formation was not observed in the control plants. As in embryo formation and haploid plant number, significant differences were observed in the transformation rate to plant depending on the genotype. The transformation rate to plant varied between 88.9% and 0. While the highest transformation rate to plant was determined as 88.8% (30 plants) in genotype B1, no transformation was observed in 12 genotypes (B3, B7, B9, B16, B17, B20, G1, G4, G5, C1, C2, and C3). The B1 genotype was followed by B6 with 71.4% and B15 with 68%. The transformation rate of plant genotypes collected from Besni was found to be significantly higher than the genotypes collected from Gölbaşı and the control varieties. All genotypes produced a total of 491 embryos, of which 162 (33%) developed into plants. At the same time, embryo formation and plant formation were not observed in genotypes B7, G5, C1, and C3. Embryo formation was observed in genotypes B3, B9, B16, B17, B20, G1, G6, and C2, but transformation to plant was not observed. In the present study, genotypes that responded to anther culture at high levels (45%) (B11) were identified among Besni pepper genotypes. Significant differences were detected between the varieties collected from the two districts and the control varieties.

The success of anther culture is influenced by many factors, including plant and environmental (*in vivo* and *in vitro*). The plant factors include the genotype of the donor plant (Kim et al., 2004; Supena et al., 2006; Ari et al., 2016), the developmental stage of flower buds (microspore stage) (Parra-Vega et al., 2013; Mangal and Srivasatava, 2019), and the physiological state of the donor plant (Ercan et al., 2006; Ata et al., 2019), while the plant growth conditions (Ata et al., 2019), pretreatments (Dumas de Vaulx et al., 1981), nutrient medium (Supena et al., 2006; Irikova et al., 2011a, b; Bat et al., 2020), plant nutrient and carbon starvation, *in vitro* culture conditions (temperature, lighting and photoperiod), application of different additives and plant growth regulators (Niazian and Shariatpanahi, 2020) and growing season (Ercan et al., 2006; Rodeva and Cholakov, 2006) are the main environmental factors (Taskin et al., 2011).

The most important factor affecting the response to androgenesis is the genotype of the donor plants, and it varies considerably not only from species to species (inter-specific) but also within species (intra-specific). (Phippen and Ockendon, 1990; Taskin et al., 2011; Asif, 2013; Irikova et al., 2016). A variation between genotypes has been demonstrated for many field crops and horticultural crops, including the species representing *Capsicum*, *Solanum,* and *Brassica* genus (Rodeva et al., 2004, Nowaczyk et al., 2006, Lantos et al., 2012). Studies have shown that genetic differences in microspore embryogenesis can occur not only among various species, cultivars, and hybrid forms but also among individual plants of a single cultivar, depending on the plant's current physiological state (Kristiansen and Anders, 1993, Irikova and Rodeva, 2004, Nowaczyk et al., 2009). Genç (2023) reported that embryo formation induced by anther culture showed significant differences in different pepper types (fruit shape) and even in different genotypes. Atasoy et al. (2021) studied the anther culture response of a population consisting of 23 pepper genotypes of green pepper, capia, Charleston, and bell pepper. Significant differences were found among the genotypes and the embryo formation rate of the genotypes in anther culture varied between 22% and 74%. A significant portion of the genotypes used by Atasoy et al. (2021) had higher embryo formation rates (60-74%) than the genotypes we used. In a study investigating the effect of genotype on anther culture, it was reported that 11 different pepper species anther were cultured and embryos were obtained from all varieties except Kandil and Yalova Charleston (Ercan and Şensoy, 2011).

The pepper genotypes used in Ercan and Şensoy (2011) and this study were cultured under the same conditions using the same nutrient medium. However, Besni pepper genotypes formed more embryos and produced more plants. This once again demonstrated that the genotype effect is important in anther culture, as stated by Ercan and Şensoy (2011). Among the three genotypes tested by Niklas-Nowak et al. (2012), the embryo formation rate in F_2 population plants of *C. annuum* showed a significant difference (0-16%) among the genotypes. In agreement with the present study, the response to anther culture differed among the genotypes, but the genotypes used in this study produced significantly higher embryos (about three times more) compared to the cited study.

