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Research Article

Effects of Colchicine Applications on Embryo Yield and Spontaneous Chromosome Doubling in Pepper (*Capsicum annuum* L.) Anther Culture

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ABSTRACT

The haploidy technique starts with the production of haploid embryos and continues with the doubling of the chromosome set. Androgenic embryos can be either haploid (H) or spontan doubled haploid (SDH). Since haploid plants are sterile, anti-mitotic agents are applied at appropriate doses and durations to restore diploid status and fertility. Chromosome doubling can be induced in vivo with anti-mitotic agents, but this method is expensive, complex and time consuming. Therefore, spontaneous doubling of chromosomes during culture is preferred. In this study, the effects of colchicine use on embryo yield and embryo development in pepper (*Capsicum annuum* L.) anther culture were investigated. Colchicine is a chemical agent that changes the cell division process by inhibiting microtubule formation in plant cells. Colchicine was applied to pepper anthers at concentrations of 0.3%, 0.4% and 0.6% in semi-solid and double-layer media for 14 and 21 days. Embryo and plant regeneration rates obtained according to media, colchicine dose and application duration were examined. According to the trial results, the highest embryo formation rate (21.03%) was obtained in the group treated with 0.6% colchicine dose for 14 days in semi-solid media. The development rates of embryos into plants varied depending on both colchicine doses and nutrient media used. Plants developed from embryos were grouped as haploid (H) and spontaneous double haploid (SDH) by examining the presence of pollen in flowers, seeds in fruits, stomata number and stomata characteristics. It was observed that the addition of colchicine to the medium had a very significant effect on the SDH plant ratio. The highest SDH plant ratio (74.28%) was obtained from the application of 0.6% colchicine for 21 days in semi-solid medium. On the other hand, the highest SDH plant ratio (69.05%) in double-layer media was determined in the application of 0.6% colchicine dose for 14 days. The SDH plant ratio obtained from embryos grown in colchicine-free medium was found to be 48.89% (14 days) and 44.74% (21 days). It can be concluded that colchicine application significantly enhances the production of SDH plants in pepper anther culture, with the most effective treatment being 0.6% colchicine for 21 days in a semi-solid medium.

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INTRODUCTION

Pepper is one of the vegetables with high economic value worldwide. Various biotechnological methods are used to improve traits such as high yield, disease resistance and quality in pepper production. It requires great effort and time to obtain 100% homozygous pure lines in plant breeding programs. The ability of haploid plants to reach full homozygosity in a single generation by chromosome doubling has become a valuable tool in terms of shortening the time in plant breeding (Yaman & Karaca Sanyürek, 2023). Haploid induction from male gamete (androgenesis) and female gamete (gynogenesis) are prominent methods in this process. Methods such as parthenogenesis are also used, and techniques such as insufficient or irrated pollen use during pollination and chromosome elimination are also preferred approaches in this field (Zhang et al., 2020; Ebrahimzadeh et al., 2021; Marin-Montes et al., 2022; Salehian et al., 2023). Successful results were obtained with the anther culture method in studies conducted specifically on pepper plants.

The first step of the haploidy technique is to obtain haploid embryos and the second step is to double the chromosome set. Androgenic embryos can be haploid (H) or spontaneously doubled haploid (SDH) during culture. Double haploids are very important for plant breeding because they can produce completely homozygous plants within one generation, making qualitative and quantitative phenotypic selection more efficient (Hooghvorst & Nogués, 2021).

Haploid embryos formed during the natural process of androgenesis usually fail to complete their development. Haploid plants are generally smaller and have a weak development compared to their diploid counterparts. Haploid plants are sterile due to the improper pairing of chromosomes during gamete formation and cannot produce pollen and seeds. In order to restore diploid status and fertility in haploids, they should be subjected to some anti-mitotic agents at appropriate doses and durations (Segui-Simarro & Neuz, 2008).

In androgenetic embryos, chromosome doubling is critical for ensuring developmental stability, maintaining genetic balance and creating homozygous lines for research. Obtaining haploid plants with in vitro techniques and transforming them into diploid plants offers an effective way to develop plant varieties with new and superior characteristics. Embryos with diploid chromosome structure are obtained by doubling the chromosomes and these embryos are generally stable and suitable for development.

Spontaneous or in vitro induced chromosome duplication during culture has the advantage of reducing the time and cost of DH production by eliminating the need for in vivo chromosome doubling. One way to increase the viability of haploid embryos and improve plant development in anther culture method is diploidization of embryos at the beginning of culture (Comlekcioglu, 2021) . Although the mechanism of SDH in androgenesis has not been fully explained, three main mechanisms have been mentioned in the literature; endoreduplication, nuclear fusion and c-mitosis (Seguí-Simarro & Nuez, 2008). The use of various antimitotic agents for chromosome doubling in haploid plants is evaluated as c-mitosis mechanism. C-mitosis leads to chromosome doubling by affecting the metaphase stage of cell division with antimitotic agents. Antimitotic agents are generally known as metaphase inhibitors that are effective during metaphase. Colchicine, colcemid, vinblastine, acenaphthene, dintroanilines, phosphoroamidates, pyridines, benzamides, benzoic acid compounds affect the metaphase stage of cell division and cause chromosome doubling. Compounds such as colchicine in particular prevent cell division by altering microtubule dynamics and cause doubling of chromosome number (Dewitte & Murray, 2003; Vaughn, 2006; Dhooghe et al., 2011).

