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Effects of Different Nutrient Media on Embryo Induction and Plant Regeneration in Cucumber (*Cucumis sativus* L.) Ovary Cultures

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ABSTRACT

Haploids and doubled haploids play a crucial role in the process of plant breeding. Ovary culture has proven to be an effective method for generating double haploid (DH) plants in cucumbers. The objective of this study was to optimize culture media conditions for gynogenesis induction *in vitro* to broaden morphogenetic possibilities and accelerate the creation of homozygous lines. The effects of seven different induction culture media on embryo and plant development in the ovary culture of Beith Alpha-type and Silor-type cucumber (*Cucumis sativus* L.) were investigated. Ovary culture experiments were conducted during the fall and spring seasons with 26 Beith Alpha-type and 11 Silor-type cucumber genotypes at the F_2 and F_3 generations. The results revealed that the most successful outcomes were obtained from genotypes coded HTB20, HTB24 and HTB25 of the Beith Alpha-type and HTS4 and HTS5 of the Silor-type. The findings also demonstrated that explants cultured in the autumn season showed higher regeneration efficiency compared to those cultured in spring. Overall when compared to Silor types, Beith Alpha types exhibited a more favorable response in terms of plant regeneration and embryo development, depending on genotype.

Keywords: Gynogenesis, medium compositions, haploid, Beith Alpha, Silor-Type

Introduction

Cucumber (*Cucumis sativus* var. *sativus* L.) is an important species with significant economic value within the Cucurbitaceae family. Of the 97 million tons of cucumber produced annually in the world, 80 million tons belong to China. The second country that produces the most cucumber is Türkiye (1.87 million tons). Russia comes in 3rd with a very close value (FAO, 2023). In Türkiye, cucumber is the most produced vegetable after tomatoes and watermelons (Anonymous, 2024). Originating from India, cucumber is among the Asian/Australian group species in the *Cucumis* genus (Renner et al., 2007; Renner and Schaffer 2008). Cucumber is one of the oldest cultivated vegetables. It is thought that cucumber, which completed its evolution process in India, passed from the Himalayas to China from the north, or was carried to China and Europe via the "Silk Road" (Staub et al., 2008). Cucumber was brought to Syria and Anatolia via Iran in the 6th-7th centuries (Paris et al., 2012). Günay (2005) reports that the famous Çengelköy, Langa and Maltepe local cucumber varieties were grown in vegetable gardens in Istanbul from the 1500s until the 1960s. In addition to these monoic varieties, many landraces specific to different regions such as Kilis (Tombul) and Dere have been grown all over Anatolia until recently (Çağlar and Şensoy 2021). Anatolia has a diversity of cucumber genetic resources. However,

improved quality OP varieties are almost nonexistent and old. Hybrid cucumber production has become increasingly widespread globally due to its potential for higher yields, improved disease resistance, and better marketability. The hybrid cucumber market is projected to continue expanding, as growers seek varieties that offer both enhanced productivity and resilience against environmental stresses. Crop productivity can be significantly improved by creating hybrids through the crossing of pure lines with specific characteristics. Consequently, pure lines are crucial for plant breeding programs. However, producing 100% homozygous lines through traditional breeding methods demands considerable time and resources due to multiple cycles of self-pollination and selection. On the other hand, biotechnological alternatives are considerably more efficient and sustainable compared to conventional approaches.

For cucurbits, there are three primary methods frequently employed in modern breeding programs to generate haploids and homozygous doubled haploids (DHLs): in situ haploid parthenogenesis (mainly induced by pollination with irradiated pollen), *in vitro* gynogenesis (occurring during *in vitro* ovule or ovary culture), and *in vitro* androgenesis (taking place during *in vitro* anther or microspore culture) (Dong et al., 2016). Among them, to date, *in vitro* unfertilized ovule/ovary culture remains the most successful and widely used method for haploid induction in cucumber (Gémes-Juhász et al., 2005).

