scientific reports

Check for updates

OPEN

Effect of medium composition on in vitro ovary culture of cucumber

Fildaus Nyirahabimana[1,3]✉, Mansur Hakan Erol[2], Yıldız Aka Kaçar[1,3] & İlknur Solmaz[1,3]

Ovary culture is among the most effective techniques for haploid induction in horticultural crops, including cucumber. The application of doubled haploid technology can significantly accelerate cultivar development by enabling the rapid production of homozygous inbred lines. This study evaluated the effects of culture medium combinations of plant growth hormones (2,4-D, NAA, IAA, IBA, PUT, BAP, KIN, TDZ) on haploid induction in cucumber via ovary culture. Four induction and five regeneration media were tested across four cucumber varieties: Beith Alpha, PTK40, Botanik, and Sardes. The highest embryo formation rate (88%) was observed in the Botanik variety, followed by Sardes, PTK40, and Beith Alpha, particularly on induction medium supplemented with 2,4-D and PUT. Plant regeneration was achieved on a medium containing IAA and BAP. Flow cytometric analysis of twenty randomly selected plants confirmed that all were diploid, with no haploids detected. This outcome indicates that the primary objective of inducing haploid plants was not successfully accomplished. These findings highlight substantial limitations in the current induction protocol and underscore the need for further optimization to improve haploid recovery efficiency. The authors recommend more research to develop and improve ploidy level stability for cucumber ovary culture.

Keywords Cucumber, Cytokinins, Double haploid, Gynogenesis, Tissue culture

Recently, haploids (HP) and doubled haploids (DH) have gained considerable importance in plant breeding programs[1]. A haploid is a plant with the gametophytic chromosome number (n), whereas doubled haploids (2n) are haploid plants in which chromosomes have been duplicated through various techniques, including the use of chemical agents such as colchicine. The production of haploid and DH plants represents a particularly valuable biotechnological approach, and advancements in haploidy technology have greatly contributed to the development of pure homozygous lines[2]. Cucumber (*Cucumis sativus* L.) is a member of the *Cucurbitaceae* family. It is a diploid plant of 2n = 14 chromosomes in the *Cucumis* genus. Generally, the *Cucurbitaceae* family consists of about 1000 species in around 100 genera that include various economically important vegetables and popular fruits in daily human nutrition[3,4]. The basis of vegetable breeding is the production of inbred pure lines. Due to the traditional breeding of the vegetable species that belong to the cucurbit plants, breeding studies take a very long time[5]. Gynogenesis is a pivotal approach used to produce haploid and DHs as homozygous plants[1,6]. Numerous factors have been reported to influence ovule and ovary culture in haploid plant production studies of cucumber, as well as donor plant genotype, growth regulators, temperature treatments, developmental stage of the female gametophyte, and various environmental and culture conditions[7–10].

Furthermore, studies on haploid embryo and in vitro plant production through gynogenesis in cucumber have intensified currently[10–21]. The effects of growth retardants in different media have been evaluated in cucumber through micropropagation of nodal explants to develop new varieties[22]. It is caused by the number of simultaneous stress reactions that occurred from abnormal environmental conditions imposed in vitro culture[23]. Various explants, as well as ovaries and anthers and different culture conditions, such as temperature pretreatment (cold: 4 °C, heat: 35 °C), have been tested on haploid plant induction in cucumber[24]. Cucumber breeding research is highly intensive, with significant efforts directed toward the development of new varieties. However, haploidization technology, which aims to shorten breeding programs, has not yet achieved the desired level of efficiency. In this study, we hypothesized that haploid and doubled haploid (DH) plants could be produced by refining the protocol through the application of pre-treatments such as cold and heat shocks, along with the use of different nutrient media combinations. The objective was to evaluate the effects of distinct culture media compositions on in vitro ovary culture for haploid induction in four cucumber varieties, with the broader goal of advancing future cucumber breeding programs.

[1]Department of Biotechnology, Institute of Natural and Applied Sciences, Çukurova University, 01330 Adana, Türkiye. [2]Biotechnology Research and Application Center, Çukurova University, 01330 Adana, Türkiye. [3]Department of Horticulture, Faculty of Agriculture, Çukurova University, 01330 Adana, Türkiye. ✉email: f.nyirahabimana@outlook.com



Fig. 1. Used female flower buds (**a**: Botanik, **b**: PTK40, **c**: Beith Alpha, **d**: Sardes).

	Standard: MS, 30 g/L sucrose, 7 g/L agar, and pH 5.8				
Medium code	**2,4-D (mg/L)**	**BAP (mg/L)**	**TDZ (mg/L)**	**PUT (µM)**	**KIN (mg/L)**
IM1			0.06		
IM2	0.15				1.5
IM3	0.1	1			
IM4	5			40	

Table 1. Used nutrient media component. IM1: Induction medium 1, IM2: Induction medium 2, IM3: Induction medium 3, IM4: Induction medium 4.

Material and method

This study was carried out in the year 2022–2023 in the research plastic greenhouse of the Horticulture Department, Faculty of Agriculture, Çukurova University. The cucumber ovary tissue culture studies were performed in Prof. Dr. Saadet Büyükalaca's tissue culture laboratory in the Department of Horticulture, Çukurova University (Adana, Türkiye). The area of the study is located between the 35°21'58.2" East longitude and 37°01'47.5" North latitude.

Plant materials

The total of 4 cucumber varieties [PTK40 (mini-group from Petektar Tohum Company), Sardes (long group from Bayer Tohum Company), Botanik (pickling group from Yüksel Tohum Company), and Beith Alpha (one open-pollinated group from Antalya Tarım Company)] were used as donor plant materials. The seedling process was performed by Antalya Seed Company. On 19 April 2022, fifty healthy seedlings were transferred to the greenhouse (the research plastic greenhouse of the Horticulture Department, Faculty of Agriculture, Çukurova University) in double rows with (100 – 50) x 50 cm spacing distance. Plant materials were grown on their main stem. All greenhouse plant growth requirements, as well as disease and pest control, irrigation, pruning, and weeding, were performed. Female flower buds were collected when donor plants reached maturity to collect the healthier female flower buds' samples for ovary tissue culture. In this study, female flower buds were collected at 2 weeks after the second female flower was observed[8,21]. The fresh unpollinated ovaries were taken one day before anthesis when the color of the corolla was greenish-yellow (Fig. 1).

