Pak. J. Bot., 53(3): 1001-1005, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-3(14)

EFFECT OF COLCHICINE ADDITION TO CULTURE MEDIUM ON INDUCTION OF ANDROGENESIS IN PEPPER (*CAPSICUM ANNUUM* L.)

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Abstract

Anther culture generate completely homozygous line in single generation whereas 6–8 cycles of self-pollination is required to develop homozygous line through conventional breeding method. Experiment on the anther culture in pepper was carried out to determine effect of colchicine on haploid embryo. Semi-solid and double layer culture media were studied. MS (Murashige & Skoog, 1962) medium containing 4 mg l^{-1} NAA(naphthalene acetic acid), 0.5 mg l^{-1} BAP (6-benzylaminopurine), 2 g l^{-1} activated charcoal, 30 g^{-1} sucrose, 10 mg l^{-1} $AgNO_3$ (silver nitrate) and 0.05 mg l^{-1} biotin, 0.5 mg l^{-1} ascorbic acid, 7 g l^{-1}agar was used as control (M1). Semi-solid M2 was % 0.3 colchicine containing M1. The third medium (M3) was double layer. The semi-solid layer was the same of the M1 and liquid layer was % 0.3 colchicine solution. The experiment was carried out in randomized block design with 4 replications. 5 Petri dishes per repetition and 10 anthers per Petri dishes were cultured. Frequency of embryos increased by 89.5% and 36.8% for M2 and M3 medium, respectively, as compared to control (M1).The chromosome doubling of pepper haploids is necessary to restore diploid status and restore fertility. Spontaneous double haploid (SDH) ratios were 33.3% in M1 medium, 57.6% in M2 and 47.3% in M3 medium. Colchicine application significantly affects the number of embryos obtained, embryos rate, the number of regenerated plants and SDH plant numbers.

Key words: Anther culture, Chromosome doubling, Colchicine, Haploidy, Homozygosity, Plant breeding

Introduction

Haploid plant technology provides important advantages for obtaining homozygous pure lines to be used in breeding studies in a shorter time and for the purification of mixed varieties or populations grown in a short time. Gametic cells in plants restore the path of gametophytic development it has the capacity to irreversibly switch to the sporophytic pathway (androgenesis or gynogenesis) (Germana, 2011). Complete homozygous lines can be produced in one generation after culturing gamete cells.

In vitro haploid plants in pepper can produce by two methods, one is anther culture and other is microspore culture. The anther culture method was the best known and most widely used one in the genus *Capsicum*. Factors that can effect *In vitro* haploid production such as genotype, physiological status and growth conditions of donor plants, stage of pollen development, and *In vitro* culture medium and conditions (Comlekcioglu *et al*., 2001; Wang & Zhang, 2001; Boyaci, 2001; Rodeva *et al*., 2004; Buyukalaca *et al*., 2004; Simsek, 2009; Çömlekçioğlu & Ellialtıoğlu, 2018; Vural & Ari, 2020). Studies and problems related to anther culture, successful embryo formations and the effect of critical factors for androgenesis in *Capsicum* genus has been documented by Irikova *et al*., 2011; Segui-Simarro *et al*., 2011; Çömlekçioğlu & Ellialtıoğlu, 2018.

Androgenic embryos can be either haploid or spontaneously doubled haploid (SDH). Different rates of SDH have been reported in pepper anther culture. In studies conducted on this subject, the frequency of spontaneous diploidization of pepper regenerations derived from anther culture has been reported to differ significantly depending on the genotype (Çömlekçioğlu & Ellialtıoğlu, 2018; Simsek, 2011). It is more difficult to double the chromosomes by treating plants later *In vivo*. Segui-Simarro & Neuz (2008) reported that genotype, gamete (microspor or ovule) development stage and pre-treatment and culture conditions are factors that affect the frequency of spontaneous chromosome doubling. Mityko *et al*., (1995) and Gyulai *et al*., (2000) found that 65% of regenerated plants were SDH. Supena *et al*. (2006) observed that SDH rich up to 13 to 51%. Niklas-Nowak *et al*., (2012) determined that the frequency of SDH is almost 50%. Ercan *et al*., (2006) determined that all of the 76 plants that have developed in the anther culture were haploid. Alremi *et al*., (2014) reported that 94% of 40 plants were haploid, while Ari *et al*., (2016) found that 63 of 122 plants (51.6%) were SDH, 52 (42.6%) were haploid and 7 (5.73%) were mixoploid. In a comprehensive study on this subject carried out by Keleş *et al*., (2015) it was determined that out of total 611 plants, 373 (60%) were haploid and 238 (39%) were SDH, whereas Gemesne *et al*., (2001) established similar frequency of spontaneous diploidization among regenerated embryos (68.5% haploid plants; 29.8% spontaneous DH plants, 0.7% tetraploid plants and 1% aneuploid plants consisting one chimera). Gałązka & Niemirowicz-Szczytt (2013) reported that while plants derived from cucumber parthenogenetic embryos are predominantly haploid, the use of ovule or ovary culture and anther or microspore culture allows both haploids and SDH to be induced.