The process of haploid regeneration through unpollinated female gametophytes is commonly referred to as "gynogenesis." Chambonnet and Dumas de Vaulx (1985) were the first to successfully obtain haploid embryos and plants from squash via *in vitro* unfertilized ovule/ovary culture. Since then, gynogenesis is a viable method for haploid production in various cucurbits, including pumpkin (Kwack and Fujieda 1988; Sun et al., 2009; Min et al., 2016), squash (Metwally et al., 1998b), melon (Beharav and Cohen 1995; Ficcadenti et al., 1999), cucumber (Gémesné et al., 1997; Gémes-Juhász et al., 2002; Moqbeli et al., 2013; Özsan et al., 2017; Pınar et al., 2021), and others (Rakha et al., 2012; Dong et al., 2016). Numerous studies have demonstrated that the successful recovery of haploids and doubled haploids in ovule/ovary culture is influenced by several factors, such as the genotype of donor plants, temperature pre-treatment, the developmental stages of the female gametophyte, growth regulators, and other components of the culture media. Specific efforts have been made to optimize these conditions for cucumber (Suprunova and Shmykova 2008; Diao et al., 2009; Li et al., 2013; Moqbeli et al., 2013; Plapung et al., 2014; Tantasawa et al., 2015; Baktemur et al., 2022) and squash (Xie et al., 2006; Shalaby 2007), aiming to maximize the number of haploid plantlets.

This study aimed to investigate the impact of various factors influencing *in vitro* gynogenesis, including the genotypes of donor plants and the induction media, on embryo and plant formation in the unpollinated ovary culture of 26 Beith Alpha-type and 11 Silor-type of cucumber genotypes.

Materials and Methods

Plant Material

26 Beith Alpha-type and 11 Silor-type cucumber genotypes, at the F_2 and F_3 generations, were used as donor plants. The plants were cultivated in greenhouses at Antalya Technopark, Hektaş Seed Co., and maintained using conventional horticultural practices. The study was conducted in three consecutive growing seasons, the spring and autumn seasons of 2023 and the spring season of 2024, each genotype was represented by 40 plants (Table 1). Fertilization included MAP (mono-ammonium phosphate) at a rate of 750 g/da applied throughout the ovary collection period. Irrigation was performed every 4 days via drip system, delivering 750 cc per plant per irrigation event. No pest control treatments were applied in order to preserve embryo quality from donor plants.

In the study, female flowers were harvested from donor plants for about two to three weeks after the emergence of the first female flowers. Unpollinated ovaries were collected early in the morning, six hours before anthesis as described by Gémes-Juhász et al., (2002). Petals, sepals, and styles were removed, and the ovaries were surface sterilized in 70% ethanol solution for 1 min and then soaked in 20% commercial bleach solution supplemented with 1-2 drops Tween20 for 15 min, and finally rinsed with sterile dH_2O three times for 5 min. The peels of the sterilized ovaries were removed and they were cut lengthwise into two halves (Nyirahabimana and Solmaz 2021), which were then placed into seven different culture media. Each ovary explant was placed in a single petri dish with two ovary slices (Table 1).

Unpollinated Ovary Media and Culture Conditions

The induction media used in this study were based on Murashige and Skoog (1962) (MS) medium, with 30 g/L sucrose and 7 g/L agar added to each (Diao et al., 2009). The media were coded from C1 to C7 and consisted of different plant growth regulators as follows: C1: 0,1 mg/L 2,4-D and 1 mg/L kinetin; C2: 0.04 mg/L TDZ, 0.05 mg/L NAA, and 0.2 mg/L BAP;

C3: 0,1 mg/L 2,4-D, 1 mg/L kinetin, 0,05 mg/L NAA and 0.2 mg/L BAP; C4: 0.04 mg/L TDZ; C5: 0,1 mg/L 2,4-D, 1 mg/L kinetin and 0.02 mg/L TDZ; C6: 0.05 mg/L 2,4-D and kinetin, with 0.02 mg/L TDZ; C7: 0.7 mg/L 2,4-D, 1 mg/L kinetin, 0.5 mg/L NAA, 1 mg/L BAP, and 10 mg/L $AgNO_3$.