Used nutrient media composition

The induction media The induction media (IM1: 4.4 g MS, 30 g L^{-1} sucrose, 7 g/L agar, 0.06 mg/L Thidiazuron (TDZ); IM2: standard medium with 0.15 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1.5 mg/L Kinetin (KIN); IM3: 0.1 mg/L 2,4-D and 1 mg/L BAP; IM4: 5 mg/L 2,4-D and 40 µM PUT (Table 1) and regeneration media (RM1: 0.05 mg/L NAA and 0.2 mg/L BAP; RM2: 0.5 mg/L NAA and 0.5 mg/L BAP; RM3: 0.01 mg/L IAA and 1 mg/L BAP; RM4: 0.1 mg/L IAA and 1 mg/L BAP; RM5: 0.1 mg/L IAA and 2 mg/L BAP) were tested in this study (Table 2). Before sterilization in the autoclave at 121 °C for 15 min, the pH of all nutrient media was adjusted to 5.8 with the aid of 1 NaOH and 1 HCl.

To evaluate the effects of induction medium combinations on unpollinated ovary culture, a two-factor factorial experiment was conducted using a completely randomized design (CRD). The factors included four cucumber varieties and different induction media. For each variety, five ovary slices were placed in each 90-mm plastic Petri dish, with a total of five Petri dishes used per variety[11]. The growing chamber was under a lux intensity light of 3000 to 4000 Lx for 16 h/8 hr light/dark at 25℃ temperatures.

Ovaries surface sterilization

After the collection of the unpollinated ovaries, they were directly brought to the laboratory of plant biotechnology for tissue culture processes. Female flower buds were separated from their calyx for surface sterilization. The

Medium code	Standard: MS, 30 g/L sucrose, 7 g/L agar, and pH 5.8		
	NAA (mg/L)	BAP (mg/L)	IAA (mg/L)
RM1	0.05	0.2	
RM2	0.5	0.5	
RM3		1	0.01
RM4		1	0.1
RM5		2	0.1

Table 2. Regeneration media combination. RM1: Regeneration medium 1, RM2: Regeneration medium 2, RM3: Regeneration medium 3, RM4: Regeneration medium 4, RM5: Regeneration medium 5.

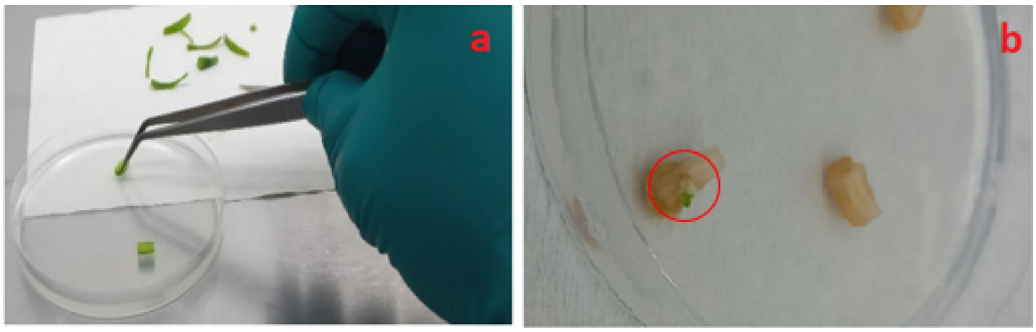


Fig. 2. (**a**) Ovary culture, (**b**) Direct embryo development.

collected ovaries were washed with autoclaved water three times. Then, the ovaries were taken into the cabinet, and using sterile pens, the ovaries of each cucumber variety were transferred into sterilized jars. The ovaries were kept in 70% ethanol solution for 1 min and rinsed thrice with autoclaved water. Later, explants were kept in 10% sodium hypochlorite with 2 to 3 drops of Tween 20 for 10 min. After that, the ovaries were rinsed four times with sterile distilled water. The excessive water was removed by putting wet ovaries on filter paper.

Ovary culture on the haploid induction media

The plant growth regulators (PGRs), particularly auxins, have been frequently employed to induce gynogenesis, and their optimum doses differ significantly from species to species or variety to variety[25]. Collected cucumber female flower buds were kept at 4 °C for 4 days to apply the cold shock pre-treatment. Following the sterilization steps as mentioned above, the ovaries were peeled without damaging the ovules with sterile pens and scalpels. The ovaries were sliced horizontally into five slices based on their size under sterile conditions. Then, the explants were cultured on the prepared induction nutrient media (Table 1) in 90 mm plastic Petri dishes (Fig. 2). Each Petri dish contains 5 ovary slices. Twenty-five ovary slices were cultured into the used nutrient medium for each variety. After culture, all cultures were transferred to 25℃ under a lux intensity light of 3000–4000 Lx for 16 h/8 h light/dark in a growing chamber. After 3 weeks of the initial culture, the induction media were changed to the regeneration media (Table 2). Following, the fresh nutrient regeneration media was changed every 4 weeks. Therefore, all Petri dishes stayed at 25℃ under the same conditions in the growing chamber. The embryo induction rate was determined after 30 days of initial culture, and the plant regeneration rate was evaluated five months after embryo-like structures (ELS) were observed.

Data collection and statistical analysis

The experiment contained 3 replicates, and each replication consisted of 5 Petri dishes. The results were analyzed into percentage values. Moreover, the embryo development percentage (EDP) was calculated based on differentiating parts, not the total cultured explants. While embryogenic callus percentage (ECP) was calculated based on the total number of cultured slices (explants), and the JMP Statistical Package program version 17.0.0.

