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In vitro ovary culture of cucumber (*Cucumis sativus* L.) for haploid plant production

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ABSTRACT

In order to produce haploid plants through ovary culture in cucumber (*Cucumis sativus* L.), a factorial experiment based on randomized design was conducted with three replicates under *in vitro* conditions. The ovaries of Sina, a hybrid variety of cucumber were harvested one week after flowering. They were cultured in the MS medium containing different concentrations of (0, 0.8 and 1.2 mg/L) TDZ and BAP, respectively. The best calli and the highest percentage of calli were obtained in the culture medium containing 0.8 mg/L TDZ and BAP. Then, 3 week-old explants were transferred to the differentiation medium; MS medium, supplemented with NAA, IBA, IAA, 2,4-D, Pic at 0.05 mg/L and 3 cytokinins including TDZ, BAP, Kin with 0.5, 1 and 1.5 mg/L, respectively, with a total of 15 treatments and 3 replicates. They were subcultured once every two weeks. The highest percentage of callus formation was obtained in the presence of 0.05 mg/L NAA and 1.5 mg/L TDZ. The highest percentage of embryogenesis (93.33%) was observed in the presence of IAA at 0.05 mg/L and 0.5 mg/L Kin. Application of IBA at 0.05 mg/L with 0.5 mg/L Kin increased the embryogenic calli formation. Also, the highest regeneration percentage (83.33%) was found in all 4 treatments including 0.05 mg/L IBA, with 0.5 mg/L TDZ, 0.05 mg/L IBA with 0.5. 0 mg/L BAP, 0.05 mg/L 2,4-D with 1.5 mg/L BAP and 0.05 mg/L Pic, with 1.5 mg/L BAP. After transferring the explants to the second step culture media, the embryo-like structures (ELS) developed into plantlets. Finally, complete plantlets were obtained after rooting the plantlets on the MS medium with no supplement. In addition, the results of chromosome counting showed that among 10 regenerated plantlets, one plantlet was haploid, and 9 plantlets were diploid.

Key words: Callus, Haploid, Hormone, Ovary, Tissue culture.

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INTRODUCTION

Cucumber (*Cucumis sativus* L.) belongs to Cucurbitaceae family (Zieliński *et al.*, 2017). Cucumber can produce female, male and hermaphrodite flowers. Due to the influence of genetic factors and environmental conditions, production of male or female flower organs may be selective and result into female or male flowers, or this growth may be non-selective and result into hermaphrodite flowers. (Kalloo and Bergh, 1994). Cucumber is a cross-pollinate and also self-pollinate plant (Burg and Burg, 1966; Delaplane and Mayer, 2000; Stanghellini *et al.*, 2002).

Pollination in this plant is done by insects, mainly bees, however, wind does not play a role in cucumber pollination (García-Lara *et al.*, 2019). Haploids have great value for plant breeders, because they can be easily doubled and pure homozygous lines can be obtained in a short period of time (Basu *et al.*, 2011).

Recessive genes like disease resistance genes (Basavaraju, 2011) or any other traits are expressed through haploids. In laboratory conditions, haploids can be obtained through anthers, pollen, ovules, etc culture. (Basu *et al.*, 2011). Haploids provide valuable materials for basic research and plant breeding (Faris *et al.*, 2000). Currently, the fastest way to doubling of a haploid is the production of cucumber haploid plants and multiply their chromosomes to create double haploids (Asadi and Seguí-Simarro, 2021). In cucurbit family, most of the plants are obtained as haploid, and then chromosome multiplication takes place under the influence of many factors. The plants obtained from developed, parthenogenetic embryos are genetically stable and mainly haploid, while *in vitro* culture of cucumber and anthers leads to the formation of both haploids and DHs (Gałązka and Niemirowicz-Szczytt, 2013). Chromosome doubling steps of cucurbits haploids are mainly based on colchicine (Yetisir and Sari, 2003). The first haploid plants from the Cucurbitaceae were obtained in 1950s. In many cucurbit plants, pollination with irradiated pollen is the most popular method of haploid induction, which is constantly being optimized and applied to an increasing number of cucurbit species (Gałązka and Niemirowicz-Szczytt, 2013). The production of inbred parental lines via repeated self-pollination takes 4–8 years, and the creation of a commercial hybrid can take as long as 10 years. However, the use of doubled haploid technology allows obtaining inbred lines in one generation, shortening the time needed for hybrid production (Blinkov *et al.*, 2022).