Following previous studies (Bajaj,1980; Başay and Ellialtıoğlu, 2013; Ellialtıoğlu et al., 2015; İlhan and Kurtar, 2022), this study confirmed once again that genotype is one of the most important factors affecting the success of anther culture in pepper. Denli et al. (2022) reported at least two genes control the androgenic response in pepper and that it may be heritable. Conical and bell pepper genotypes had higher embryo formation rates.

Table 4. Responses of different Besni pepper genotypes to anther culture
Çizelge 4. Farklı Besni biber genotiplerinin anter kültürüne tepkileri

Genotypes	Number of anthers cultured	Number of embryos formed	Embryo formation rate (%)	Number of plants	Transformation rate into plant (%)
B1	150	9	6.0	8	88.9
B2	150	23	15.3	10	43.5
B3	150	15	10.0	0	0.0
B4	150	42	28.0	11	26.2
B5	150	6	4.0	1	16.7
B6	150	14	9.3	10	71.4
B7	150	0	0.0	0	0.0
B8	150	7	4.6	1	14.3
B9	150	4	0.26	0	0.0
B10	150	42	28.0	21	50.0
B11	150	68	45.3	20	29.4
B12	150	45	30.0	23	51.1
B13	150	21	14.0	3	14.3
B14	150	19	12.6	9	47.4
B15	150	44	29.3	30	68.2
B16	150	6	4.0	0	0.0
B17	150	8	5.3	0	0.0
B18	150	29	19.3	2	6.9
B19	150	10	6.6	6	60.0
B20	150	6	4.0	0	0
Besni Genotypes $\bar{X}$		20.9	13.1	7.8	37.3
G1	150	1	0.6	0	0
G2	150	5	3.3	1	20.0
G3	150	8	5.3	5	62.5
G4	150	11	7.3	2	18.2
G5	150	0	0.0	0	0
G6	150	47	31.3	0	0
Gölbaşı Genotypes $\bar{X}$		12	7.08	1.3	10.8
C1	150	0	0.0	0	0
C2	150	1	0.6	0	0
C3	150	0	0.0	0	0
Control Cultivars $\bar{X}$		0.3	0.3	0	0
Total	4350	491	11.3	163	33.2

Similarly, Grozeva et al. (2021) found a higher embryo formation rate in conical, bell, and pumpkin-shaped pepper genotypes than in long peppers, while Keleş et al. (2015) and Pınar et al. (2020) found a higher gynogenesis response in capia and bell peppers. On the other hand, Ercan and Falk (2011) reported a higher androgenic response in the long pepper variety Demre-8 compared to the other pepper varieties they used. This shows that the productivity of anther culture in pepper is affected by a series of factors such as genotype, culture conditions, pretreatments, and environmental conditions in which the plant is grown (Asif, 2013).

In this study, medium components that were found promising in previous studies were used. However, as reported in previous studies (Ercan and Şensoy, 2011; Ata et al., 2019; Genç, 2023), significant differences depending on the medium were reported in anther culture studies. Alremi et al. (2014) reported that different pepper genotypes (B, 151, 171, and Alfajer) responded differently to eight different nutrient medium combinations. It has been determined that B5 medium without silver nitrate gives a better response in embryo formation. In the current study, significant variations were observed in the rate of embryo formation and transformation to plant among pepper genotypes collected from a relatively narrow area (two districts of Adıyaman province). The results of this study also showed that anther culture was affected by the interaction of

many factors, especially genotype and culture medium. İlhan and Kurtar (2022) reported that the nutrient medium and genotype significantly affected embryo induction, formation, and plant transformation rate and that the B5 medium produced more successful results compared to the MS medium. Similarly, Özsoy (2019) reported that 209 embryos and 134 plants were obtained from the MS medium and 218 embryos and 100 plants were obtained from the B5 medium. In this study, MS was used as a basal medium, and approximately twice as many embryos were obtained compared to the results of Özsoy (2019). Obtaining different results in the same nutrient medium and culture conditions once again reveals the importance of the genetic structure and physiological status of the donor plant. The response of Elazığ pepper (Kofik), a local pepper variety, to anther culture was investigated using the same culture media and conditions used in this study and it was reported that 106 embryos were produced from a total of 1206 Petri dishes and 45 of these embryos developed into plants. It was also reported that embryo formation was not observed in 11 genotypes collected from Elazığ (Duruk 2023). Approximately four times more embryos were obtained from the Besni pepper genotypes used in this study than from the Elazığ pepper genotypes. The conclusion from the studies and previous reports is that the effect of genotype and nutrient medium has a decisive role in the success of anther culture in pepper. The data of the study revealed that a single standard anther culture protocol does not produce the same productivity in different pepper genotypes and that appropriate protocols should be determined according to type/genotype. In addition, Pınar et al. (2020) reported that the response rate of genotypes in the culture medium may be different and emphasized that genotypes should be optimized according to time in order to obtain satisfactory results.