Ploidy level analysis

Flow cytometry (Sysmex Cube 8) was used to determine the ploidy level. Fresh and young leaf samples of selected twenty plants developed from in vitro embryogenic calli-derived plantlets (regenerants) were collected and transported directly to the laboratory of IDA Yaşam Teknolojileri for ploidy identification. Concerning leaf samples (0.5 cm^2 of leaf tissue per plantlet in individual glass tubes), they were chopped with a razor blade, and 400 µL of extraction buffer was added. Subsequently, all samples were incubated for 30 to 60 s. Afterward, these samples were filtered using a special filter (Partec 50 m Cell Trics®). The addition of 1.6 ml of staining buffer to

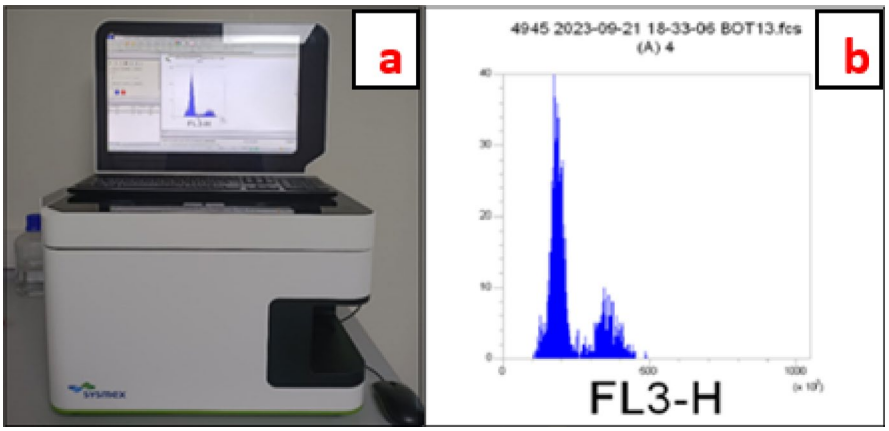


Fig. 3. (**a**) Flow cytometry machine, (**b**) Recorded peak from the analyzed samples.

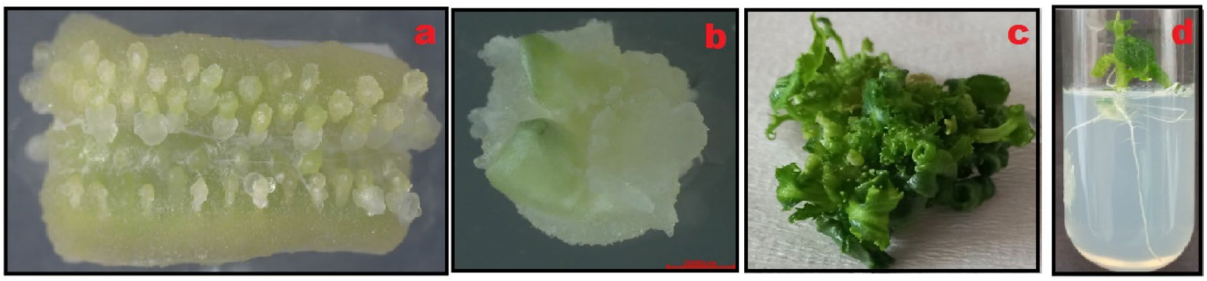


Fig. 4. Developed structures: (**a**) ovule enlargement (ELSs), (**b**) embryogenic callus, (**c**) plantlet like structure, (**d**) plant in glass tube.

the sample tube for a brief incubation of 30 to 60 s before taking readings on a flow cytometer machine was performed (Fig. 3).

Results

Embryo and plant regeneration

Throughout the haploid plant production, cold and heat stress pre-treatment has been applied as an essential factor to raise the efficiency of gynogenesis in various species[12,26]. In particular, the different rates of plant embryo induction were induced from fresh and four-day cold (4℃) shock pretreated female flower buds. When the effect of the media on the callus rate was examined, the highest embryo induction rate (88%) was obtained from Botanik in 5 mg/L 2,4-D with 40 µM PUT medium combination, appearing within two weeks from the first day of culture in this study. The observations indicated that cold treatment of ovaries was effective for the direct formation of haploid embryos. According to the observation of this study, the ovule enlargement, change in color, and maturation of callus, embryo-like structures, and plantlet-like structures were evaluated (Fig. 4). Callus formation appeared on the surface of cultured ovary slices at almost 2 to 3 weeks of initiation culture. In the experiment, the embryo germination media [RM2 (0.5 mg/L NAA and 0.5 mg/L BAP), RM3 (0.01 mg/L IAA and 1 mg/L BAP), RM4 (0.1 mg/L IAA and 1 mg/L BAP), and RM5 (0.1 mg/L IAA and 2 mg/L BAP) (details are not presented in this manuscript)] were tested. The embryos and embryogenic calli developed from the ovaries' slices cultured for 4–6 weeks were recorded. Then, all ovaries' pieces were transferred to the fresh medium. For good rooting, plant-like structures (plantlets) were developed and subcultured every four weeks to a fresh MS medium supplemented by 0.1 mg/L indole-3-butyric acid (IBA). Observations on the development of ovary slices were made, and the plant regeneration frequency was recorded and evaluated five months after ELS was observed.

Differentiation rates showed notable variation across varieties and media conditions (Table 3). The highest differentiation rates (88% and 60%) were observed in the Botanik and Sardes varieties, respectively, under the IM4 medium. In IM3 medium, only the PTK40 variety exhibited the development (24%). In IM2, the low rate (8%) of development was recorded exclusively in the Botanik variety. Beith Alpha revealed moderate differentiation only under IM1 and IM4 media combinations, while PTK 40 showed consistent development across three media, with its highest rate (52%) recorded in IM1 medium. The IM2 medium was largely unsuccessful within all varieties, showing no differentiation except in PTK 40, which also still failed to respond meaningfully. From a medium perspective, IM4 emerged as the most effective culture medium combination, which significantly enhanced the differentiation across all varieties. In terms of variety performance, PTK40 and Botanik stood out

Variety	Medium				
	IM1	IM2	IM3	IM4	Variety Average
Beith Alpha	22 [fg]	0	0	20 [g]	10.50 [d]
PTK 40	52 [c]	0	24 [f]	32 [d]	27 [a]
Botanik	0	8 [h]	0	88 [a]	23.25 [b]
Sardes	28 [e]	0	0	60 [b]	22 [c]

Table 3. Differentiation rates (%) of cucumber varieties under different induction media. Differentation Rate ($LSD_{variety}$: 3.75, $LSD_{variety*medium}$: 1.02). Values followed by different letters indicate statistically significant differences according to the LSD test ($p < 0.05$).