Haploid plants with a doubled set of chromosomes (doubled haploid (DH)) significantly speed up the selection process by the fixation of genetic traits in each locus in the homozygous state within one generation. Doubled haploids are mainly attained by, the formation of plants from *in vitro* cultured gametophytic (haploid) tissues and cells, or by targeted reduction in the parent chromosome during intra- or interspecific hybridization (Zargar *et al.*, 2022).

Three induction media (M1: Murashige and Skoog (MS), 0.04 mg/L Thidiazuron (TDZ); M2: MS, 0.15 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 1.5 mg/L Kinetin; M3: MS, 0.1 mg/L 2,4-D, 1 mg/L 6-Benzylaminopurine (BAP) and one regeneration medium (MS, 0.2 mg/L BAP, 0.05 mg/L 1-Naphthaleneacetic acid (NAA), and 31 cucumber genotypes were used in a research carried out by Baktemur *et al*. (2022) to investigate the effects of genotype and nutrient medium on obtaining haploid plants through ovary culture in cucumber. At the end of study, in terms of embryo formation, M3 (33.41 embryos per 100 cultured ovaries, 99.61 embryos per 100 developed ovariesovaries) and M2 (30.70 embryos per 100 cultured ovaries, 122.05 embryos per 100 developed ovaries) were found to be better than M1 (17.54 embryos per 100 cultured ovaries, 68.34 embryos per 100 developed ovaries).

Gynogenesis induction through unfertilized ovary/ ovule culture is an effective way to accelerate breeding schemes by obtaining homozygous materials in a short period, and great progress has been made with cucurbitaceous crops in recent years. However, limited studies are available for *Cucurbita* plants, especially winter squash (*Cucurbita maxima*) and pumpkin (*Cucurbita moschata*) (Zou *et al.*, 2020)

Unpollinated ovules cultured at one day prior to anthesis were the most suitable developmental stage for efficient induction of embryo like structures (ELS) and haploids (Pradeepkumara *et al*., 2023).

Most of studies conducted in the field of haploids have been conducted on anther and pollen grains and a limited number of studies have been conducted on ovary. Therefore, in order to further investigate the effect of different hormones on the production of haploid plants from ovary, a research was performed. This study included a cucumber cultivar, unfertilized ovary explants, 5 types of auxins (IBA, NAA, 2, 4-D, IAA and Pic) and 3 types of cytokinins (TDZ, BAP, KIN) were carried out in a factorial experiment based on completely randomized design with three replications. In this research, various attributes were also investigated including callus formation percentage, embryogenesis

percentage and regeneration percentage.

MATERIALS AND METHODS

In the present study, cucumber seeds (Sina hybrid variety) were planted in the greenhouse located in Imam Khomeini International University (IKIU) in light soil (40% cocopeat, 30% peat moss, 15% field soil and 15% perlite) in 25×20 cm size plastic pots and irrigated in 10-14 day intervals. Cocopeat was immersed in a pan of water to absorb water and gradually open up and its moisture content raised up to the desired level. Then peat moss was immersed in water in another pan to moisten. Then, farm soil was sieved and finally all the components were mixed together. Seeds germinated after 4-7 days and pots were watered once every 3 days. Weeds were eradicated using Fuzitel Aluminum as soil fungicide (0.5 g/liter in irrigation twice), N.P.K fertilizer was applied (20.20.20) (0.5 g/l) as foliar spraying in early mornings, once every 14 days, during the plant growing period. One month upon planting, the grown plants initiated flowering, and 3 weeks after appearance of the first female flowers, unfertilized ovaries were harvested in the early morning, placed in glass petri dishes covered by aluminum foil and taken to the tissue culture lab immediately.