The main objective of this research was to examine impact of colchicine application on androgenic responses of pepper. The study also aimed to determine the frequency of spontaneous chromosome doubling (diploidization frequencies), in order to find a practical solution to the chromosome doubling process of haploid plants.

Materials and Methods

Cultivation of donor plants and flower buds collection: This experiment was conducted in Eskisehir Osmangazi University, Faculty of Agriculture, Department of

Horticulture (Eskisehir, Turkey). Donor plants, cv. Diyar F1 (*Capsicum annuum* L) (AntemaTarım), were planted in 15 liter plastic pots containing peat in the not heated greenhouse at 50x60 cm spacing.

Although the morphological characteristics of the buds and anthers, which have the most appropriate microspore development stage for the initiation of embryogenesis, vary between the pepper species and varieties, flower buds were collected and brought to the laboratory in the morning at the stage of petals started to be visible but do not exceed the level of the petals more than 1-2 mm and almost 1/3 of the anthers have anthocyanin.

Flower bud disinfection: The flower buds were surface sterilized by 15% calcium hypochlorite for 15 minutes and then rinsed 4 times with sterile distilled water. The anthers were removed without their filaments and placed in Petri dishes under aseptic conditions.

Nutrient media: Two semi-solid and double layer culture media were studied. The contents of the studied media were as follows;

M1(control); semi solid MS (Murashige & Skoog, 1962); 4 mg l^{-1} NAA(naphthalene acetic acid), 0.5 mg l^{-1} BAP (6-benzylaminopurine), % 0.2 active charcoal, 30 gl^{-1} sucrose, 10 mg l^{-1} AgNO3 (silver nitrate), 0.05 mg l^{-1} biotin, 0.5 mg l^{-1} ascorbic acid and 7 g l^{-1} agar.M2; semi- solid M1 containing % 0.3 colchicine. M3; the third medium was bilayer. The semi-solid layer was the same of the M1 and liquid layer was % 0.3 colchicine solutions.

Incubation conditions of anthers: Planted anthers were subjected to high temperature at 35ºC under continuous dark conditions for 2 days. Then they were taken to a low light, average 500 lux (Testo 540 luxmeter, Germany) in climate chamber set at 25ºC and 16/8 hours photoperiod. Anthers cultured in M2 were transferred to M1medium after 21 days (3 weeks).

Regenerated embryos were transferred into phytohormone-free MS medium and incubated in incubation room set at 25 ºC, average 4000 lux and 16/8 hours photoperiod. The mature plants were grown in the greenhouse.

Determination of ploidy level: The ploidy level of samples, stained with DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride), were measured by flow cytometry (using a CyFlow® Ploidy Analyser, Partec).

Experiment design and statistical analyses: The study was carried out with 4 replications and 5 Petri dishes (7 cm diameter) per repetition. Ten anthers obtained from two flower buds were planted in each Petri dishes (20 Petri dishes and 200 anthers were used in each medium).

Experiments were arranged in a completely randomized design. Data were subjected to analysis of variance (ANOVA, Tarist Statistical Software) and means were separated by Tukey's HSD test (A probability level $P < 0.01$ was used to test significance of differences between means).

Results and Discussion

The effect of colchicine application on haploid embryo formation and spontaneous (or stimulated) double haploid embryo in pepper anther culture was investigated.