Ovary pieces were incubated in the dark at 35°C for 2 days, followed by 3 more days at 25°C in the dark. After the induction period, they were transferred to growth chambers with a 16/8 light/dark photoperiod. Cultures were visually monitored daily, and when embryo-like structures first appeared -typically between 2 and 3 weeks- they were transferred to regeneration medium (coded as R), which contained an MS-based medium with 30 g/L sucrose, 0.05 mg/L NAA, and 0.2 mg/L BAP (Gémes-Juhász et al., 2002). For rooting, the plants were rooted in a hormone-free MS medium. For plants that did not root, they were transferred to MS medium containing 0.1 mg/L IAA. Plants showing sufficient root development were then acclimatized to external conditions in pots containing a 1:1:1 mixture of peat:perlite:vermiculite, and their cultivation continued in the greenhouse. The pH of the C, R and rooting medium was adjusted to 5.8 and autoclaved at 121°C for 20 min. and then poured on sterile plastic petri dishes (60 mm in diameter), tubes or jars. The hormones were filter-sterilized and added to the media after autoclaving, once the temperature of the medium had dropped to 40-50°C.

Statistical Analysis

The experiment began in the spring season of 2023 with 11 Beith Alpha-type and 6 Silor-type F_2 cucumber genotypes, using seven different induction media. The results were analyzed, and three prominent C media were selected to establish the experiments for the autumn season of 2023, where 11 Beith Alpha-type and 7 Silor-type genotypes were used. Finally, in the spring season of 2024, only the C1 medium was used for the culture of 14 Beith Alpha-type genotypes at the F_3 stage. After two weeks of culture, embryo observations were carried out for another ten weeks. The plants that developed were then rooted and acclimatized to greenhouse conditions. The culture initiation procedures were carried out over three weeks. A total of 2665 Beith Alpha-type and 863 Silor-type cucumber genotypes' ovaries were cultured. The data on the number of developing embryos and plant regeneration were recorded at the end of 12 weeks following the initiation of the culture process. The embryo development frequency, expressed as the percentage of embryos per ovary, and the plant conversion frequency, defined as the percentage of embryos successfully developing into plants, were calculated.

One-way analysis of variance (ANOVA) was performed to evaluate differences between applications and genotypes. The general linear model procedure of SPSS (Statistics 20) software (IBM Corp., Armonk, NY, USA) was used for data analysis. All main effects were considered fixed effects. Tukey's multiple range post hoc test was employed for multiple comparisons of the genotypes and media and t-test for seasons with an alpha level of 0.05.

Results and Discussion

In the study, embryo structures (ES) began to appear on the surface of ovules approximately 2 to 3 weeks after culture initiation. The experiments were completed in approximately 16 weeks, including the acclimatization of the plantlets. (Figure 1).

The studies carried out in the spring of 2023 with 11 Beith Alpha-type and 6 Silor-type genotypes across seven different induction media showed no statistically significant effect of either genotype or medium on embryo development frequency ($P<0.05$). However, based on observations and mean values of embryo development frequencies, the responses of the C1, C4, and C6 media were found to be more successful compared to the other media. The data from the first phase of the study (2023 Spring), among the 17 genotypes, C1, C4, and C6 induction media proved to be effective in promoting embryo development, with C1 medium standing out as the most promising (Table 2). In contrast, the genotypes did not respond to the C7 medium, which contained $AgNO_3$. Moreover, in this medium, the ovary explants exhibited the development of high-mass callus formations (data not shown).