In order to surface sterilize, the ovaries were washed with water for 20 minutes to remove dust and other contaminations. Then, ovaries were washed 3 times with sterile distilled water inside a laminar airflow hood and disinfected with 70% (v/v) ethanol for 20 seconds and washed 3 times with sterile distilled water. Then, ovaries were immersed in 1% (w/v) sodium hypochlorite solution for 20 minutes and finally washed 3 times with sterile distilled water (Tantasawat *et al*., 2015a, 2015b). Then, the explants were placed on the culture medium in sterilized petri dishes. Ovaries were cut longitudinally (in size of 1 mm) and then placed on the MS culture medium supplemented with 0.8 mg/L BAP and 0.8 mg/L TDZ. Then, the explants were placed in a 35 C incubator in darkness for 3 days. After three weeks, the explants were subcultured (Sorntip *et al*., 2017) and then explants transferred to the differentiation medium containing MS medium supplemented with 5 types of auxins (NAA, IBA, IAA, 2, 4-D and Pic) with of 0.05 mg/L and 3 types of cytokinins (TDZ, BAP and Kin) with 3 different concentrations (0.5, 1 and 1.5 mg/L). The explants were subcultured once every other week. After embryos turned into seedlings, healthy and fresh seedlings were transferred into MS_0 medium (hormone-free MS medium). When rooting, seedlings were transferred into pots containing a sterilized mixture of soil, perlite, peat moss and cocopeat to adapt the regenerated plants and finally they were transferred to the field soil (Sorntip *et al.*, 2017).

In order to determine the ploidy level of the regenerated plants, one week after seedlings rooting, the roots with 1.5-2 cm length were separated and placed in 0.05% colchicine for 3 h. Afterwards, the roots were washed in distilled water and immediately transferred to Levitsky's fixative solution (1:1 volume ratio of 10% formaldehyde and 1% chromic acid) and kept for 24 h at 4 °C. Then, they were washed for 3 h under the tap water, and roots were transferred in 70% ethanol at -20 °C. These explants were hydrolyzed in 1N hydrochloric acid for 20 min at 70 °C in a hot water bath. Acetoferric hematoxylin solution was used for staining the roots for 16 h at room temperature. In order to completely soften root tissue and preparation of slides, roots were placed in 45% acetic acid for 5 min (Farjaminezhad *et al*., 2012). Metaphase cells were observed and photographed with a Michrome 20 model of Michrome microscope.

RESULTS

Embryogenesis and regeneration of seedlings

One millimeter pieces of unfertilized ovaries were cultured on the induction medium containing TDZ and BAP at 0.8 mg/L. After 10 days, the explants swelled and then turned into callus (Figure 1A). In the next step, the created calli were transferred to the differentiation medium, and about 2 weeks later, spherical embryos were observed on the callus tissue (Figure 1B) and subsequently these embryos were transformed into heart-shape embryos (Figure 1C) and torpedo shapes were distinct (Figure 1D). Three weeks later, first shoot-like structures were observed (Figure 1E). Then the explants were transferred to the MS culture medium without growth regulators for rooting (Figure 1F). Finally, 10 different plants were regenerated from ovary culture, and these plants were treated with TDZ 0.5 mg/L+IBA 0.05 mg/L, BAP 0.5 mg/L+IAA 0.05 mg/L, 0.5 mg/L Kin+IBA0.05 mg/L, Kin 0.5 mg/L+NAA 0.05 mg/L, BAP 0.5 mg/L+IBA 0.05 mg/L, TDZ 1mg /l+IBA 0.05, TDZ 0.5 mg/L+0.05 mg/L Pic. Plants number of 2, 1, 1, 1, 2, 1 and 2 were regenerated in each treatments, respectively (Figures 1I and 1J).

Then, rooted seedlings (Figures 1G to 1J) were transferred to the small pots containing autoclaved soil (25% cocopeat, 25% peat moss, 25% perlite and