Variety	Medium				
	IM1	IM2	IM3	IM4	Variety Average
Beith Alpha	40 [h]	0	0	62.17 [e]	25.54 [d]
PTK 40	130 [b]	0	75 [d]	47 [g]	63 [a]
Botanik	0	50 [f]	0	110 [c]	40 [c]
Sardes	46.67 [g]	0	0	150 [a]	49.25 [b]

Table 4. Embryo development percentage (EDP) on differentiating parts of cucumber varieties under different induction media. EDP ($LSD_{variety}$: 0.59, $LSD_{variety*medium}$: 1.74). Values followed by different letters indicate statistically significant differences according to the LSD test ($p < 0.01$).

Variety	Medium				
	IM1	IM2	IM3	IM4	Variety Average
Beith Alpha	0	0	0	8 [c]	0
PTK 40	0	0	16 [c]	24 [c]	0
Botanik	0	0	0	272 [a]	40 [b]
Sardes	0	0	0	68 [b]	93 [a]

Table 5. Embryogenic callus percentage (ECP) of cucumber varieties under different induction media. $LSD_{variety*medium}$: 15.62. Values followed by different letters indicate statistically significant differences according to the LSD test ($p < 0.01$).

with superior average differentiation rates (27% and 23.25%, respectively, for variety average), while Beith Alpha remained the least responsive overall. These findings highlight the critical role of both variety and medium composition in driving successful regeneration, with IM4 consistently supporting high differentiation across diverse genetic backgrounds.

Embryo formation from differentiated structures (EDP) exhibited distinct patterns across cucumber cultivars and medium combinations (Table 4). The Sardes cultivar showed the most pronounced embryogenic response, reaching 150% under IM4, followed by PTK40 with 130% in IM1 and Botanik with 110% in IM3. This indicates that embryo formation from differentiated ovary slices often exceeds the initial differentiation rates. Such outcomes reflect a strong regenerative capacity and efficient embryogenesis within the same medium. PTK40 exhibited a 75% differentiation rate in IM3, while Botanik demonstrated notable embryogenic potential (50%) in IM2. In IM1, PTK40 maintained a 130% differentiation rate, whereas Botanik showed no regeneration. The result emphasizes the genotype's ability to initiate embryogenesis despite suboptimal differentiation conditions. For instance, Beith Alpha displayed low and variable embryo formation across media, except for a 62.17% EDP response in IM4. Overall, IM4 emerged as the most effective medium for supporting embryo development across all cultivars, particularly in Botanik, Sardes, and PTK40. IM1 followed, with responsive outcomes in PTK40, Sardes, and Beith Alpha. These findings underscore the importance of maintaining differentiated structures within the same medium to maximize embryogenic transition. While genotype effects remained evident, Sardes and Botanik consistently outperformed others in embryogenic formation capacity.

In the calculation of embryogenic callus percentage (ECP) in cucumber cultivars, the interaction between cultivar and medium was found to be significant (Table 5). The Botanik variety demonstrated the highest embryogenic (ECP) response at 272%, followed by 68% of Sardes under IM4 and moderate levels under the IM1 and IM3 medium combinations. This pattern indicates the strength of Botanik in terms of regenerative potential and responsiveness to all tested media in this study. The PTK40 variety showed embryogenic callus formation (16%) only under IM3, despite showing high differentiation and embryo formation in other media. This suggests that the embryogenic transition in Botanik may depend on the medium-specific and not directly correlated with initial differentiation rates. In contrast, Beith Alpha and PTK40 did not produce any embryogenic callus

Variety	Medium	Number of plants obtained from embryo	Number of plants obtained from embryogenic callus	Number of albino plants obtained	Ploidy level of the obtained plants
PTK 40	IM3	2	0	0	Diploid
Botanik	IM4	22	70	10	Diploid
Sardes	IM1	6	0	6	-
	IM4	0	4	4	Diploid

Table 6. Regenerated plant counts, albino occurrence, and ploidy levels of cucumber varieties. Only variety–medium combinations that yielded plants are shown. Conditions with zero regeneration were excluded for clarity.

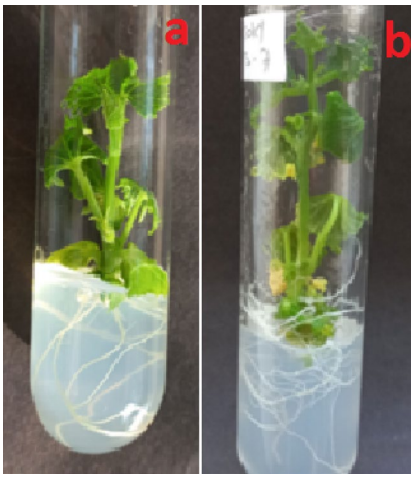


Fig. 5. (a-b) Developed and grown plantlets in glass tube.

under any condition, indicating limited conversion capacity despite earlier differentiation or embryo formation. Overall, IM3 and IM4 emerged as the most effective media for promoting embryogenic callus, particularly in responsive genotypes like Sardes. These findings reinforce the importance of genotype–medium interaction in achieving successful regeneration and highlight the need for tailored protocols based on variety-specific responses.