Analysis of variance results is presented in Table 1. The effects of colchicine application methods was found to be significant ($p \leq 0.01$) in terms of the numbers of embryos obtained, embryos per 100 anthers, regenerated plants and SDH plants (Table 2). It was found that the rates of embryo formation (% 18 for M2 and % 13 for M3) were higher in both colchicine applications compared to control (% 9.5). The average frequency of formed embryo increased by 89.5% and 36.8% for M2 and M3 medium, respectively, compared to control (M1). Colchicine application also affected the embryos development. Among regenerated embryo (Fig. 1); 15, 26, and 19 embryos were fully developed (Fig. 2) into mature plantlets for M1, M2 and M3 medium respectively and adopted to greenhouse climatic conditions. Plants regenerated on from both M2 and M3 were mostly SDH when compared to plants obtained on from M1, which were mostly haploid. Colchicine treatment significantly increased the SDH rate. The ability of microspores to spontaneously double the number of chromosomes was determined to be 33% in the control medium whereas the SHD ratios were 57.6% and 47.3% for M2 and M3 medium, respectively. The average frequency of SDH plants increased by 17.3 % and 14.1% for M2 and M3 medium respectively, compared to control.

Table 1. Summary of ANOVA for androgenetic parameters.

Source of variation	df	Total embryos		Embryos per 100 anthers		Regenerated plant no	
		Mean square	*F*-value	Mean square	*F*-value	Mean square	*F*-value
Treatment	2	19.750	23.700**	79.000	23.700**	7.750	10.731**
Error	9	0.833		3.333		0.722	
Total	11	4.273		17.091		2.000	

** Significant at alfa level 1%; df= Degrees of freedom

Table 2. Androgenic embryos rate, regenerated embryos and spontaneous double haploid plants from different culture media.

Medium	Planted anthers no	Total embryos no	Embryos per 100 anthers	Regenerated plant no	SDH plant no	Frequency of SDH %
M1(Control)	200	19 b	9.5 b	15 b	5 b	33.3 b
M2	200	36 a	18 a	26 a	15 a	57.6 a
M3	200	23 b	11.5 b	19 ab	9 ab	47.3 ab

Means were separated by Tukey's test at $p \leq 0.01$. Column having different letter(s) are statistically significant

Fig. 1. Developing embryos from cultured anther.

Fig. 2. Fully developed plantlets.

In this study, colchicine was applied to the anther by two different methods. Colchicine-free semi-solid medium (M1) was used as control. In the first experiment, the anthers were incubated in M2 nutrient medium with % 0.3 of colchicine for 3 weeks and transferred to M1. In the second experiment, bilayer nutrient medium (M3) was used and after anther planting on semi-solid M1 medium was performed, 2 ml % 0.3 colchicine solution per Petri dish was added as the liquid phase. The first developing embryo was observed after 4 weeks of culture. Embryos were transferred to semi-solid hormone-free MS medium. All haploid embryos and plants were obtained by regeneration of microspores and germination of embryos without a callus phase. Addition of colchicine to culture medium resulted with positive effects on viability of embryo, regeneration and growth into full developed plantlets.

In the anther culture of Capsicum, the numbers of embryos obtained from different genotypes and their growth to normal plant rates are different. Most of the obtained embryos have problems in their germination and growth into full plantlets. Although many nutrient media composition has been studied in pepper anther culture and successful protocols have been developed, the common disadvantage mentioned in the studies is that the haploid embryo induction, development and conversion to full plantlet rate is low and this rate should be increased (Comlekcioglu *et al*., 2001; Wang & Zhang, 2001; Rodeva *et al*., 2004; Koleva-Gudeva *et al*., 2007; Liu *et al*., 2007; Olszewka, 2014). This is a factor that limited the use and success of anther culture in breeding studies (Irikowa *et al*., 2011).

An effective chromosome doubling method is an important consideration in the production of haploid plants (da Silva Dias, 2003; Yuan *et al*., 2015). Shim *et al*., (2006) reported that the use of antimitotic agents for embryo induction during the first few hours of microspore culture results with high frequency in chromosome doubling. Factors such as antimitotic agent concentration, temperature during the treatment, and duration of treatment are critical for individual species.

Kasha (2005) reported that the ideal time for doubling the chromosome number to obtain a double haploid from haploid plants is when the cell divisions in the gametes at the first pair stage. When chromosome doubling is required, da Silva Dias (2003) suggested that *In vitro* colchicine treatment instead of plant application to increase the frequency of chromosome doubling of haploid plants. The researcher reported that at low concentrations (0.01-0.02%) colchicine only inhibits the cell division cycle for a short time, then cells are able to continue into mitosis when they contain a doubled chromosome set. The optimal time for *In vitro* antimitotic agent application was determined as the first 12 hours after microspore isolation. Similar to results obtained in this study, Supena *et al*., (2006) reported that *In vitro* application of colchicine during the first week of culture increased (almost by two fold) embryos produced and SDH ratio in Capsicum.