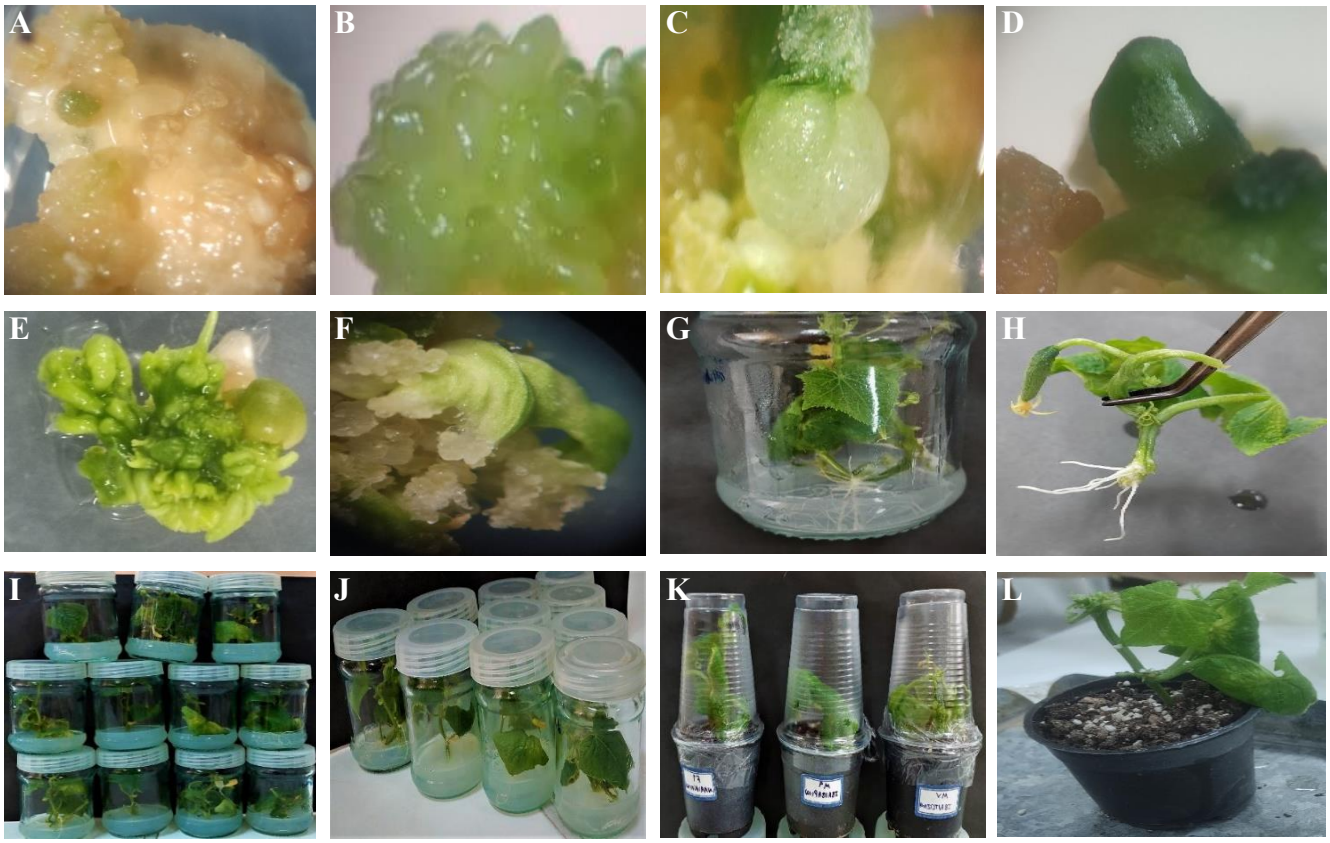

Figure 1. Embryogenesis and regeneration of seedlings. **A:** Callus, **B:** Spherical embryo, **C:** Heart-shaped embryo, **D:** Torpedo-shaped embryo, **E:** Shoot-like structures, **F:** Regenerated plant, **G:** Root development, **H:** Rooted seedling, **I & J:** Regenerated plants, **K:** 48 h after transfer to soil, **L:** Transfer of rooted plant to Pot.
*Figures **A** to **H** were developed in the presence of TDZ 0.5 mg/L+IBA 0.05 mg/L treatment hormones.

25% field soil) for adaptation (Figure 1K) and they were kept inside a growth chamber at 25 °C and 16/h light and 8/ h of dark by transferring into a 10 cm pot (Figure 1L).

Ploidy level determination of regenerated plants

To count chromosomes and determine the ploidy level of plants, fresh root tips of the obtained plants were used (Deng *et al*., 2020). In this study, ploidy level in 10 regenerated plants was evaluated by chromosome counting. These regenerated plants included haploid (n=x=7) and diploid (2n=2x=14) seedlings. One plant obtained (10%) was haploid (Figure 2B) and 9 other plants (90%) were diploid, out of 10 regenerated plants (Figure 2A).

Effect of TDZ and BAP treatments on callus formation percentage, embryogenesis percentage and regeneration percentage

There was no significant difference in the variance analysis results of TDZ and BAP treatments on the percentage of callus formation, and percentage of embryogenesis and regeneration percentage (Table 1).

The effects of auxin and cytokinin hormones on the percentage of callus formation, percentage of embryogenic calli and regeneration.

Based on the results of analysis of variance (Table 1), main effects of auxin and cytokinin and also interaction effect of auxin and cytokinin for the traits: the percentage of embryogenic calli, the percentage of embryo-formed calli and the percentage of regeneration were significant at 1% probability level, while the percentage of callus formation in the presence of auxin and cytokinin and their interaction effect did not show a significant difference.

