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Effects of Biotin and Ascorbic Acid Applications on Haploid Embryo Induction in Semisolid and Double Layer Nutrient Media in Pepper (*Capsicum annuum* L.) Anther Culture

Burcu Demirkaya[1] **Nuray Comlekcioglu**[2,*]

[1]Eskişehir Osmangazi University, Institute of Science, Eskişehir / Turkey

[2]Eskişehir Osmangazi University, Faculty of Agriculture, Department of Horticulture, Eskişehir / Turkey

[*]Corresponding Author: ncomlekcioglu@ogu.edu.tr

Abstract

Generation of homozygous double haploid (DH) lines by androgenesis is a promising alternative to selfpollination programs across generations. Despite the routine use of anther culture in peppers, there are still many bottlenecks and improvements in methodology are required. The aim of this study was to determine the effects of the structure of the nutrient medium (semi-solid and double layer) and the addition of biotin and ascorbic acid to the nutrient media on obtaining haploid embryos by anther culture method. MS (Murashige and Skoog 1962) medium containing 4 mg l^{-1} NAA, 0.1 mg l^{-1} BAP, 0.25% activated charcoal, 30 g l^{-1} sucrose, and 10 mg l^{-1} AgNO3 (silver nitrate) were used as the basal nutrient medium. A total of 8 nutrient media compounds were studied using 0.05 mg l-1 biotin and 0.5 mg l^{-1} ascorbic acid separately or together in semi-solid and bi-layer (double-phase) nutrient media. Solidification of nutrient media was achieved with 7 g l^{-1} agar. The cultured anthers were subjected to high-temperature pre-treatments at 35 °C in continuous dark conditions for 2 days. Then they were taken to a climate chamber at of 25 °C temperature adjusted to 16/8 hour photoperiod. It has been observed that the success of obtaining embryos of semi-solid medium was higher than double-layer medium. The addition of biotin and ascorbic acid to the nutrient medium provided 8.8 fold increases in embryo regeneration compared to the control medium. In the presence of only one of biotin or ascorbic acid in the nutrient medium, the number of embryos increased compared to the control.

Keywords: Androgenesis, Vitamin, Breeding, Pure line

Introduction

Androgenesis (anther or isolated microspore culture), which is the formation of sporophytes from immature male gamete cells in vitro, is the most widely used method for the production of double haploid (DH) lines in vegetable breeding. Fully homozygous lines are produced in a single generation, thus avoiding the large number of selfing cycles required in traditional breeding programs by this method. Studies on anther culture in peppers have shown that embryo induction by androgenesis and the success achieved are under influence of so many factors. Some of these factors are related to the composition and structure of the nutrient medium, growth regulators, genotype and growing conditions of the donor plant, stress pre-treatments, incubation conditions of anthers, microspore development stage, and the conditions during the application of the culture technique (Irikova et al., 2011, Çömlekçioğlu & Ellialtıoğlu, 2018).

Capsicum is considered as one of the recalcitrant species for androgenesis (Heidari-Zefreh et al. 2019, Pinar et al. 2020).Anther culture has been practiced for a long time in

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Orcid: Burcu Demirkaya: 0000-0002-3390-4669 and Nuray Çömlekçioğlu: 0000-0001-7189-613X
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crops including Capsicum genus, nevertheless, many factors are limiting the frequency of embryogenesis and regeneration in Capsicum. Overcoming the factor limiting success in androgenesis requires extensive studies and new strategies focusing on optimizing in vitro factors and improving current protocols.

The stress treatments to anthers cultures are an essential factor in androgenic response. It has been shown that stress pre-treatments applied to anthers and microspores, inducing the sporophytic pathway, inhibit the gametophytic pathway (fertile pollen development), and increase embryogenic potential (Sanchez et al. 2020). Several pre-treatments protocols have been reported to induce microspore embryogenesis and plant regeneration in different varieties. Pretreatments of anther culture result in an increased androgenic response (Irikova et al., 2011, Perez-Perez 2019, Sanchez et al. 2020). Although stress pre-treatments are necessary to initiate the sporophytic development of microspores, these pre-treatments could also cause the cells to lose their viability.