The haploid embryo induction potential of cucumber varieties was strongly influenced by both variety and induction medium. Among the tested media, IM4 consistently supported the highest differentiation rates, embryo formation, and embryogenic callus development, confirming its crucial role in effective in vitro haploid induction. The Botanik variety demonstrated superior performance across all parameters, reaching up to 88% differentiation rate, 110% EDP, and 272% ECP. Sardes produced promising results of a 60% differentiation rate, 150% EDP, and 68% ECP, followed by PTK40, which demonstrated moderate (32% differentiation rate, 47% EDP, and 24% ECP) regenerative capacity, while Beith Alpha remained the least responsive variety. The data emphasize the importance of maintaining differentiated structures within the same medium to enhance embryogenic transition and highlight the variety-specific nature of regeneration responses. Moreover, for regenerating plants (Table 6) from embryogenic callus and embryos to plantlets, a medium containing different concentrations of NAA (0.05 and 0.5 mg/L), BAP (0.2, 0.5, 1, and 2 mg/L), and IAA (0.01 and 0.1 mg/L) medium combinations (Table 2) was applied, and regenerant plants were developed into strong plant structures (Fig. 5). In this study most successful medium combinations were 0.1 mg/L IAA + 1 mg/L BAP and 0.1 mg/L IAA + 2 mg/L (detailed results are not presented in this manuscript). Then, the obtained plants were developed and multiplied in MS medium supplemented with 0.1 mg/L IBA. These findings offer a strategic framework for optimizing haploid plant induction protocols in cucumber, with implications for genetic transformation and breeding applications.

Furthermore, from all the regenerant plants, twenty of them were randomly selected for flow cytometry analysis to determine ploidy levels. In this study, the flow cytometry results confirmed that plants obtained from cucumber ovary culture were identified as diploid. All regenerated plants exhibited diploid chromosome numbers. Therefore, diploid plants could originate from the regeneration of diploid explant tissue during callus formation. Due to the difficulty of cytological tracking in developing callus tissues, both scenarios remain plausible. Table 6 presents only the variety–medium combinations that yielded regenerated plants, excluding conditions with zero regeneration. The data clearly demonstrate that IM4 was the sole medium capable of supporting plant regeneration across the used cucumber varieties. Botanik exhibited the highest regeneration capacity, producing 70 plants from embryogenic callus and 22 from embryos and maintaining a relatively

low albino frequency (10 plants). Additionally, PTK40 and Beith Alpha showed moderate regeneration, with PTK40 also yielding two embryogenic-derived plants, while Sardes produced 6 plants from embryos and 4 from embryogenic callus, these regenerants maintained diploid integrity and a manageable albino rate. These results reinforce the critical role of variety–medium interaction in successful plant regeneration. The consistent performance of IM4 across varieties highlights its effectiveness in promoting differentiation, embryogenesis, and plant regeneration. The absence and low value of plant regeneration induction in other medium combinations further underscores the specificity required for variety selection and optimal in vitro outcomes.

Discussion

In this study, plant growth regulators such as auxins (IBA, 2,4-D, NAA, IAA), cytokinins (BAP, KIN, TDZ), and polyamines, particularly putrescine, are commonly used in in vitro ovule/ovary and anther/microspore culture studies in cucurbits[27,28]. Recently, in the literature, it is stated that 2,4-D promotes embryo development and callus formation in haploid induction studies. Therefore, in the present study, four induction [IM1 (0.06 mg/L TDZ), IM2 (0.15 mg/L 2,4-D + 1.5 mg/L KIN), IM3 (0.1 mg/L 2,4-D + 1 mg/L BAP), and IM4 (5 mg/L 2,4-D + 40 µM PUT)] and five regeneration [RM1 (0.05 mg/L NAA and 0.2 mg/L BAP), RM2 (0.5 mg/L NAA and 0.5 mg/L BAP), RM3 (0.01 mg/L IAA and 1 mg/L BAP), RM4 (0.1 mg/L IAA and 1 mg/L BAP), and RM5 (0.1 mg/L IAA and 2 mg/L BAP) (detailed results are not presented in this manuscript)] medium combinations were tested. Recently, in the literature, it is stated that 2,4-D promotes embryo development and callus formation in haploid induction studies. Therefore, in the present study, four induction [IM1 (0.06 mg/L TDZ), IM2 (0.15 mg/L 2,4-D + 1.5 mg/L KIN), IM3 (0.1 mg/L 2,4-D + 1 mg/L BAP), and IM4 (5 mg/L 2,4-D + 40 µM PUT)] and five regeneration [RM1 (0.05 mg/L NAA and 0.2 mg/L BAP), RM2 (0.5 mg/L NAA and 0.5 mg/L BAP), RM3 (0.01 mg/L IAA and 1 mg/L BAP), RM4 (0.1 mg/L IAA and 1 mg/L BAP), and RM5 (0.1 mg/L IAA and 2 mg/L BAP) (detailed results are not presented in this manuscript)] medium combinations were tested. Furthermore, Kara and Sari[27] investigated the effects of polyamines (spermidine and putrescine) on haploid plant production through ovule culture in squash varieties. In their study, a medium containing 5 mg/L 2,4-D and 40 µM PUT was tested, and the results demonstrated successful embryo development under this medium combination.

Additionally, several promising results were observed in the use of the 2,4-D growth regulator during haploid induction of different horticultural crops. For instance, Metwally et al.[29] reported that summer squash anthers placed on MS medium containing 5 mg/L 2,4-D showed regeneration levels of up to 1.9 plants per anther. In the study conducted by Ashok Kumar et al.[25], it was indicated that optimum embryogenic calli/embryos in cucumber were obtained from B5 medium supplemented with 0.44 mg/L 2,4-D and 0.25 mg/L BAP. Song et al.[30] demonstrated that 2,4-D was a relatively important contributing factor for embryogenic callus induction in anther culture. The best medium for inducing cucumber embryogenic callus was MS medium supplemented with 0.5 mg/L 2,4-D, 1.0 mg/L BAP, and 1.2 mg/L KIN. Furthermore, Hamidvand et al.[31] found that the application of 0.44 mg/L 2,4-D together with 0.11, 0.23, and 0.34 mg/L BAP and 0.12, 0.25, and 0.37 mg/L KIN positively affected embryogenic calli in two cucumber cultivars (Beta Alfa and Esfahani). The highest number of embryos per anther (1.03 for Beta Alfa and 1.00 for Esfahani) was obtained from the medium combinations of 0.44 mg/L 2,4-D and 0.23 mg/L BAP which were used together in both cultivars. However, our study's findings showed that IM3 (0.1 mg/L 2,4-D + 1 mg/L BAP) has a positive impact only on the PTK40 variety (24 embryos per 100 cultured slices, 75 embryos per 100 developed slices), whereas IM2 (0.15 mg/L 2,4-D + 1.5 mg/L KIN) responded only in the Botanik variety (8 embryos per 100 cultured slices, 50 embryos per 100 developed slices) (Table 3). In distinct studies, the application of TDZ and temperature pre-treatments was mentioned for promising results in cucumber haploid induction via ovary culture. In the study conducted by Diao et al.[8], various factors, including genotype, thermal pre-treatment (35 °C for 2, 3, and 4 days), and different concentrations of (0.01, 0.02, and 0.04 mg/L) TDZ and 10 mg/L $AgNO_3$ (silver nitrate), were evaluated in cucumber ovary culture. Among thermal treatments, the 35 °C for 3 days treatment displayed a positive impact on embryo formation in most varieties. The study further reported that the highest embryogenic response was obtained from the medium supplemented by 0.04 mg/L TDZ, with embryo formation rates ranging from 20% to 72.7% across the tested varieties.