A successful protocol is required for efficient chromosome doubling in haploids induced in vivo by the use of anti-mitotic agents. Multiple factors must be adjusted to maximize the rate of genome duplication. Successful outcomes can vary depending on antimitotic agent application efficiency, especially concentration, exposure time, application method and conditions. It is also critical for plant developmental stage and plant survival. Therefore, SDH is desired during culture. When haploid plants are exposed to colchicine, oryzalin, trifluralin and other chemicals with antimitotic effects in vitro or in vivo, chromosome doubling occurs (Vural et al., 2019). These applications can be done in vitro, at the explant, callus or plantlet periods. Chromosome duplication is difficult in haploid plants with a single chromosome set; usually application of antimitotic agents is used. In vivo application of antimitotic agents is costly, difficult and time-consuming.

During the androgenesis process, in vitro chromosome doubling varies significantly among different species and genotypes. The success rates vary depending on factors such as genotype, culture conditions and

chromosome doubling method, and it is a labor-intensive work (Mishra et al. 2021, da Silva Dias, 2003; Yuan et al., 2015).

Shim et al. (2006) reported that factors such as the concentration of antimitotic agents, temperature during application and exposure time are critical for the species. Kasha (2005) reported that chromosome duplication also depends on the haploidy method, and that the first pollen mitotic division stage of the microspore in androgenesis and Supena et al. (2006) reported that the first week of culture are the ideal times for chromosome duplication. Da Silva Dias (2003) reported that low concentrations (0.01-0.02%) of colchicine only inhibit the cell division cycle for a short time, and then the cells can continue mitosis when they contain a doubled set of chromosomes. The most suitable time for in vitro antimitotic agent application was determined as the first 12 hours after microspore isolation. Therefore, SDH should be intensively investigated in addition to the possibilities of increasing the frequency of embryo formation and transformation into a normally developed plant in the anther culture technique, which has become a routine method in Capsicum breeding studies.

It is stated that factors such as culture conditions, pretreatments, use of colchicine and similar antimitotic substances are determinants of in vitro chromosome doubling (Zhao & Simmonds, 1995). In addition, it is stated that there is a direct relationship between the use of plant hormones in in vitro cultures and DNA duplication (Joubes & Chevalier, 2000). It is stated that the type of explant used in the androgenesis process may also affect the SDH rate (Sato et al., 2005). Comlekcioglu and Ellialtıoğlu (2018) reported that SDH rates obtained from pepper anther culture differed significantly among genotypes. It was emphasized that the gamete developmental stage and culture conditions also affect this process (Segui-Simarro & Nuez, 2008). Niklas-Nowak et al. (2012), as a result of cytometric analysis of 63 regenerated pepper plants, it was determined that 32 plants were haploid (H) and 31 plants were SDH. Keleş et al. (2015) investigated the effect of pepper fruit type on obtaining SDH plants through anther culture and the highest rate was observed in the bell pepper type with 53.4%. Grozeva et al. (2021) reported the SDH formation rate in pepper anther culture as 40.14%, while Misal and Das (2023) determined this rate as 40.70% and Arı et al. (2016) as 51.60%. In his study examining the effect of colchicine, Comlekcioglu (2021) reported that SDH rates were 33.3% in control medium, 57.6% in semi-solid medium and 47.3% in double-layer medium.

Published protocols can be used as a basis for experimental design, but different application methods should be tested for best results. Unfortunately, many published DH protocols do not report antimitotic agent application details and treatment efficiency. The current methods can be used to further refine the genome doubling methodology for major vegetable species. Genome duplication tests will provide other researchers with valuable information for the use and further development of genome doubling protocols.

This study aimed to determine the effects of in vitro colchicine applications on embryo yield, embryo development rates into full plants and the increase in the number of spontaneous dihaploid plants in pepper anther culture.

MATERIAL AND METHOD

Plant material

The study was conducted at Petektar Tohumculuk Seed Company in Antalya. The 45-coded bell pepper genotype in the company's gene pool was selected as the donor plant. Seed sowing was carried out in the autumn growing season. Seedlings were planted in the greenhouse in double rows at 50x50x100 cm intervals in the 2-3 leaf period. Anther culture was started with the buds formed 30 days after planting.

In previous studies, in order to determine the development period suitable for anther culture of pepper genotypes, the acetocarmine staining method was used in the study of Gursoy et al. (2022), as well as the research findings of Comlekcioglu et al. (2001) and Alremi et al. (2014) were taken as reference. Accordingly, the stage was selected when the bud size was 5 mm and the petals were 1-2 mm longer than the sepals, the anther color was cream-yellow, and the tips turned slightly purple with anthocyanin accumulation (Figure 1).