In this study, it was concluded that the differences detected between pepper genotypes may be the effect of a physiological variability characteristic for *in vitro* plant cultures. In addition, Şahin et al. (2022) reported that the pepper genotypes used in this study had significant morphological diversity, including flower bud size. In this study, the appropriate microspore stage study was not conducted on pepper genotypes, and since the flower size and morphology recommended in previous studies were used, the correct microspore stage for each genotype may not have been cultured. It was concluded that this also contributed to the difference between genotypes. By determining the correct flower bud stage for each genotype that did not respond to anther culture or gave a very low response (N7, G1, G5, C1, C2, and C3), and by using different media and additives, a satisfactory androgenic response can be obtained from these genotypes. Recently, many additives, including phytohormones (Khan et al., 2020; Hale et al., 2022), growth retardant hormones, stress hormones, compatible solutes, polyamines, histone deacetylase inhibitors, cellular antioxidants (enzymatic and non-enzymatic), and arabinogalactan proteins, which are endogenously produced organic compounds required to regulate plant growth and development, have been used to increase the efficiency of *in virto* haploid induction by enhancing tolerance to embryo-stimulating stresses (Niazian and Shariatpanahi, 2020; Hale et al., 2022). By using the additives listed above, it may be possible to produce androgenic haploid plants in genotypes with low or no response to anther culture in this study.

DNA methylation, histone methylation, and acetylation are important processes that control the functional state of chromatin and subsequently regulate gene expression during cell division, proliferation, and differentiation (Cedar and Begrman, 2009). One of the cellular processes that occurs during stress-induced embryogenesis is epigenetic reprogramming; essentially a general reduction in DNA methylation (Testillano, 2019). Low H3K9 methylation levels are positively correlated with microspore reprogramming from gametophytic to sporophytic development and the initiation of embryogenesis (Testillano et al., 2010; Testillano, 2019). In *Brassica napus*, high levels of acetylated histones H3Ac and H4Ac were reported in vacuolated microspores, a sign of reprogramming (Rodríguez-Sanz et al. 2014). Therefore, the addition of DNA demethylating agents and histone deacetylase inhibitors to the medium may increase the efficiency of androgenic haploid induction. Application of a DNA demethylating agent, 5-Azacytidine (AzaC) increased embryogenesis induction in isolated microspore culture of oilseed rape and *Hordeum vulgare* (í). In the same species, the application of BIX-01294, a small molecule that prevents H3K9 methylation, improved microspore reprogramming and embryogenesis (Berenguer et al., 2017).

Antioxidants, which can be enzymatic and non-enzymatic, are one of the most important components that provide ROS balance by scavenging cellular ROS accumulation (Chen et al., 2020; Hale et al., 2022). The positive effect of low-molecular-weight antioxidants glutathione and ascorbic asit on microspore embryogenesis and an increase in the number of embryo-like structures has been reported in isolated microspore cultures of triticale (Zur et al. 2019). Other materials with antioxidant characteristics, such as L-ascorbic acid, can increase the antioxidant enzyme activities and antioxidative response of treated cells (Chen et al., 2020). The ascorbic acid application under carbohydrate starvation and heat shock treatment (32 °C) caused a significant increase in the number of cotyledon embryos produced in isolated microspore culture (Heidari-Zefreh et al., 2018) and anther culture (Doğangüzel et al., 2021) in pepper. Confirming these two studies, Zeng et al. (2017) stated that the embryogenesis efficiency in isolated microspore culture of broccoli was increased by 1.2-fold and 2.5-fold with the

addition of reduced ascorbate and glutathione, respectively. In the microspore culture of flowering Chinese cabbage, a 10.33-fold increase in the frequency of embryogenesis was reported when L-ascorbic acid sodium salt was added to the NLN-13 medium (Niu et al., 2019). Methylene blue is another type of antioxidant that was reported to have a positive effect on the androgenic response of ornamental kale (Chen et al., 2019). Cell wall modification agents such as AGPs (Arabinogalactan protein) have been reported to be effective during both somatic embryogenesis (Pérez-Pérez et al. 2019) and microspore embryogenesis (Testillano, 2019; Camacho-Fernández et al., 2021). The addition of gum arabic as an AGPs carrier to the medium caused 2.8 times higher androgenesis in barley (Makowska et al., 2017), while in tomato it was found to be more effective than cold treatment and kinetin application in anther culture (Niazian et al., 2019). Different agents mentioned in this literature and whose positive effects on embryogenesis in different species have been reported can be used to obtain responses from pepper genotypes with low androgenic responses.