This study was conducted to investigate the effects of the structure of the nutrient medium (semi-solid and double layer) and the addition of vitamin 7 (biotin) and vitamin C (ascorbic acid) to the nutrient medium on androgenic embryogenesis of pepper.

Material and Method

This research was carried out in the tissue culture laboratory of the Department of Horticulture, Faculty of Agriculture, Eskisehir Osmangazi University (Eskisehir, Turkey). In this study, Diyar F1 capia pepper variety was used as a donor. Donor plant seedlings were planted in 15-liter pots containing peat and grown in an unheated polycarbon greenhouse.

Flower buds were harvested early morning when the corolla and calyx were the same sizes or when the corolla was slightly above the calyx. These buds have anthocyanin production up to 1/3 of the anthers and the anthers mostly contain late uninucleate, mid uninucleate and young binucleate stage microspores (Çömlekçioğlu and Ellialtıoglu 2018).

Sterilization of flower buds; the flower buds were rinsed first with water and then in 70% ethyl alcohol. Then it was kept in 10% commercial bleach (containing 10% sodium hypochlorite) and one drop of Tween-20 solution for 15 minutes. Rinsed 3-4 times with sterile distilled water.

Nutrient media; four semi-solid and four double-layer nutrient media combinations were formed by addition biotin and ascorbic acid together or separately. Table 3.1 shows the nutrient media combinations studied.

Vitamins including MS (Murashige and Skoog 1962) nutrient medium containing 4 mg l^{-1} NAA, 0.1 mg l^{-1} BAP, 0.25% activated charcoal, 30 g l^{-1} sucrose and 10 mg l-1 $AgNO_3$ was used as control treatments. In the experiments, by using 0.05 mg l^{-1} biotin and 0.5 mg l^{-1} ascorbic acid separately and together, semi-solid and bilayer media were created and a total of 8 nutrient media compounds were studied. Both the solid and the liquid layer of the bilayer nutrient medium contained the same components. Solidification was achieved with 7 g of l^{-1} agar for the semi-solid medium and the solid phase of the double-layer medium. The sterilization of the media was carried out by keeping in an autoclave at 121 °C for 15 minutes under 1.2 atm pressure. Obtained embryos were transferred to hormone-free semisolid MS media.

The filaments were carefully cut with the help of a scalpel and forceps inside a laminar flow cabinet, and the anthers removed from the buds were placed on the nutrient medium as the dorsal surface in contact with the medium without immersion in it.

The sterile Petri dish (7 cm) was used for semi-solid media and a 100 ml glass jar was used for double-layer media. Ten anthers obtained from 2 buds were planted in each petri dish (or glass jar). The edges of the petri dishes, which have been planted, were covered with parafilm.

Incubation; anthers planted on media were subjected to continuous dark conditions and high-temperature application at 35 ºC for 2 days. Then the cultures were taken to a climate chamber adjusted to 25 ºC temperature and 16/8 hour photoperiodic arrangement with the light intensity of approximately 1000 lux.

Experiment design and statistical analyses; the study was carried out with 4 replications and 5 Petri dishes (or glass jar) per repetition. Ten anthers obtained from two flower buds were planted in each dish (200 anthers were used for each medium). Experiments were arranged in a completely randomized design. The data were subjected to analysis of variance (ANOVA) using the TARIST (Açıkgöz et al., 2004) statistical software The least significant difference (LSD) test was used to separate of differences in means.

Table 1. Nutrient media studied and structure and their contents.

Medium		Content
Semi solid	M 1 (CONTROL)	MS + 4 mg l^{-1} NAA, 0.1 mg l^{-1} BAP, % 0.25 Activated charcoal, 30 g l^{-1} Sucrose, 10 mg l^{-1} $AgNO_3$, 7 g l^{-1} Agar, pH 5.6-5.8
	M2	M1 + 0.05 mg l^{-1} Biotin + 0.5 mg l^{-1} Ascorbic Acid
	M3	M1 + 0.05 mg l^{-1} Biotin
	M4	M1 + 0.5 mg l^{-1} Ascorbic Acid
Double layer	M5	M1
	M6	M2
	M7	M3
	M8	M4

Results and Discussion

The effect of ascorbic acid and biotin in the culture medium to optimize the production of anther-derived haploid embryos of the pepper was investigated in this experiment. The total number of embryos obtained, the number of embryos per bud and per 100 anther (Table 2) showed significant differences ($P \leq 0.01$) according to the nutrient media and contents.