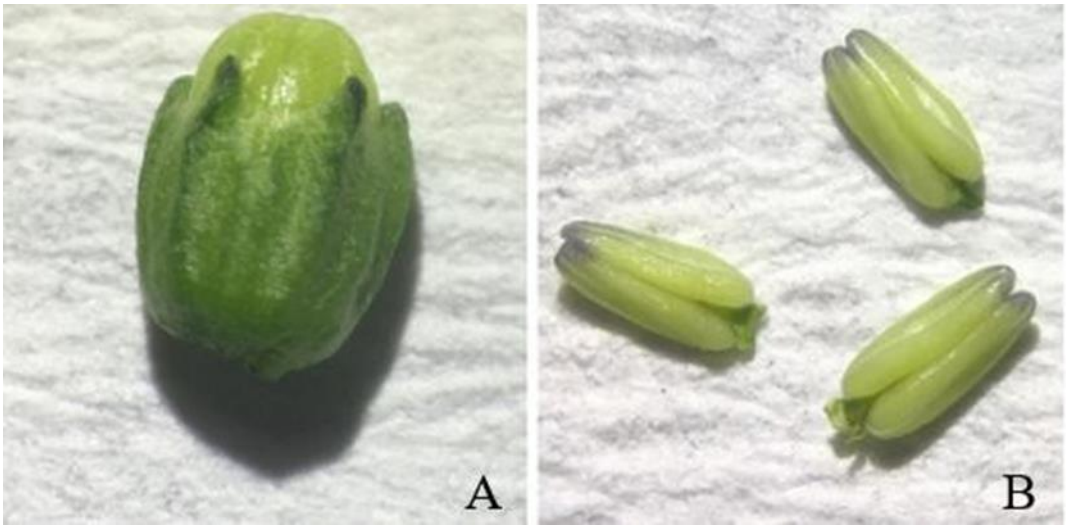

Figure 1. *A; bud at suitable period for anther culture, B; anther at the appropriate period for anther culture*

Sterilization of buds

The buds collected in the morning and brought to the laboratory were shaken in a solution prepared with 15% commercial bleach (approximately 5% sodium hypochlorite content) and 1-2 drops of Twin-20 for 15 minutes. After this process, they were rinsed with sterile distill water 3 times for 5 minutes each.

Nutrient medium

MS (Murashige & Skoog, 1962) containing 4 mg l^{-1} NAA (Naphthalene Acetic Acid), 0.5 mg l^{-1} BAP (Benzyl Amino Purine), 0.25% activated charcoal, 30 g l^{-1} sucrose, 15 mg l^{-1} $AgNO3$ (Silver Nitrate) and 8 g l^{-1} agar was used as the primary culture medium. Regeneration medium was prepared as primary culture medium without activated charcoal and $AgNO_3$. The media coming out of the autoclave were poured into sterile 9 cm diameter glass Petri dishes in a sterile cabinet.

Preparation of colchicine applications

Semi-solid media were created by adding 0.0%, 0.3%, 0.4% and 0.6% colchicine doses to the primary nutrient medium. In double-layer media, while the primary nutrient medium formed the semi-solid phase, 0.3%, 0.4% and 0.6% colchicine doses was added as the liquid phase after anther inoculation. Anthers cultured in the prepared media were transferred to the regeneration medium on the 14th and 21st days of culture (Table 1).

Table 1. *Applications and their contents*

Applications	Contents
Control (Basic medium)	MS + 30 g l^{-1} Sucrose, 0.25% Activated Charcoal, 4 mg l^{-1} NAA, 0.5 mg l^{-1} BAP, 15 mg l^{-1} $AgNO_3$, 8 g l^{-1} Agar
0.3% Colchicine	Control + 0.3% Colchicine (Semi-solid)
0.3% Colchicine	Control + 0.3% Colchicine (Double-layer)
0.4% Colchicine	Control + 0.4% Colchicine (Semi-solid)
0.4% Colchicine	Control + 0.4% Colchicine (Double-layer)
0.6% Colchicine	Control + 0.6% Colchicine (Semi-solid)
0.6% Colchicine	Control + 0.6% Colchicine (Double-layer)
Regeneration medium	MS + 30 g l^{-1} Sucrose, 4 mg l^{-1} NAA, 0.5 mg l^{-1} BAP, 8 g l^{-1} Agar

Placing anthers in the medium

Anthers were cut from their filaments using forceps and scalpels and placed in Petri dishes so that their dorsal surfaces were in contact with the medium (Figure 2). Each Petri dish contained 25 anthers, and 24-27

(600 to 675 anthers) Petri dishes were used for each application. For double-layer applications, after placing the anthers in the Petri dish, filter-sterilized colchicine solutions were added (Figure 3).

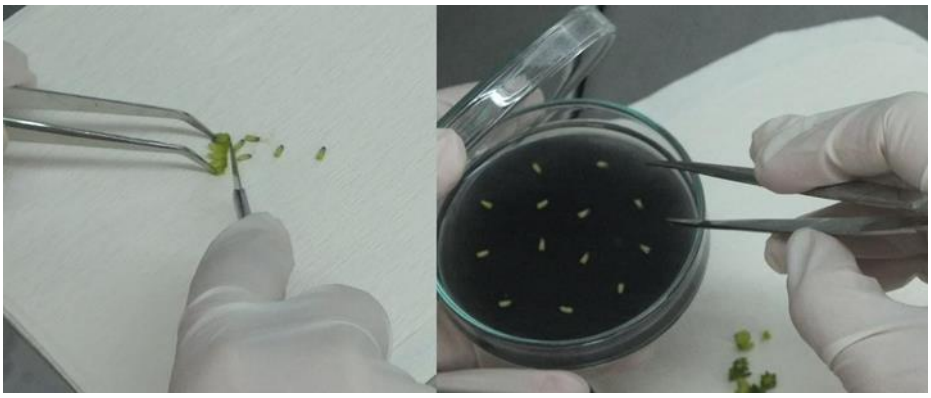

Figure 2. *Cutting the anthers from their filaments and placing them in the nutrient medium*

Figure 3. *Adding filter-sterilized colchicine onto anthers for biphasic applications*

Incubation conditions

Anthers placed in the nutrient medium were exposed to a 35ºC temperature shock in the dark for two days. After being removed from the incubator, they were moved to the climate chamber kept at 25ºC. Anthers kept in the dark in the climate chamber were transferred to the regeneration medium on the 14th and 21st days of culture (Figure 4). After being kept in the dark for 35 days, the cultures were continued to be incubated in the climate chamber with a 16-hour photoperiod at 2300 lux. The embryos developing here were moved into sterile jars filled with hormone-free MS medium.