In the 2023 fall season, the genotypes of the donor plants and the induction media had significant effects on the formation of ES and plant development ($P<0.05$) (Table 3). Differences in embryo structure and plant development were observed depending on the induction medium used. Although no significant differences were observed among the genotypes, an analysis of the mean values indicated that the Beith Alpha-type genotypes HTB20, HTB24 and HTB25 and Silor-type genotypes HTS4 and HTS5 exhibited the highest embryo development frequency. According to the calculations of embryo percentage per ovary, these genotypes achieved approximately 167%, 118%, and 125% success in Beith Alpha-type and 18% and 14% in Silor-types, respectively. The influence of these factors on embryo formation and subsequent plant growth highlights the importance of genotype and media selection in the successful induction of gynogenesis in cucumbers. Domblides et al., (2020) reported that unpollinated ovule culture can reliably induce

doubled haploid plants in Cucurbitaceae, highlighting that similar protocols can effectively shorten breeding cycles in cucumber species-our findings also support the efficacy of ovary culture for embryo induction, even if ploidy states were not assessed. When evaluating the frequency of healthy embryo-to-plant conversion, the C4 induction medium exhibited significantly higher success in Beith Alpha-type genotypes, while the conversion frequency was found to be the lowest in the C1 medium (Table 3). Given the high incidence of abnormal embryo development in cucumbers (Li et al., 2013), this result is particularly meaningful. Similarly, Özsan et al., (2017) reported abnormal embryo development in a comparable induction medium in their study. However, other factors such as genotype and pre-treatments should also be considered when assessing embryo-to-plant conversion frequencies.

These findings are consistent with the results reported by Diao et al., (2009) and Moghbeli et al., (2013) in cucumber, Dumas and Chambonnet (1986) and Shalaby (2007) in squash. A recent study evaluated genotype-specific responses and heat shock pretreatment for induction of embryo-like structures, reporting rates of 16-20% across commercial cucumber varieties (Nyirahabimana and Solmaz, 2024). This aligns with our observed high induction frequencies in selected genotypes under C1, C4, and C6 media, reinforcing the importance of both media composition and pre-treatment regimes. These studies have demonstrated the effects of genotype and medium composition on embryogenesis.

This study also investigated the effect of the spring and fall seasons on embryo development. Przyborowski and Nlemirowicz-Szgzytt (1994) reported a significant impact of the growing season on haploid embryo development in cucumbers in their study, where they examined various parametric factors influencing embryo formation. In the present study, based on the embryo development frequencies and embryo-to-plant conversion frequencies of the cultivars subjected to ovary culture in both seasons (Table 4), a statistically significant difference was observed between the two seasons ($P<0.05$).

In the case of *Cucurbita pepo*, the highest number of embryos was obtained from ovaries harvested one day before anthesis (Dumas de Vaulx and Chambonnet, 1986; Metwally et al., 1998a), whereas in cucumber, the maximum number of embryos was observed in ovaries collected 6 hours before to anthesis (Gémes Juhász et al., 2002). Although the timing of donor plant collection in the study was determined based on these results, the effects of ovaries collected at different time intervals before anthesis could be further explored.

As reported by Li et al., (2013), gynogenesis was triggered using unpollinated ovules from Chinese long cucumbers, with the most effective induction treatment involving CBM medium containing 0.03 - 0.07 mg dm^{-3} TDZ. In contrast, in this study, the best hormonal combination for induction was obtained from an MS-based medium containing 2,4-D and kinetin. Çetinkaya (2015) also found these PGR concentration rates and types to be successful for the induction of gynogenesis in cucumber. Nevertheless, the C4 induction medium containing TDZ has yielded successful results in embryo development compared to some other media. To enhance the efficiency of these results, further studies could be conducted on the ovarian developmental stage and pretreatment conditions.

Conclusions

Haploids can be obtained using the ovary as well as other sexual explants. The current study reveals various factors for efficient ovary embryogenesis induction in cucumbers using different culture media. Our findings demonstrated that the rate of embryo production induction varied among genotypes and culture media. Future recommendations include the use of SSR markers, flow cytometry, and other methods to detect spontaneously regenerated plants and integrate them directly into breeding programs. Additionally, identifying the response of genetic resources in the existing gene pool to ovary culture and conducting more productive studies with varieties from this pool will provide valuable insights. Optimization of genotypes and culture media plays a significant role in hybrid production and enhancing genetic diversity. In this context, further detailed studies on additional genotypes and culture conditions will contribute to improving cucumber productivity. Future research is expected to advance with comprehensive genetic analyses and applied techniques, building upon the findings of this study.