Moreover, Ozsan et al.[10] evaluated eight nutrient media in three genotypes in cucumber ovary culture. Cucumber basal medium (CBM) containing different doses of kinetin, 2,4-D, and TDZ was used, and the ovaries were cut into four pieces. Preincubation was performed for 3 days at 35 °C in the dark, and then the explants were kept in the dark again for two days at 25 °C. After that period, the explants were transferred to a 16/8 light/dark photoperiod condition at 25 °C. The embryo count of 36.95 embryos in 100 developed ovaries and 31.40 embryos in 100 cultured ovaries was reported to be higher in the medium composition of CBM (cucumber basal medium) supplemented by 0.03 mg/L TDZ. Yet, the results of this study revealed that the induction medium containing TDZ (IM1: 0.06 mg/L TDZ) responded to embryo formation in all varieties [PTK40 (52 embryos per 100 cultured slices, 130 embryos per 100 developed slices), Sardes (28 embryos per 100 cultured slices, 46.67 embryos per 100 developed slices), and Beith Alpha (22 embryos per 100 cultured slices, 40 embryos per 100 developed slices)] except the Botanik variety (Table 3). Based on the previous studies that performed to enhance haploid induction in cucumber, Baktemur et al.[13] analysed the effects of variety and nutrient medium on obtaining haploid plants via ovary culture in 37 cucumber varieties and three induction medium combinations of MS supplemented with 0.04 mg/L TDS); 0.15 mg/L 2,4-D, 1.5 mg/L KIN; 0.1 mg/L 2,4-D, 1 mg/L BAP and one regeneration (0.2 mg/L BAP, 0.05 mg/L NAA) medium were applied. The authors determined the embryogenesis success for both the number of embryos per 100 cultured ovaries and the number of embryos per 100 developed ovaries. It was reported in their study that the embryogenesis percentages for cultured ovaries and developed ovaries among varieties varied between 0% and 104%, and 0% and 416.66% respectively. Flow cytometry-based ploidy analysis displayed that 34.72% of regenerated plants were haploid, 37.5% diploid, 22.22% mixoploid, and 5.55% tetraploid. Yet, in the present study, diploid plants were obtained in two varieties (Botanik and Sardes). Throughout the formation of plant-like structures, some of the plants from Sardes were albino; the plant gained

from PTK40 perished at an early stage of its life, and there was no plant formation from Beith Alpha. According to previous studies on producing haploid plants in cucurbit crops, it is crucial to select the appropriate genotype or variety, effective medium composition, and culture conditions, as well as temperature pre-treatments during the ovary culture process[11,15,32,33].

Besides in the current study, the application of cold shock pre-treatment (4 °C) for four days to the ovaries was performed. In the results, the embryo development to the plant-like structure, Botanik, was a successful variety in the IM4 medium combination, within the application of cold pre-treatment. In the comparison of the study carried out by Deng et al.[14] to investigate the simple and effective method of directly producing cucumber plants via unpollinated ovary culture, the experiment analyzed the factorial interactions between genotypes, nutrient medium containing TDZ, and application of cold pre-treatment in cucumber to enhance haploid induction. The results indicated that applying cold pre-treatment at 4 °C for 4 days, in combination with 0.06 mg/L TDZ medium and genotype interaction, serves as an effective strategy to enhance gynogenesis efficiency. This approach achieved the highest regeneration induction rate (79.3%). The results of this study showed that the cold (4 °C) pre-treatment contributed the highest embryo induction rate (52%) from the PTK40 variety. In a study by Majed et al.[34], the effects of different culture media and temperature treatments on cucumber ovary culture were investigated. The induction medium contained TDZ, 2,4-D, and KIN, while the maturation medium included NAA and BAP as plant growth regulators were tested. Temperature treatments were applied at 25 °C for four weeks or at 35 °C and 4 °C for three days, followed by 25 °C for the remaining culture period, to promote callus induction in two cucumber varieties (Kros and Silah) at different floral stages. The results indicated that the medium containing TDZ and floral buds collected 12 h before anthesis produced the highest callus and embryogenic-like structures (ELS) formation via ovary culture. The optimization of gynogenesis induction media conditions according to growing seasons was conducted to accelerate the production of homozygous lines from 26 Beith Alpha and 11 Silor cucumber varieties. The results indicated that the most responsive genotypes were Beith Alpha types (HTB20, HTB24, and HTB25) and Silor types (HTS4 and HTS5). Moreover, explants cultured during the autumn season exhibited significantly higher regeneration efficiency compared to those cultured in spring. Overall, Beith Alpha genotypes showed a more favorable response in terms of plant regeneration and embryo development than Silor types, highlighting a strong genotype-dependent effect[35].