Figure 4. *Transfer of anthers to regeneration medium*

Acclimatization of plantlets to external conditions

Plantlets were removed from the nutrient medium and planted in small pots (100 ml) filled with a 1:2 peat:perlite mixture. The pots were bagged to maintain humidity and placed in an acclimatization room at 25°C under 2300 lux light and a 16-hour photoperiod. Rooted plants were transplanted into 2.5-liter pots and taken to the greenhouse. The plants, which were kept in pots in the greenhouse for a week, were planted in the greenhouse soil.

Determination of haploid and spontaneous double haploid plants

Pollen in flowers and seed in fruits were examined for this purpose. Stomata count, stomata length and stomata width measurements were made with a microscope (Soptop CX40-T) in the preparations prepared by taking leaves from three different regions of 10 plants of each application. Stomata count and measurements were made in three different regions per unit area (0.08 mm^2) with a 40 magnification objective and a 10 magnification eyepiece.

Data evaluation

Experiments were carried out according to the randomized plot design with three replications (600-675 anthers in total) with 8-9 Petri dishes (200-225 anthers) in each replication. Embryo rate was calculated as the ratio of the number of embryos obtained to the number of anthers cultured. Plantlet rate was calculated as the ratio of the number of plantlets developing from embryos after culture to the number of embryos obtained. The significance level of the differences between the means was determined using the LSD test.

RESULT AND DISCUSSION

In the study, a total of 9100 anthers were cultured and a total of 1022 embryos were obtained (Figure 5.) In 1022 embryos, 495 of the obtained embryos successfully developed into plants (Figure 6). Embryo formation and plant transformation rates varied according to the medium, colchicine dose and application duration (Table 2).

Table 2. *Numbers of anthers, embryos and regenerated plants cultured according to medium, colchicine dose and application time*

Medium	Application Duration (Days)	Colchicine Dose (%)	Anther (Number)	Embryo (Number)	Plantlet (Number)
Semi-solid	14	0,0	650	124	65
		0,3	675	100	59
		0,4	675	80	48
		0,6	675	142	60
	21	0,0	675	96	55
		0,3	650	84	39
		0,4	675	134	54
		0,6	675	104	55
Double-layer	14	0,3	675	26	5
		0,4	600	29	11
		0,6	600	10	1
	21	0,3	675	35	12
		0,4	600	51	29
		0,6	600	7	2

When embryo formation rates were evaluated, it was determined that semi-solid medium was more successful than double-layer medium. While the embryo formation rate in semi-solid medium was 15.91%, it was 4.1% in double-layer medium. The highest embryo formation rate of 21.03% was obtained from anthers cultured in semi-solid medium at a dose of 0.6% colchicine for 14 days. The lowest rate of 1.17% was determined in the application of 0.6% colchicine in double-layer medium for 21 days (Table 3).

Tablo 3. *Embryo formation rate according to different medium structure, colchicine doses and colchicine application duration(%)*

Medium	Colchicine Dose (%)	Application duration (Day)	
		14	**21**
Semi-solid	**0.3**	14.77a	12.40b
	0.4	11.87b	20.00a
	0.6	21.03a	15.40b
Double-layer	**0.3**	3.87a	5.20a
	0.4	4.83b	8.50a
	0.6	1.67a	1.17a
LSD%1	1.693		

The average values indicated with different letters in each line are statistically different from each other.