In the present study, the transformation rate of plantlets to mature plants was found to be 33%. It was concluded that the low transformation rate was due to inadequate laboratory/greenhouse conditions for the acclimation stage of the plantlets, acclimation being carried out on a single plant, and not all plants having the same physiological maturity. Although this rate is consistent with the results of many studies (Keleş et al., 2015; Pınar et al., 2020; Grozeva et al., 2021; Duruk, 2023; Shana et al., 2024) conducted on pepper, the loss of potential haploid plantlets produced with great effort was considered a bottleneck that needed to be overcome. This study has shown that it is risky to acclimate plants produced *in vitro* to the outside environment from a single plant without multiplying them. Instead of proceeding from a single plant, if multiple plantlets were produced to be transferred to the outdoor environment with several subcultures, the risk of a high loss rate could be overcome. Since not all plantlets formed after embryogenesis in anther culture are at the same physiological maturity (weak/strong), some weak plantlets may be lost during the acclimation phase. Plantlets with strong root and shoot development have a higher rate of forming mature plants that can be transferred successfully to the outdoor environment. In *in vitro* conditions, plants can be produced from very different tissues and organs due to the totipotency characteristics of plants, and incomplete and problematic embryos (aged, endospermless, and haploid) can be converted into mature plants (Chandra et al., 2010; Saskin et al., 2022). However, one of the disadvantages of *in vitro* plant propagation is that the survival rate of *in vitro* plants, which are produced with intensive labor and cost, is low when transferred to external conditions. One of the most important factors limiting the success of the *in vitro* plant production method is the process of acclimating the obtained plants to outdoor conditions. The success of transferring *in vitro* grown plants to external conditions is generally determined by the physiological state of the acclimatized plant (weak/strong) and the acclimatization conditions. Depending on the factors above, high losses occur during the acclimatization phase due to different factors such as light intensity, temperature, and water stress (Kumar and Rao, 2012). The main reasons for this are the inability of the plants to uptake sufficient water due to weak root development after transplanting, excessive water loss due to insufficient cuticle formation, transplanting shock, various pathogenic attacks, poor photosynthesis, and similar factors occurring in the post-transplanting period (Krishna et al., 2005; Kara et al., 2022). For any micropropagation protocol, successful rooting of plantlets is a prerequisite to facilitate acclimation to soil conditions. Only plantlets longer than 1.5 cm and with dense roots can be considered usable for acclimation (Khalafalla et al., 2011; Copetta et al., 2023). The low plant conversion rate observed in this study could be eliminated to a certain extent by increasing the number of plants to be acclimated to the external environment and by pre-transplanting applications increasing root quantity and quality. In many studies, basic media supplemented with only IBA and NAA or combinations of these hormones in the range of 0.2-2.0 ppm have been used successfully. While the addition of IBA to the medium increases primary/secondary root formation, NAA increases root hair formation. It has been reported that decreasing the concentration of inorganic salts is also beneficial in increasing root volume. Hardening of plants by exposing them to high light intensity, nutrient starvation, and low relative humidity before transplantation may contribute to reducing post-transplant mortality (Chacal and Gosal, 2002; Velasco and Watson, 2020).

Correlation analysis was performed between the morphological data produced in our previous study (Şahin et al. 2022) and the responses of genotypes to anther culture. A significant positive relationship was found between fruit shape ($r=0.533$; $n=29$), fruit cross-section shape ($r= 0.519$; $n=29$), and response to anther culture at 1% significance level (Figure 5).