A total of 9 embryos were obtained from the M1 medium that was studied as a control in this experiment. On the other hand, the highest number of embryos (80 embryos) developed from M2 medium containing both 0.05 mg l^{-1} biotin and 0.5 mg l^{-1} ascorbic acid. In the presence of only one of biotin or ascorbic acid in the nutrient medium, an increase in embryo formation was achieved compared to the control medium. This increase was found higher in the presence of only biotin (M3) than in the presence of ascorbic acid (M4). 13 and 11 embryos developed from semi-solid M3 and M4 media, respectively. Biotin and ascorbic acid added to MS media significantly affected embryogenesis positively. In this study, semi-solid media were generally more successful than double-layer media.

Biotin containing M7 (11 embryos) was found to be more successful than others double-layer media. Embryo regeneration could not be achieved in M5 medium. A total of 125 embryos from semi-solid media and a total of 31 embryos from double-layer media regenerated. The number of embryos obtained from semi-solid media increased by 403% (fourfold) compared to double-layer media.

Table 2. Total Number of Obtained Embryos, Embryos per Bud and Embryos per 100 Anther Obtained by the Media.

Media		Total Embryo Number	Embryo Number per Flower Bud	Embryo Number per 100 Anthers
Semi-solid	M1	9.0 bc	0.9 bc	3.6 bc
	M2	80.0 a	8.0 a	32.0 a
	M3	13.0 b	1.3 b	5.2 b
	M4	11.0 b	1.1 b	4.4 bc
Double-layer	M5	0.0 c	0.0 c	0.0 c
	M6	5.0 bc	0.5 bc	2.0bc
	M7	11.0 b	1.3 b	4.4 bc
	M8	5.0 bc	0.5 bc	2.0 bc
	LSD $P \leq 0.01$	1.88	0.94	3.76

The column having a different letter(s) are statistically significant

Between 0.0 and 8.0 embryos per flower bud were obtained. It was determined that the highest performance was obtained from M2 medium with 8.0 embryo number per flower bud.

The embryos obtained from the media studied were also calculated as the number of embryos per 100 anthers. The highest embryo ratio was obtained from the M2 with 32 embryos/100 anthers. The addition of biotin and ascorbic acid to semi-solid media provided approximately 8.8 fold higher number of embryos compared to the control (M1) medium.

In the presence of only biotin or only ascorbic acid in the nutrient medium, the number of embryos increased by 4.4 and 2.2 fold, respectively, compared to the control. In double layer M6 and M8 media, 0.55 decrease was observed in the rate of embryos obtained compared to semi-solid M1 media. The embryo could not be obtained from dual-phase medium (M5) without biotin and ascorbic acid. The highest rate of embryos was obtained in nutrient medium (M7) containing ascorbic acid in double-layer media.

Even if all the necessary conditions of the plant are provided in anther culture, the structure of the nutrient medium content is very important in reaching the result. The semi-solid medium was found to be more successful in terms of embryo induction than the dual phase medium.

In this study, the addition of biotin and ascorbic acid to the nutrient medium together created synergy and improved embryo productivity. Vitamins, known for their antioxidative properties, increased haploid embryo development. It has been determined that antioxidants such as biotin and ascorbic acid are necessary in anther culture to improve the haploid embryogenesis of pepper. A higher embryo was obtained from the medium to which biotin was added (M3) compared to the medium to which only ascorbic acid (M4) was added. In this case, a more positive effect of biotin on obtaining embryos in anther culture compared to ascorbic acid was determined.

Androgenic embryogenesis is based on the ability of microspores, after exposure to various stresses, to differentiate and deviate from normal pollen development into sporophytic development and transform into a whole plant.