Spontaneous doubling of the chromosome number during anther culture occurs without the inductive effect of an anti-mitotic agent (eg. colchicine). When anti-mitotic agents are used to double the number of chromosomes, stimulated double haploids (and plants with higher ploidy levels) are occurs. In this study all diploid plantlets obtained were called SDH. The increase in the spontaneous double haploid ratio in colchicine added culture media was commented to the formation of double haploid stimulated by colchicine.

Yuan *et al*., (2015) found that spontaneous chromosome doubling occurs randomly and depends on the genotype. The spontaneous doubling in the microspores-derived cabbage population ranged from 0 to 76.9% and in the broccoli population from 52.2% to 100%. Especially after 1 or more years in tissue culture, it has been reported that the chromosomes of most haploids are doubled and transformed into spontaneous double haploid or mixed ploidy plants.

Supena *et al*., (2006) reported that the frequency of spontaneous diploidization changed from 14 to 51% depending on genotype. Irikova *et al*., (2011) recorded that the ratio of haploid to spontaneous doubled haploid plants varies from 1:1 to 1:2 in large fruit pepper, and from 3:1 to 2:1 in pepper. The rate 25% of spontaneous diploidization was obtained by Lantos *et al*., (2012) while 31 of 63 (49%) SDH plants were established by Niklas-Nowak *et al*., (2012). Olszewska *et al*., (2014) founded frequencies average 45.6 % haploid plants and 54.3 % SDH plants by average.

Galazka *et al*., (2015) reported that haploidy technique is two-stage method. Haploid embryos or plants obtained in the first stage should be converted into DH by chromosome doubling in the second stage. Therefore chromosome doubling becomes the most important factor.

Haploid plants are smaller, plant viability, *In vitro* regeneration, development and growth is lower compared to diploid donor plants. They are sterile since the chromosomes cannot match during meiosis. To include haploid plants in breeding programs, their fertility must be restored, either spontaneously or by induced chromosome doubling (Murovec & Bohanec, 2012; Aleza *et al*., 2009; Riddle *et al*., 2006). Therefore, chromosome doubling is necessary in order to use newly obtained plants in breeding programs. For this, haploids should be subjected to some anti-mitotic agents with appropriate doses and durations to restore diploid status and restore fertility (Segui-Simarro & Neuz, 2008).

Chromosome doubling can occur spontaneously during culture or can be induced later by the use of anti-mitotic agents *In vivo*. In order to develop fertile homozygous plants, a successful protocol is required for doubling the chromosomes of the haploid plants obtained. Because of *In vivo* colchicine application to haploid plants is costly, difficult and time consuming, spontaneous doubling of chromosomes in anther culture is highly desirable.

Conclusions

Colchicine application to nutrient medium in the early stages of culture results with significantly increase the success of androgenetic response in pepper. The yield of embryos obtained, the number of regenerated plants and SDH plant numbers were increased.

Present study shows that the use of colchicine to improve both haploid and SDH embryos production in pepper anther culture has a significant effect. Although it was not detected in this study, when using colchicine in the nutrient medium, it is possible to obtain plants with DNA content higher than 2n. This results should be useful to plant breeders because *In vitro* spontaneously or stimulated chromosome doubling has advantage to reduce the time and cost of DH production. The occurrence of spontaneous haploids during culture eliminates the need to double the chromosome.

One of the ways to increase the viability of haploid embryos and improve plant development in the anther culture method is the diploidization of embryos at the beginning of culture.

For this reason, in the anther culture technique, which has become a routine method in breeding studies in Capsicum, the possibilities of increasing embryo formation and conversion frequencies to the normal developed plant should be studied intensively.

Acknowledgements

This work was supported by Scientific Research Projects Commission of Eskisehir Osmangazi University. Project No. 2013-191. The author would like to thank the Dikmen Tarım Ürünleri Ltd. Şti. (Bilecik, Turkey) for providing their flow cytometer to carry out the ploidy analysis of regenerated plants.

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(Received for publication 11 May 2019)