The effects of fruit shape, fruit cross-sectional shape, and fruit neck formation on embryo number, embryo formation rate, plant number, and plant conversion rate parameters were evaluated in detail with eta squared and confidence intervals, where $r>0.5$ as a result of correlation analysis (Table 5 and 6). The eta squared value of fruit shape on embryo number is $\eta^2=0.285$, embryo formation rate is $\eta^2=0.279$, and plant formation rate is $\eta^2=0.268$. Fruit shape has a high correlation with embryo number, embryo, and plant formation rate ($\eta^2>0.138$). Eta squared value of fruit cross-section shape on embryo number is $\eta^2=417$, embryo formation rate is $\eta^2=0.417$,

and plant formation is η^2=0.240. As in fruit shape, fruit cross-section shape was found highly correlative with embryo number, embryo formation, and plant formation rate (η^2>0.138). Since the eta value of neck formation on fruit on all parameters (number of embryo, embryo, and plant formation rate) is greater than 0.138, the effect on the parameters is significant (Table 5).

Figure 4. Plants acclimatized to the outdoor environment and reached transplanting size (shown with green arrowheads), plantlets not grown (shown with red arrowheads).

Şekil 4. Dış ortama alıştırılmış ve dikim büyüklüğüne ulaşmış bitkiler (yeşil ok uçlarıyla gösterilmiştir), büyümemiş bitkicik (kırmızı ok ucuyla gösterilmiştir).

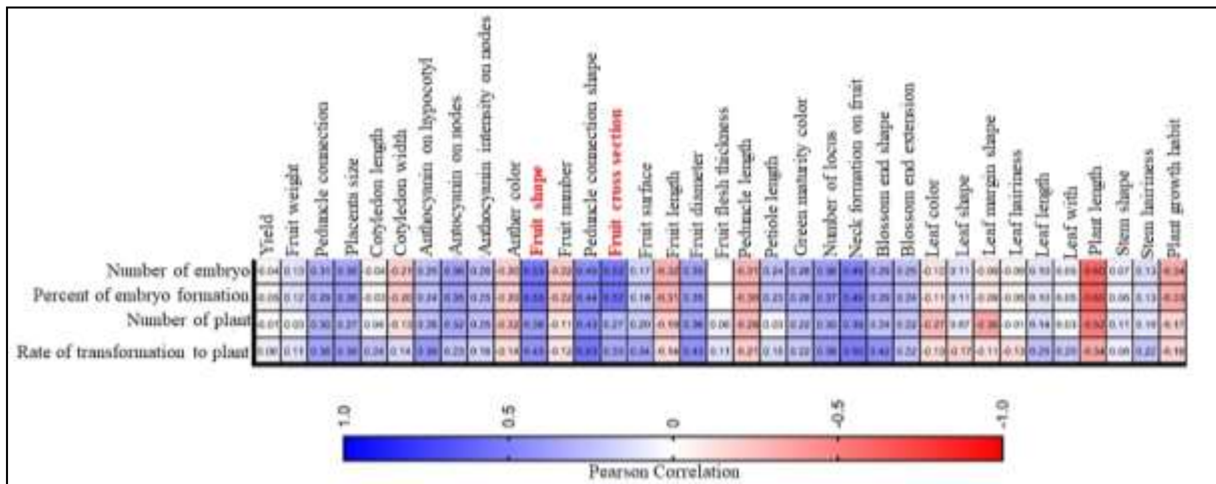

Figure 5. The correlation matrix heat map shows the values of the Pearson correlation coefficient between embryo number, embryo formation rate, plant number, and plant formation rate with plant morphological characteristics (Şahin et al., 2022), the positive values in blue and the negative in red. It ranges from -1 to 1, whereby - 1 means a high negative linear relationship between variables, 1 indicates a high positive linear relationship between variables and 0 indicates that there is no relationship between studied variables.

Şekil 5. Korelasyon matrisi ısı haritası, embriyo sayısı, embriyo oluşum oranı, bitki sayısı ve bitki oluşum oranı ile bitki morfolojik özellikleri (Şahin et al., 2022) arasındaki Pearson korelasyon katsayısının değerlerini, pozitif değerleri mavi ve negatif değerleri kırmızı olarak gösterir. -1 ile 1 arasında değişir, burada -1 değişkenler arasında yüksek negatif doğrusal ilişki anlamına gelir, 1 değişkenler arasında yüksek pozitif doğrusal ilişki anlamına gelir ve 0 incelenen değişkenler arasında ilişki olmadığını gösterir.