Microspores can be encouraged to deviate from their gametic development and transition to embryogenesis by in vitro specific stress applications (Shariatpanahi 2006, Hosp et al.2007, Seguí-Simarro and Nuez 2008; Munoz-Amatriain 2009, Rodriguez-Serrano et al.2012, Ahmadi and Ebrahimzadeh, 2020, Perez-Perez 2019).

Certain stresses such as low or high temperature applications, osmotic stress and mutagenic substances, carbohydrate or nitrogen starvation, drought stress, gamma irradiation, oxidative stress, low atmospheric pressure have been reported

to play a vital role in microspores reprogramming microspores in many plant species (Irikova et al., 2011, Shahinul Islam 2012, Sanchez et al.2020).

Exposing the anthers in the culture to high temperature pre-treatment (35 °C) is the most commonly used stress condition in pepper. Cheng et al. (2020) reported that stress is considered to be the inducer for microspore embryogenesis, heat stress is indispensable in pepper androgenesis and green plants could not be obtained without heat pretreatment.

However, the applied stress conditions are important factors that negatively affect the viability of microspores in the early stages of culture. The sudden increase in the levels of intracellular reactive oxygen species (ROS) of microspores exposed to a high-temperature pre-treatment causes the death of the microspores (Zur et al. 2009, Gill and Tuteja, 2010, Varnier et al., 2009).

While high ROS level causes a decrease in microspore viability, it also affects the metabolism in many microspore cells that survive, triggers the transition to sporophytic development, and changes microspore development (Shariatpanahi et al. 2006, Zur et al 2009, Ceyhan and Aktaş, 2020).

Reactive oxygen species are natural byproducts of cellular metabolism but can be harmful to the cell if not detoxified. However, vitamins can activate defense reactions against oxidative stress (Zur et al. 2009).

Vitamins are considered important components that induce plant cell growth, and their role as an antioxidant has also been reported (El sharabasy 2019). With the addition of ascorbic acid, the removal of reactive oxygen species is achieved to a great extent (Becker et al. 2014).

Plant cells grown in vitro can synthesize essential vitamins in insufficient quantities. For this reason, culture media are often supplemented with vitamins to increase growth. Therefore, endogenous or exogenous antioxidants are thought to increase the efficiency of embryogenesis by increasing the viability of microspores. As ascorbic acid is an antioxidant, it provides tolerance to osmotic and oxidative stress in plants. Ascorbic acid reduces oxidative stresses in the plant and ensures the survival of microspores. This situation has a positive effect on the increase of embryos obtained. It has been reported that ascorbic acid increases embryogenesis by increasing microspore viability, whether it is found in the plant endogenously or when applied exogenously (Habibi et al. 2009, Hoseine et al., 2013, Zeng et al., 2015).

One of the most important functions of B vitamins is that they play a role as a cofactor in enzyme-catalyzed reactions and contribute to many metabolic activities in plants. Vitamin B tolerates osmotic and oxidative stresses in plants (Roje, 2007).

Considering the importance of biotin in plants, a significant increase in the rate of embryos was detected In this study by the addition of biotin to nutrient media compared to the control group, and it was determined that biotin had a positive effect.

Al-Khayri (2001) found that the effects of biotin and thiamine concentrations on callus growth and somatic embryogenesis vary according to species and doses and have a positive effect on embryogenesis. Ozsan and Onus (2017) reported that the best androgenic results were obtained from media containing biotin and folic acid among MS media which different types of B vitamins (biotin, folic acid, and cobalamin) were added.

DH methods have great importance in plant breeding and agriculture. Breeding new varieties is an activity that requires a long time, but also requires high labor and cost. Increasing embryo productivity in anther culture can reduce the duration and cost of plant breeding and increase the use of DH technology in breeding programs.

As a result, it has been determined that it is important to increase the tolerance of microspore cells against oxidative stress caused by the stress pre-applications applied to anthers, and adding vitamins to the culture medium due to their anti-oxidant properties is a factor that increases the success. The necessity of using which vitamin at which dose against different stress pre-applications with different genotypes should be determined experimentally and further studies should be conducted.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

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