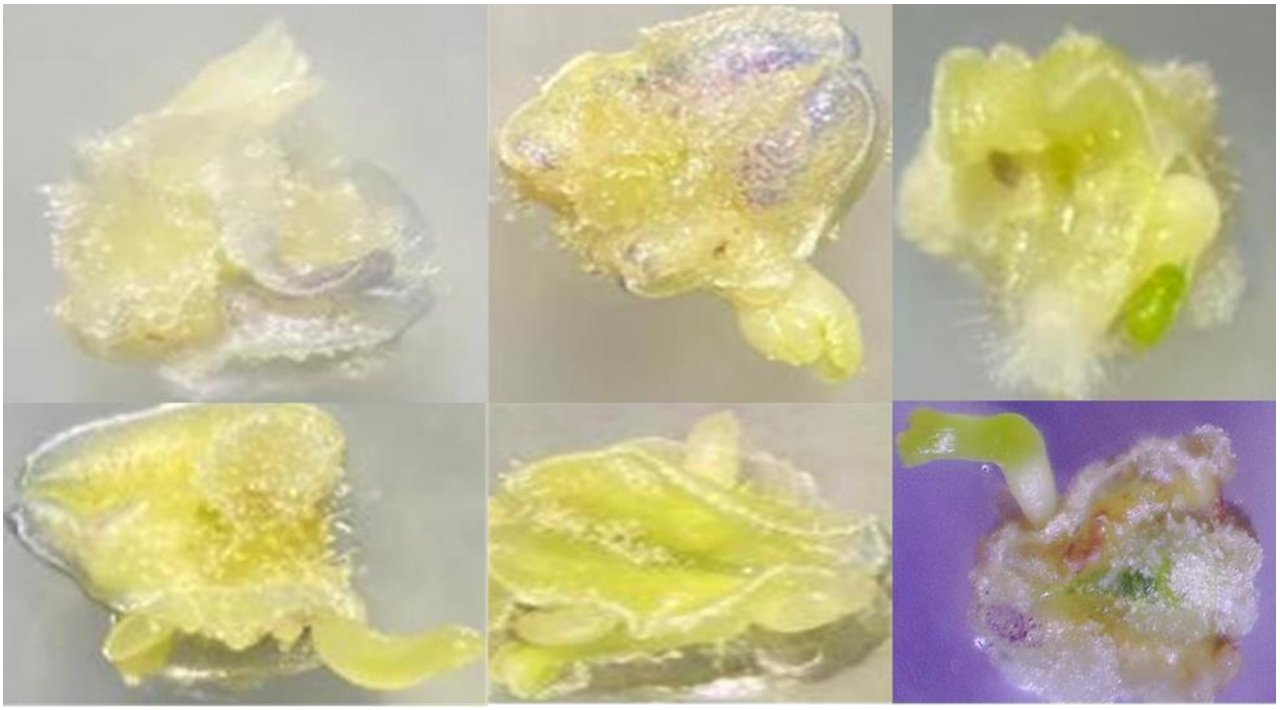

Figure 5. *Some embryos obtained in the study*

Media and colchicine application duration had significant effects on plant regeneration rates. The highest plant regeneration rate was obtained as 53.74% in colchicine application for 14 days in semi-solid medium. This was the most successful plant regeneration result obtained in the study. Application of colchicine for 21 days resulted in similar plant regeneration rates in both media structures (Table 4).

Table 4. *Effect of aplication period X medium on plant regeneration rate (%)*

Application Period (Day)	Medium	
	Semi-solid	**Double-layer**
14	53.74a	27.90b
21	46.68a	45.03a
LSD%1	13.033	

The average values indicated with different letters in each line are statistically different from each other.

In semi-solid medium, the plant regeneration rates varied between 47.56% and 52.75% and the difference was found to be statistically insignificant. In double-layer medium, the highest plant regeneration rate (47.32%) was observed at 0.4% colchicine dose (Table 5).

Table 5. *Effect of medium X dose on plant regeneration rate (%)*

Medium	Colchicine Dose (%)		
	0.3	**0.4**	**0.6**
Semi-solid	52.75a	50.33a	47.56a
Double-layer	27.21b	47.32a	36.11ab
LSD%5	11.626		

The average values indicated with different letters in each line are statistically different from each other.

Figure 6. *Some plantlets developed from the obtained embryos*

Comlekcioglu (2021) determined that the addition of colchicine to the nutrient medium in pepper anther culture increased the embryo formation frequency by 89.5% in semi-solid medium and 36.8% in double-phase medium compared to the control medium. In his study, 19, 36 and 23 embryos were obtained from the control without colchicine, semi-solid and double-layer media containing 0.3% colchicine, respectively. The number of regenerated plants from the embryos was reported as 15, 26 and 19, respectively. In a study

comparing anther culture and shed-microspore culture, it was reported that the anther culture method was a more reliable and successful option than shed-microspore culture to obtain haploid plants in pepper varieties (Kanmaz, 2021).

At the end of the experiment, the plants that were fully developed from the embryos obtainedand transferred to the greenhouse were grouped as H and SDH according to the presence of pollen in the flowers, the presence of seeds in the fruits and the number of stomata on the leaves. 10 plants were evaluated from each application. In the applications made in double-layer medium, 2-7 plants were transferred to the greenhouse and observations were made on these plants. The number of stomata, stomata width and length in donor, H and SDH plants are presented in Table 6 and shown in Figure 7, Figure 8, and Figure 9.

Table 6. *Average stomata number, stomata width and length in donor, haploid and SDH plants*

Ploidy Level	Stomata Number (0.08 mm^2)	Stomata Width (µm)	Stomata Length (µm)
Donor	11.25b	23.83ab	29.78a
Haploid	21.75a	21.73b	25.38b
SDH	13.25b	26.43a	31.38a
LSD%1	3.447	3.374	3.346

The average values indicated with different letters in each line are statistically different from each other.