Table 5. Eta square values for the effect of fruit shape, fruit cross-sectional shape, and fruit neck formation on embryo number, embryo formation percentage, plant number, and transformation rate (η^2:eta square)
Çizelge 5. Meyve şekli, meyve enine kesit şekli ve meyve boyun oluşumunun embriyo sayısı, embriyo oluşum yüzdesi, bitki sayısı ve bitkiye dönüşüm oranı üzerindeki etkisi için eta kare değerleri (η^2:eta kare)

Parameters	Fruit shape		Fruit cross-section shape		Neck formation on fruit	
	Sig.(p)	η^2	Sig.(p)	η^2	Sig.(p)	η^2
Number of embryo	0.013	0.285	0.001	0.417	0.008	0.236
Embryo formation rate	0.014	0.279	0.001	0.417	0.006	0.245
Number of plants	0.114	-	0.372	-	0.0357	0.153
Plant formation rate	0.017	0.268	0.028	0.240	0.006	0.247

$\eta^2<0.01$ small, $\eta^2<0.059$ medium, $\eta^2>0.138$ high, $p>0.05$, the eta squared of the parameters are insignificant.

When the confidence interval values of fruit shape on embryo number, embryo formation percentage, plant number, and transformation rate were examined, the confidence interval values of the genotypes with fruit shape 3 scale value did not contain zero value, so the reliability level was higher than the genotypes with the other two fruit shapes (1-4). When the confidence interval values of the genotypes with fruit cross-sectional shape 5 scale value did not include zero value, the level of reliability was higher than the genotypes with the other two fruit cross-sectional shapes (3-7). When the confidence interval values on fruit neck formation; embryo number, embryo formation percentage, plant number, and plant transformation rate were examined, the confidence interval values of the genotypes with fruit shape 1 scale value did not include zero value, so the level of reliability was higher than the genotypes with the other scale value (0) (Table 6).

CONCLUSIONS and RECOMMENDATIONS

In this study, 26 pepper genotypes of Besni pepper, a local pepper landrace, collected from Besni and Gölbaşı districts, and Yalova Çorbacı, Sera Demre, and Cırgalan pepper varieties as controls were used to investigate their responses to androgenic embryogenesis. As a result, Besni pepper responded positively (24 genotypes from 26 genotypes) to anther culture. While the embryo formation rate varied between 45.3 and 0.6, 11 of the pepper genotypes had embryo formation rates of 10% and above. This rate is higher than the rates reported in most previous studies. B11 (45.3%), B12 (30%), B10 (28%), B4 (28%) and G6 (31.3%) had significantly higher (>25%) embryo formation rates. The transformation rate of the embryos into plants varied between 0% and 89%. The highest rate was obtained in the B1 genotype, while no transformation into plants occurred in the 6 genotypes that formed embryos. It was concluded that it would be possible to produce pure lines from the Besni pepper population in a short time. As can be seen, there is a significant variation both in the rate of embryo formation and the rate of embryos turning into mature plants. The physiological and genetic basis of this difference can be investigated using genotypes with high androgenic response and genotypes with very low or no response. In addition, the effects of different flower bud sizes (microspore stage) and different culture media supplemented with promising additives (arabinagalactone, anti-oxidants, osmotic protectors, etc.) indicated in the discussion section on the success of anther culture in very low-reactive and non-reactive genotypes can be investigated. For this reason, the inheritance of this high androgenic plant formation potential detected in the Besni pepper population and the possibility of transferring it to lines with low androgenic response but high agronomic potential are among the important research topics. Of the 490 embryos formed, 133 developed into plants. The plant formation rate of the embryos was calculated as 33%. In other words, 67% could not be turned into plants. Therefore, studies should be conducted to develop protocols for transforming plantlets into mature plants (concentration and composition of nutrients and hormones used in vitro), hardening plantlets, and improving strong root and shoot development before acclimation to external conditions.