Conclusion

The current study investigated the effect of medium composition and cucumber varieties for haploid plant induction through ovary culture. Four induction medium combinations, five embryo regeneration medium combinations, and one rooting medium were applied with four cucumber varieties. The results reported that the medium combination of 5 mg/L 2,4-D + 40 µM PUT responded splendidly to all varieties in the range of 20–88%. Gynogenesis (ovule/ovary) culture technique has been shown to have promising potential for haploid induction in cucurbit species. However, their success is strongly influenced by factors such as genotype, flower developmental stage, culture conditions, nutrient media, and temperature pretreatments like heat or cold shocks. Most cucurbit species are recalcitrant in terms of genotype, which means that the high genotype dependency remains a major limitation, where in most studies diploid, mixoploid, and tetraploid are obtained spontaneously. According to the results of this study, even if the numerous embryos, callus, and plant structures obtained from all varieties and used medium combinations, with ploid levels, all regenerants were diploids. Authors are emphasizing the importance of selecting responsive donor genotypes/varieties as the main factor affecting the efficiency of haploid plant production. For instance, some of the genotypes/varieties are low embryo producers, more recalcitrant, or affected by the composition of the induction medium, the female gametophyte developmental stage on gynogenesis induction, the impact of nutrient medium composition on plant regeneration from gynogenic callus, and pre-culture treatments of explants. Further research is needed to develop effective protocols for virtually all cucurbit haploid induction gynogenesis protocols to enhance breeding efficiency and reduce the reliance on costly irradiated pollen methods. The authors strongly recommend conducting extensive studies with advanced biotechnological approaches, particularly to analyze and resolve the issue of ploidy instability, which poses a major challenge during the haploid plant production process.

Data availability

This research article includes all supporting data.

Received: 14 June 2025; Accepted: 4 December 2025
Published online: 25 March 2026

References

1. Nyirahabimana, F. & Solmaz, İ. Haploid Plant Production Technology in Cucumber (*Cucumis Sativus* L.). 10th International Molecular Biology and Biotechnology Congress (MOLBIOTECH 04–08 2021) Proceedings Book, 28–37 (2022).
2. Germana, M. A. Anther culture for haploid and doubled haploid production. *Plant. Cell. Tissue Organ. Cult.* **104**, 283–300. https://doi.org/10.1007/s11240-010-9852-z (2011).
3. Chomicki, G., Schaefer, H. & Renner, S. S. Origin and domestication of *Cucurbitaceae* crops: insights from phylogenies, genomics and archaeology. *New Phytol.* **226**, 1240–1255. https://doi.org/10.1111/nph.16015 (2020).
4. Salehi, B. et al. Antioxidant potential of family *Cucurbitaceae* with special emphasis on *Cucurbita* genus: A key to alleviate oxidative stress-mediated disorders. *Phytother Res.* **35**, 3533–3557. https://doi.org/10.1002/ptr.7045 (2021).
5. Zheng, Y. et al. Cucurbit genomics database (CuGenDB): a central portal for comparative and functional genomics of cucurbit crops. *Nucleic Acids Res.* **47** https://doi.org/10.1093/nar/gky944 (2019). D1128-D1136.
6. Zhu, Y. Comparative transcriptome analysis of the effect of different heat shock periods on the unfertilized ovule in watermelon (*Citrullus lanatus* L). *J. Integr. Agric.* **19**, 528–540. https://doi.org/10.1016/S2095-3119(19)62777-2 (2020).