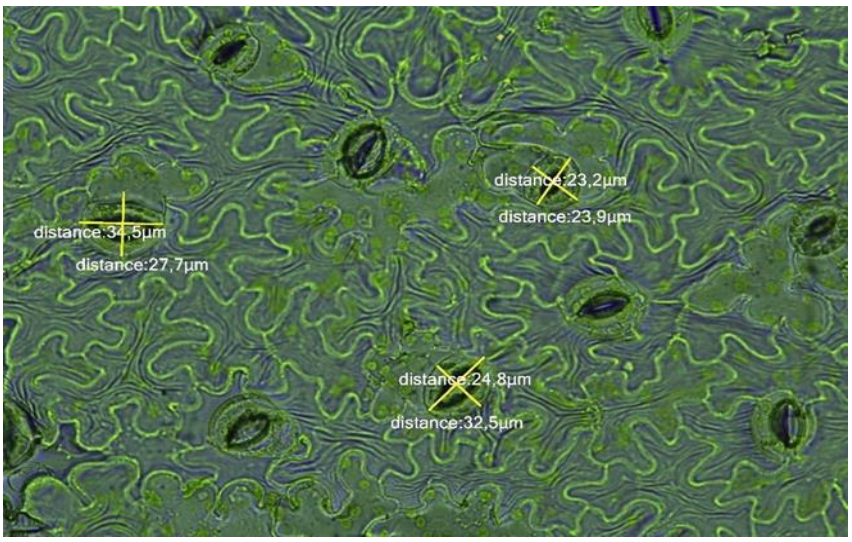

Figure7. *Donor plant stoma image*

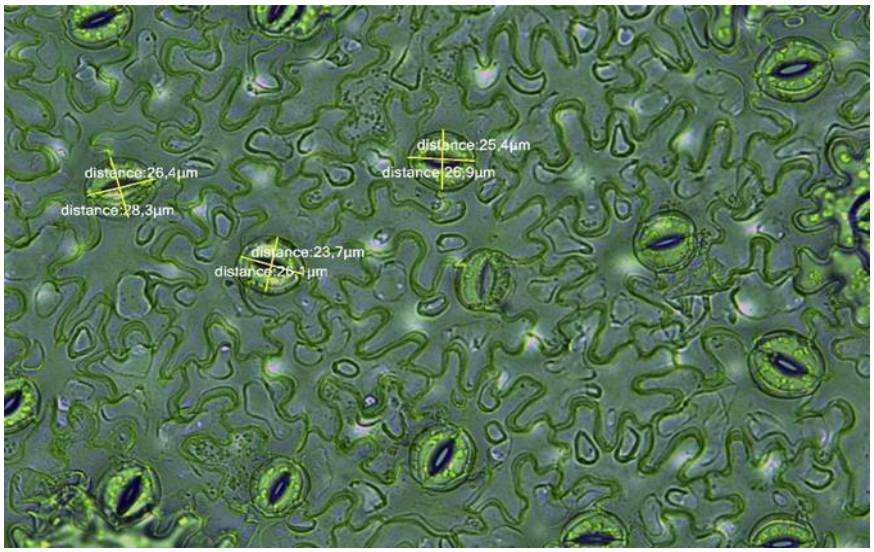

Figure 8. *Haploid plant stoma image*

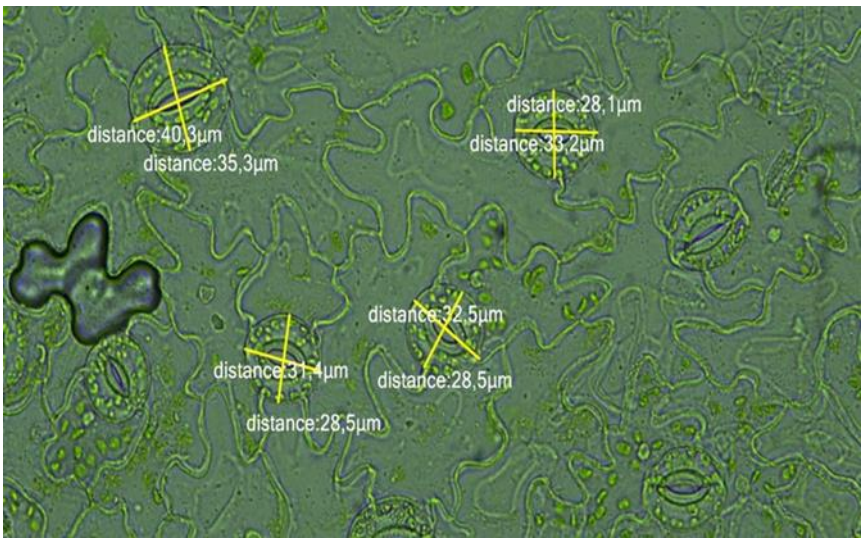

Figure 9. *SDH plant stoma image*

While haploid plants have an average of 21.75 stomata per unit area, this number varies between 11.25 and 13.25 in donor and SDH plants, respectively. Although stomatal width and length in SDH plants are larger than those in haploid plants, considering the turgor status of the plant, the number of stomata is thought to be a more decisive criterion in determining the characteristics of plants. Therefore, the number of stomata has been evaluated as an important indicator in the classification of plants as haploid and SDH. Bat et al. (2021) reported the number of stomata in the same unit area in pepper as 10.71 in haploid and 27.07 in SDH.

In the study, the relationships between variables were evaluated through pairwise correlation analyses, and the resulting correlation coefficients are presented in Table 7. A strong, negative, and statistically highly significant correlation was observed between the embryo rate and the culture media ($r = -0.885$; $P \leq 0.01$). A moderate, negative, and significant correlation was also determined between the plant regeneration rate and the media ($r = -0.528$; $P \leq 0.01$). Furthermore, a moderate, positive, and statistically significant correlation was found between the plant regeneration rate and the embryo rate ($r = 0.519$; $P \leq 0.01$). The correlation coefficients calculated for other variable pairs were not statistically significant ($P > 0.05$).