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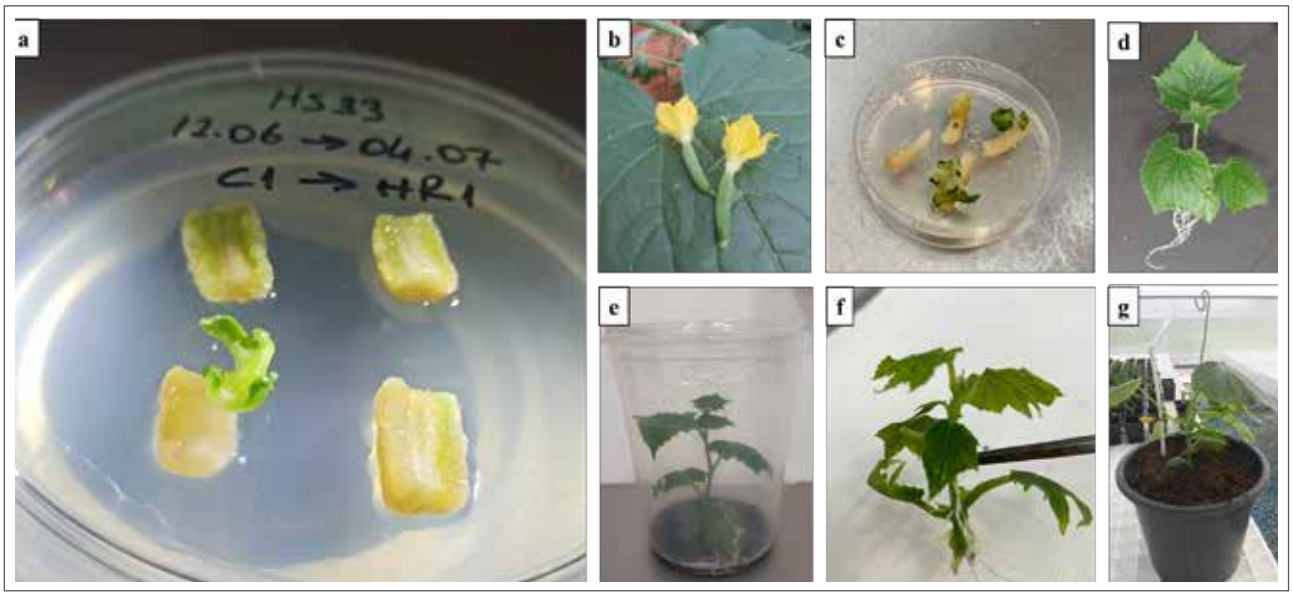

Figure 1. Stages of ovary culture in cucumber. (a) 3 weeks old ovaries in C1 media; (b) flowers collected 6 hours before anthesis; (c) embryos derived on ovaries; (d-e-f) a rooted plant; (g) an acclimatized plant (Original).

Table 1. The number of donor plants grown per genotype and the number of explants cultured in each induction medium.