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Table 6. Effect of fruit shape, fruit cross-sectional shape, and fruit neck formation on embryo number, embryo and, plant transformation rate

Çizelge 6. Meyve şekli, meyve enine kesit şekli ve meyvede boynu oluşumunun embriyo sayısı, embriyo oluşum oranı, bitki sayısı ve bitkiye dönüşüm oranı üzerine etkisi

Parameters		Scale	n	Mean	95% Confidence interval for mean		Minimum	Maximum
					Lower bound	Upper mound		
Fruit shape	Number of embryo	1	7	1.14±0.83	-0.89	3.17	0.00	6.00
		3	19	20.42±3.62	12.81	28.03	4.00	47.00
		4	3	31.67±18.69	-48.69	112.02	6.00	68.00
		Total	29	16.93±3.37	10.02	23.84	0.00	68.00
	Embryo formation rate	1	7	0.74±0.55	-0.61	2.10	0.00	4.00
		3	19	13.46±2.45	8.31	18.61	0.26	31.30
		4	3	21.10±12.44	-32.42	74.62	4.00	45.30
		Total	29	11.18±2.26	6.55	15.81	0.00	45.30
	Number of plants	1	7	0.00±0.00	0.00	0.00	0.00	0.00
		3	19	7.32±2.02	3.08	11.55	0.00	30.00
		4	3	8.00±6.03	-17.94	33.94	1.00	20.00
		Total	29	5.62±1.53	2.49	8.75	0.00	30.00
	Plant formation arte	1	7	0.00±0.00	0.00	0.00	0.00	0.00
		3	19	33.08±6.68	19.04	47.12	0.00	88.89
		4	3	20.12±4.70	-0.08	40.33	14.29	29.41
		Total	29	23.75±5.09	13.32	34.19	0.00	88.89
Fruit Cross-Section Shape	Number of embryo	3	11	9.82±4.78	-1.03	20.66	0.00	42.00
		5	16	16.75±3.26	9.79	23.71	1.00	45.00
		7	2	57.50±10.50	-75.92	190.92	47.00	68.00
		Total	29	16.93±3.37	10.02	23.84	0.00	68.00
	Embryo formation rate	3	11	6.31±3.28	-0.99	13.62	0.00	28.00
		5	16	11.14±2.18	6.50	15.78	0.60	30.00
		7	2	38.30±7.00	-50.64	127.24	31.30	45.30
		Total	29	11.18±2.26	6.55	15.81	0.00	45.30
	Number of plants	3	11	3.00±2.05	-1.58	7.58	0.00	21.00
		5	16	6.88±2.14	2.32	11.43	0.00	30.00
		7	2	10.00±10.00	-117.06	137.06	0.00	20.00
		Total	29	5.62±1.53	2.49	8.75	0.00	30.00
	Plant formation rate	3	11	8.23±4.90	-2.70	19.15	0.00	50.00
		5	16	35.56±7.34	19.92	51.21	0.00	88.89
		7	2	14.71±14.71	-172.15	201.56	0.00	29.41
		Total	29	23.75±5.09	13.32	34.19	0.00	88.89
Neck formation on fruit	Number of embryo	0	7	1.57±0.92	-0.68	3.83	0.00	6.00
		1	22	21.82±3.90	13.71	29.92	1.00	68.00
		Total	29	16.93±3.37	10.02	23.84	0.00	68.00
	Embryo formation rate	0	7	0.69±056.	-0.67	2.06	0.00	4.00
		1	22	14.52±2.60	9.11	19.92	0.60	45.30
		Total	29	11.18±2.26	6.55	15.81	0.00	45.30
	Number of plants	0	7	0.00±0.00	0.00	0.00	0.00	0.00
		1	22	7.41±1.87	3.53	11.29	0.00	30.00
		Total	29	5.62±1.53	2.49	8.75	0.00	30.00
	Plant formation rate	0	7	0.00±0.00	0.00	0.00	0.00	0.00
		1	22	31.31±5.86	19.13	43.49	0.00	88.89
		Total	29	23.75±5.09	13.32	34.19	0.00	88.89

Fruit shape (1: Elongate; 3: Conical 4: Bell); Fruit cross-section shape (3: Slightly corrugated; 5: Intermediate; 7: Corrugated) and Neck formation on fruit (0:Absent; 1: Present).

Contribution of the Authors

The data of this study were collected by Prof. Dr. Halit Yetişir, Agricultural Engineer Mh. Miraç Şahin, Assoc. Prof. Dr. Hasan Pınar. Erciyes University, Scientific Research Project Coordination Office provided support for the conduct of the study. Laboratory analyses of the study were conducted by Agricultural Engineer Mh. Miraç Şahin and statistical analyses were conducted by Dr. Faculty member Alim Aydın. The text of the article was written by Agricultural Engineer Mh. Miraç Şahin under the supervision of Prof. Dr. Halit YETİŞİR.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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