7. Gemes-Juhasz, A., Balogh, P., Ferenczy, A. & Kristof, Z. Effect of optimal stage of female gametophyte and heat treatment on *in vitro* gynogenesis induction in cucumber (*Cucumis sativus* L). *Plant. Cell. Rep.* **21**, 105–111. https://doi.org/10.1007/s00299-002-0482-8 (2002).
8. Diao, W. P. et al. Efficient embryo induction in cucumber ovary culture and homozygous identification of the regenerants using SSR markers. *Sci. Hortic.* **119**, 246–251. https://doi.org/10.1016/j.scienta.2008.08.016 (2009).
9. Gałązka, J. & Niemirowicz-Szczytt, K. J. F. H. Review of research on haploid production in cucumber and other cucurbits. *Folia Hortic.* **25**, 67–78. https://doi.org/10.2478/fhort-2013-0008 (2013).
10. Ozsan, T., Gozen, V. & Onus, A. Cucumber gynogenesis: effects of 8 different media on embryo and plant formation. *Int. J. Agric. Innov. Res. (IJAIR)*. **6**, 419–422 (2017).
11. Tantasawat, P. A., Sorntip, A., Poolsawat, O., Chaowiset, W. & Pornbungkerd, P. Evaluation of factors affecting embryo-like structure and callus formation in unpollinated ovary culture of cucumber (*Cucumis sativus* L). *Int. J. Agric. Sci.* **17** https://doi.org/10.17957/IJAB.17.3.14.257 (2015).
12. Golabadi, M., Ghanbari, Y., Keighobadi, K. & Ercisli, S. Embryo and callus induction by different factors in ovary culture of cucumber. *J. Appl. Bot. Food Qual.* https://doi.org/10.5073/JABFQ.2017.090.010 (2017). 90.
13. Baktemur, G., Keleş, D., Kara, E., Yıldız, S. & Taşkın, H. Effects of genotype and nutrient medium on obtaining haploid plants through ovary culture in cucumber. *Mol. Biol. Rep.* 1–8. https://doi.org/10.1007/s11033-022-07238-y (2022).
14. Deng, Y. et al. Direct regeneration of haploid or doubled haploid plantlets in cucumber (*Cucumis sativus* L.) through ovary culture. *Plant. Cell. Tissue Organ. Cult.* **142**, 253–268. https://doi.org/10.1007/s11240-020-01839-w (2020).
15. Sorntip, A., Poolsawat, O., Kativat, C. & Tantasawat, P. A. Gynogenesis and doubled haploid production from unpollinated ovary culture of cucumber (*Cucumis sativus* L). *Can. J. Plant. Sci.* **98**, 353–361. https://doi.org/10.1139/cjps-2017-0112 (2017).
16. Moqbeli, E., Peyvast, G., Hamidoghli, Y. & Olfati, J. *Vitro* cucumber haploid line generation in several new cultivars. *AsPac J. Mol. Biol. Biotechnol.* **21**, 18–25 (2013).
17. Li, J., Si, S., Cheng, J., Li, J. & Liu, J. Thidiazuron and silver nitrate enhanced gynogenesis of unfertilized ovule cultures of *Cucumis sativus* L. Biol. *Plant* **57**, 164–168. https://doi.org/10.1007/s10535-012-0269-x (2013).
18. Domblides, E., Shmykova, N., Belov, S., Korottseva, I. & Soldatenko, A. DH-plant production in culture of unpollinated ovules of cucumber (*Cucumis sativus* L). *Vegetable Crops Russia*. **6**, 3–9. https://doi.org/10.18619/2072-9146-2019-6-3-9 (2019).
19. Deng, Y. et al. Transcriptome analysis of ovary culture-induced embryogenesis in cucumber (Cucumis sativus L). *PeerJ* **9** (e), 12145. https://doi.org/10.7717/peerj.12145 (2021).
20. Erol, M. H. & Sarı, N. The effect of ovule-ovary culture and spermidine-putrescine applications on haploid embryo induction of cucumber (*Cucumis sativus* L). *Alatarim* **18**, 108–117 (2019).
21. Pradeepkumara, N. et al. Cucumber F1 hybrid derived from two contrasting inbreds ensures high frequency gynogenesis for induction of haploids through a modified *in vitro* based protocol. *S Afr. J. Bot.* **157**, 314–324. https://doi.org/10.1016/j.sajb.2023.03.066 (2023).
22. Konstas, J. & Kintzios, S. Developing a scale-up system for the micropropagation of cucumber (*Cucumis sativus* L.): the effect of growth retardants, liquid culture and vessel size. *Plant. Cell. Rep.* **21**, 538–548. https://doi.org/10.1007/s00299-002-0566-5 (2003).
23. Chen, J. & Ziv, M. The effect of ancymidol on hyperhydricity, regeneration, starch and antioxidant enzymatic activities in liquid cultured narcissus. *Plant. Cell. Rep.* **20**, 22–27. https://doi.org/10.1007/s002990000283 (2001).
24. Asadi, A., Zebarjadi, A., Abdollahi, M. R. & Seguí-Simarro, J. M. Assessment of different anther culture approaches to produce doubled haploids in cucumber (*Cucumis sativus* L). *Euphytica* **214**, 1–17. https://doi.org/10.1007/s10681-018-2297-x (2018).
25. Ashok Kumar, H., Murthy, H. & Paek, K. Embryogenesis and plant regeneration from anther cultures of *Cucumis sativus* L. *Sci. Hortic.* **98**, 213–222. https://doi.org/10.1016/S0304-4238(03)00003-7 (2003).
26. Nyirahabimana, F. & Solmaz, İ. Haploid induction through ovary culture in cucumber. *Vitro Cell. Dev. Biol. -Plant.* **60**, 122–130. https://doi.org/10.1007/s11627-023-10406-9 (2024a).
27. Kara, B. & Sari, N. Effects of spermidine and Putrescine applications on haploid plant obtention via ovule culture in sakız and dark green squashes (*Cucurbita Pepo* L). *Türkiye Tarımsal Araştırmalar Dergisi*. **6**, 206–213. https://doi.org/10.19159/tutad.555837 (2019).
28. Nyirahabimana, F. & Solmaz, İ. Prevailing situation of anther and ovary cultures in cucumber haploid induction. Vegetos. (2024). https://doi.org/10.1007/s42535-024-01055-3
29. Metwally, E. I., Moustafa, S. A., El-Sawy, B. I., Haroun, S. A. & Shalaby, T. A. Production of haploid plants from *in vitro* culture of unpollinated ovules of *Cucurbita Pepo* L. *Plant. Cell. Tiss Org. Cult.* **52**, 117–121. https://doi.org/10.1023/A:1005948809825 (1998).
30. Song, H. et al. Regeneration of doubled haploid plants by androgenesis of cucumber (*Cucumis sativus* L). *Plant. Cell. Tiss Organ. Cult.* **90**, 245–254. https://doi.org/10.1007/s11240-007-9263-y (2007).
31. Hamidvand, Y., Abdollahi, M. R., Chaichi, M. & Moosavi, S. S. The effect of plant growth regulators on callogenesis and gametic embryogenesis from anther culture of cucumber (*Cucumis sativus* L). *Int. J. Agric. Crop Sci.* **5**, 1089–1095 (2013).
32. Ata, A., Keleş, D., Taşkın, H. & Büyükalaca, S. Effects of season, genotype, and nutrient medium on pepper anther culture and microspore development. *Turk. J. Agric. For.* **43**, 123–137. https://doi.org/10.3906/tar-1802-35 (2019).
33. Metin, D. et al. Callogenesis, embryogenesis, and plantlet initiation in Citron watermelon (*Citrullus lanatus* var. citroides) via anther and unfertilized ovary culture. *Sci. Hortic.* **337**, 113493. https://doi.org/10.1016/j.scienta.2024.113493 (2024).
34. Majed, H., Isak, M., Yetişir, H. & Şimşek, Ö. Role of different parameters in the formation of embryo-like structure and callus through ovary culture in cucumber (*Cucumis sativus* L). *I J. N S.* **16**, 90–106. https://doi.org/10.5281/zenodo.8373169 (2023).
35. Doğan, S. et al. Effects of different nutrient media on embryo induction and plant regeneration in cucumber (*Cucumis sativus* L.) ovary cultures. *Ekin J.* **11**, 60–67 (2025).

Author contributions

IS: Conceptualized the study, FN: Performed field work, HME and FN: All experiments and analysis. FN: wrote the manuscript of the study. YAK: Revised the manuscript. And all authors revised and agreed to the final version of the article.

Funding

This study was supported by [Cukurova University Scientific Research Projects Unit with FDK-2022-15123 Project No].

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval
This study does not contain data or samples any studies of animals or humans.

Consent to publish
The final version was approved by all authors.

Additional information
Correspondence and requests for materials should be addressed to F.N.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

© The Author(s) 2026