Type of Cucumber	Genotype Code	Number of Donor Plants	Number of Explants Used						
			Medium Code						
			C1	C2	C3	C4	C5	C6	C7
Beith Alpha-type	HTB1	40	24	23	17	28	20	25	20
	HTB2	40	19	7	5	13	15	27	34
	HTB3	40	25	14	14	18	36	16	32
	HTB4	40	13	17	18	19	34	17	19
	HTB5	40	11	8	13	13	14	15	18
	HTB6	40	12	11	13	2	20	19	19
	HTB7	40	9	10	1	7	6	2	19
	HTB8	40	15	13	1	5	6	6	5
	HTB9	40	12	5	4	2	4	3	7
	HTB10	40	21	15	2	36	6	17	17
	HTB11	40	6	20	15	19	15	21	20
	HTB12	40	15	0	0	2	0	20	0
	HTB13	40	32	0	0	7	0	27	0
	HTB14	40	13	0	0	9	0	24	0
	HTB15	40	49	0	0	30	0	9	0
	HTB16	40	26	0	0	18	0	6	0
	HTB17	40	75	0	0	11	0	3	0
	HTB18	40	83	0	0	7	0	14	0
	HTB19	40	17	0	0	3	0	19	0
	HTB20	40	34	0	0	3	0	14	0
	HTB21	40	47	0	0	17	0	9	0
	HTB22	40	14	0	0	8	0	19	0
	HTB23	40	182	0	0	40	0	4	0
	HTB24	40	308	0	0	24	0	0	0
	HTB25	40	160	2	2	15	10	1	1
	HTB26	40	86	0	0	0	0	5	0
Silor-type	HTS1	40	31	6	5	12	23	6	12
	HTS2	40	14	5	20	6	17	0	14
	HTS3	40	9	13	8	2	0	0	3

Continuing Table 1

Type of Cucumber	Genotype Code	Number of Donor Plants	Number of Explants Used						
			Medium Code						
			C1	C2	C3	C4	C5	C6	C7
Silor-type	HTS4	40	13	10	20	67	25	40	28
	HTS5	40	33	0	23	32	18	1	5
	HTS6	40	22	10	12	29	19	20	12
	HTS7	40	28	0	0	14	0	7	0
	HTS8	40	17	0	0	37	0	5	0
	HTS9	40	5	0	0	35	0	6	0
	HTS10	40	11	0	0	7	0	5	0
	HTS11	40	13	0	0	35	0	5	0

Table 2. Mean values of seven induction media on the frequency of embryo structure (ES) formation and frequency of embryo to plant conversion on the 2023-Spring season.

Induction Media	Frequency of Embryo Structure Formation (%)	Frequency of Embryo to Plant Conversion (%)
C1	7.72±11.67	87.85±23.06
C2	2.40±5.26	100±0
C3	0.85±2.40	100±0
C4	7.51±16.52	88.89±14.59
C5	4.72±8.51	77.08±25.51
C6	6.62±18.70	79.05±21.44
C7	0±0	ND*

* represents “not detected”

Table 3. Effects of different induction media on the frequency of embryo structure (ES) formation and embryo-to-plant conversion on the 2023-Fall season.

Induction Media	Frequency of Embryo Structure Formation (%)		Frequency of Embryo to Plant Conversion (%)	
	Beith Alpha-type	Silor-type	Beith Alpha-type	Silor-type
C1	156.10±176.78a	19.40±24.87a	42.61±32.22b	97.5±5b
C4	11.93±27.23b	6.46±10.25b	95.83±7.22a	100±0a
C6	21.14±30.82b	13.33±32.66b	85.83±14.90ab	75±0ab

*Values are mean and standard error (SE). Means were separated by using Tukey’s multiple range post hoc test. Different letters near the means represent significant differences at $p \leq 0.05$.

Table 4. Embryo development and embryo-to-plant conversion frequencies of selected Beith Alpha-type genotypes across different seasons. All data was taken after 12 weeks of culture.

Genotype	Frequency of Embryo Formation (%)		Frequency of Embryo to Plant Conversion (%)	
	Spring	Fall	Spring	Fall
HTB13	10.00	266.67	0	34.38
HTB14	0	0	ND*	ND
HTB16	5.26	0	100	ND
HTB17	31.88	66.67	86.36	75.00
HTB20	15.38	475.00	100.00	31.58
HTB23	33.73	12.50	60.71	100.00
HTB24	53.56	268.29	60.84	14.55
HTB25	15.17	377.78	54.55	23.53

*ND: Not Detected

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