Table 7. *Correlation analysis between variables*

	Medium	Application Duration	Dose	Embryo rate	Plant regeneration
Medium	1				
Application duration	0.000ns	1			
Dose	0.000ns	0.000ns	1		
Embryo rate	-0.885**	0.058ns	0.047ns	1	
Plant regeneration	-0.528**	0.154ns	-0.160ns	0.519**	1

ns = not significant, * = significant at alfa level %5, ** = significant at alfa level %1

A total of 336 plants were examined in the study conducted to determine haploid and SDH plants. In the application carried out for 21 days at 0.6% colchicine dose in semi-solid medium, the highest SDH rate was found as 74.28% (26 plants). In the application carried out at the same dose for 14 days, the SDH plant rate was determined as 69.05% (29 plants). The SDH plant rate from embryos obtained from medium without colchicine varied between 44.74% and 48.89%. Four plants obtained from the application of 0.3% colchicine for 14 days in double-layer medium were evaluated as SDH. One plant was determined as SDH from the application of 0.6% colchicine for 14 days and two plants were determined as SDH from the application of 21 days. These results show that colchicine is effective in increasing the SDH plant rate and that SDH plant rates change in different medium conditions at different doses and times. In addition, it is understood that different medium conditions also affect the SDH plant rates. In our study, it was determined that the SDH plant rates obtained from media containing colchicine were higher than the control groups without colchicine (Table 8).

Table 8. *Numbers and rates of haploid, SDH plants obtained from applications (%)*

Medium	Application duration (Day)	Colchicine Dose %	Number of Haploid Plant	Haploid Plant Rate %	Number of SDH Plant	SDH Plant Rate %
Semi-solid	14	0.0	23	51.11	22	48.89
		0.3	20	44.44	25	55.56
		0.4	9	36.00	16	64.00
		0.6	13	30.95	29	69.05
	21	0.0	21	55.26	17	44.74
		0.3	13	43.33	17	56.67
		0.4	18	40.91	26	59.09
		0.6	9	25.72	26	74.28
Double-layer	14	0.3	-	00.00	4	100.00
		0.4	1	33.33	2	66.67
		0.6	-	00.00	1	100.00
	21	0.3	2	28.58	5	71.42
		0.4	4	26.67	11	73.33
		0.6	-	00.00	2	100.00

A total of 1020 embryos were obtained from hot pepper anthers and 516 of them developed into normal plants. In the ploidy level analyses performed on 516 regenerative plants, spontaneous chromosome doubling was observed in 40.70% (Misal & Das, 2023). In a study conducted by Keleş et al. (2015), SDH rates were compared in plants obtained by using anther culture in seven Charleston, six bell pepper, eight capia and seven pointed pepper genotypes. It was stated that SDH rates varied among different pepper species. The highest SDH plant rate was observed as 53.4% in bell pepper. 31.9% SDH plant rate was determined in Charleston pepper. 30.4% plant SDH rate was found in capia pepper. 22.2% SDH plant rate was determined in pointed pepper. These data show that SDH plant rates vary in different pepper types, with bell pepper having the highest SDH plant rate. The ability of chromosomes to self-duplicate depends on the genotype. In contrast to pepper, spontaneous chromosome doubling rates were reported as 23% in melon (Hooghvorst et al., 2020) and 63–72% in barley (Mirzaei et al., 2011).

Studies on pepper anther culture show that SDH ratios can vary widely depending on the genotype, culture medium, colchicine dose used and other conditions. SDH ratios have generally been reported ranging from 13% to 65%, and SDH ratios are generally lower than haploid ratios. Mityko et al. (1995) and Gyulai et al. (2000) reported that SDH ratios of regenerated plants varied between 65%; Gemesne et al. (2000), 29.8%; Supena et al. (2006), 13% to 51%; Ercan et al. (2006), all of the 76 plants developed in pepper anther culture were haploid; Niklas-Nowak et al. (2012), approximately 50%; Keleş et al. (2015), 39%; Alremi et al. (2014) reported that 94% of the 40 plants obtained were haploid, while Arı et al. (2016) reported that 51.6% of the 122 plants were SDH, 42.6% were haploid and 5.73% were mixoploid.

Methods to identify haploids and determine spontaneous chromosome doubling in androgenic plants play an important role in plant breeding and genetic research. Chromosome counting from root tip cells under a microscope and measuring the amount of DNA present in the cell nucleus by flow cytometry provide fast and reliable results. Haploids can also be identified using molecular markers.

Morphological and anatomical markers are easy and economical to analyze to determine the success of chromosome folding. SDH allows bypassing laborious treatments with antimitotic agents.

It is known that there is a significant and reliable correlation between stomatal numbers of haploid and SDH plants (Przywara et al., 1988; Cramer, 1999; Aryavand et al., 2003; Yuan et al., 2009; Da Silva et al., 2020; Bat et al., 2021; Tammu et al., 2021; Castañeda-Nava et al., 2023). Studies on stomatal size and stomatal

density per unit area of haploid plants show that haploid plants have smaller stomata than diploids and therefore have more stomata per unit area (Omidbaigi et al., 2010; Głowacka et al., 2010; Hannweg et al., 2013; Widoretno, 2016; Comlekcioglu & Ozden, 2019).

The distinction between haploid and diploid plants in plants obtained from anther culture in *Capsicum annuum* was tested in 170 plants. The results showed that the mean stomatal length and number were 26.4 µm and 7.4 µm in haploid, 35.2 µm and 5.8 µm in diploid, and 33.3 percent (%) and -30% higher in diploid compared to haploid. Thus, haploid and diploid were significantly different in these parameters and measurement of stomatal length is a rapid technique to determine the ploidy level in pepper (Shrestha & Kang, 2016). İlhan and Kurtar (2022) determined the ploidy levels of the plants obtained as a result of anther culture in 12 pepper genotypes by stomatal measurements (stomatal size, chloroplast number and number of stomata per unit area). As a result of stomatal characteristics of 18 plants, 13 plants were haploid and 5 plants were diploid. In the study conducted by Bat et al. (2021), higher stomatal density was found in haploid plants compared to diploid plants. The average number of stomata per unit area was 27.07 in haploid pepper and 10.71 in diploid pepper. It was determined that the average stomatal width was 22.52 µm in haploid pepper and 29.50 µm in diploid pepper and the difference between ploidy levels was significant. Haploid eggplants showed a stomatal density of 29.20 stomata per unit area, whereas doubled haploid (DH) plants had a reduced density of 12.61. The average stomatal lengths were 22.32 µm in haploids and 32.00 µm in DH plants, with corresponding widths of 17.36 µm and 22.32 µm. This suggests that DH plants possess larger but fewer stomata compared to their haploid counterparts. Haploid onion plants had stomatal lengths and widths of 27.2 µm and 25.5 µm, respectively, with a density of 157.6 stomata per mm². Diploid onions displayed larger stomata (39.1 µm in length and 31.0 µm in width) but a lower density of 129.0 stomata per mm². These findings highlight a positive correlation between ploidy level and stomatal size, and a negative correlation with stomatal density (Foshi et al., 2013). It was investigated whether morphological and cytological analyses would be sufficient for ploidy determination of haploid plants obtained by ovary culture in watermelon. For this purpose, stomatal, morphological and cytological characteristics of 15 haploid and 19 doubled haploid plants were determined. Before these analyses, the ploidy level of the plants was determined by flow cytometry. It has been reported that stomatal traits can be used as morphological markers to determine the ploidy level of plants (Kara et al., 2024). Antimitotic agent application efficiency depends on the application conditions, especially its concentration and exposure duration. Furthermore, the plant developmental stage is critical for agent accessibility and plant survival. Current methods can be used to further improve genome doubling methodology for major vegetable species (Fomicheva et al., 2024).

CONCLUSION

In pepper breeding studies, haploid plant production via androgenesis has been focused on. In pepper androgenesis, anther culture is the most effective method widely used by various researchers. The effects of many factors on obtaining haploid plants have been studied. Recently, spontaneous chromosome doubling has become the focus of attention in order to increase the plant production rate by testing and optimizing different procedures. Colchicine is a compound widely used to induce polyploidy in plant tissue culture and can have significant effects on plant development. Colchicine disrupts spindle formation during cell division, leading to an increased chance of chromosome folding. This can lead to the formation of larger and healthier embryos, increased viability and developmental potential of the embryos thus increasing embryo yield and potentially higher yields of viable plants. Since in vitro colchicine application will be carried out in a controlled environment, it minimizes the effect of external factors. It can provide higher efficiency and faster achievement of the desired effect. This is an important advantage in breding studies when time is critical. For these reasons, more experiments on in vitro colchicine applications and adoption of in vitro applications in breeding studies are necessary.

This study shows that embryo formation and plant regeneration rates in pepper anther culture vary significantly depending on the nutrient medium, colchicine dose and application times. The analyses showed that embryo formation and plant regeneration were more successful in semi-solid medium compared to the double-layer medium. It was observed that especially 0.3% and 0.4% colchicine doses were associated with higher regeneration rates. However, it was determined that plant regeneration rates decreased as colchicine dose increased. It was also determined that SDH plant rates varied depending on the duration and dose of colchicine application and that colchicine application increased the SDH rate.

As a result, colchicine applications in anther culture have been found to affect embryo formation and development. In anther culture, spontaneous chromosome doubling may occur due to stress conditions. When colchicine is applied, this may increase the probability of doubling. It has been determined that the effectiveness of colchicine in promoting embryo yield and chromosome doubling depends on the application dose and exposure time. Careful dose adjustment during application is important both to increase embryo yield and to achieve desired polyploidy levels. These applications need to be carefully optimized.

According to the results of this study, the following suggestions can be made to increase the efficiency of future studies and contribute to the wider application of haploidy techniques in pepper plants.

The highest percentage of SDH plants (74.28%) was obtained when 0.6% colchicine was applied for 21 days in semi-solid medium. Future studies could examine the effects of higher or lower colchicine concentrations and different application times on SDH rates. Determining the optimum dose is important to maximize SDH production without negatively affecting plant growth.

Although double-layered media have lower embryo formation rates than semi-solid media, trials could be conducted to improve the efficiency of these media with different nutrient compositions or hormone additions.

Considering that SDH rates vary depending on the genotype, investigating the effectiveness of colchicine applications in different pepper genotypes and conducting studies on a wide genetic base can make an important contribution.

In addition to colchicine, the efficacy of antimitotic agents such as oryzalin and trifluralin on SDH production can be investigated. It should be examined whether these agents have lower toxicity or offer higher success rates.

AUTHOR CONTRIBUTION

All authors contributed equally.

ETHICAL STATEMENT

During the writing process of the study titled "**Effects of Colchicine Applications on Embryo Yield and Spontaneous Chromosome Doubling in Pepper (*Capsicum annuum* L.) Anther Culture** ", scientific rules, ethical and citation rules were followed; No falsification has been made on the collected data and this study has not been sent to any other academic media for evaluation. Ethics committee approval is not required.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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