Mean comparison of main effect of different auxins on callus formation percentage (Table 2) showed that the highest percentage of callus formation was obtained in presence of IAA and the lowest percentage of callus formation was observed in the presence of NAA. There was no significant difference between the two hormones with other auxins. Other three auxin treatments, such as IBA, 2,4-D, and Pic, were replaced by IAA and NAA which exhibited no significant differences.

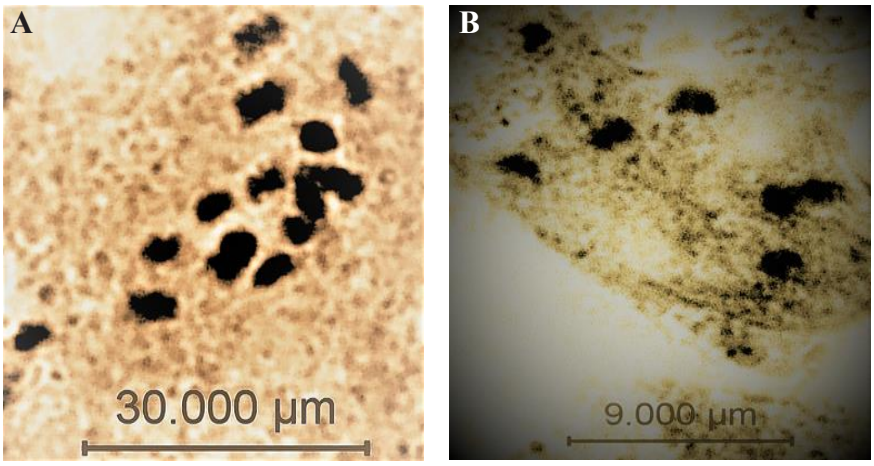

Figure 2. Chromosome count analysis in cucumber. **A:** Diploid chromosome number (2n=2x=14), **B:** Number of haploid chromosomes (n=x=7).

Table 1. Variance analysis of effects of auxin and cytokinin hormones on callus formation, embryogenesis callus, embryo callus, and regeneration percentages.

Source of variation	df	Means of square			
		Percentage of callus formation	Embryogenesis callus percentage	Percentage of embryo-formed calli	Percentage of regeneration callus
Auxin	4	180.741^{ns}	2699.583**	9675.82**	6667.05**
Cytokinin	8	76.296^{ns}	3461.9907**	788.85**	1504.77**
Auxin×Cytokinin	32	120.741^{ns}	795.208**	1065.85**	1956.78**
Error	90	94.815	126.620	50.980	4.403
Coefficient of variation (%)		10.01	18.93	20.36	16.94

** and ^{ns} are respectively significant at 1% level and there is no significant difference.

Table 2. Mean comparison of main effect of auxins on different traits.

Plant growth regulators (Auxins mg/L)	Percentage of callus formation	Embryogenesis callus percentage	Percentage of embryo-formed calli	Regeneration callus percentage
IBA	94.8148^{ab}	69.8148^{a}	56.9630^{a}	38.2099^{a}
NAA	94.0741^{b}	60.3704^{b}	46.6358^{b}	0.0000^{d}
2,4-D	98.5185^{ab}	64.5370^{ab}	32.9630^{c}	14.4444^{b}
IAA	100.0000^{a}	43.1481^{c}	6.6667^{d}	0.0000^{d}
PIC	98.5185^{ab}	59.2593^{b}	32.0370^{c}	9.2593^{c}

Means with the same letters are not significantly different at $p \leq 0.05$.

According to Table 2, the comparison of the main effect of different auxins on the percentage of embryogenic calli showed that the highest percentage of embryogenic calli was obtained in the presence of IBA and the lowest percentage of embryogenic calli was observed in the presence of IAA, and these two hormones illustrated a significant difference. Two other auxins, NAA and Pic, did not show significant differences. IBA, NAA, and Pic, did not exhibit a significant difference.

Mean comparison of different auxins on the percentage of embryo-formed calli (Table 2) showed that the highest percentage of embryo-formed calli was obtained in the presence of IBA and the lowest percentage of embryo-formed calli was obtained in the presence of IAA. Mean comparison of the main effect of different auxins on the percentage of regeneration is also presented in Table 2. The highest rate of regeneration was observed in the presence of IBA, which showed a significant difference with other

Table 3. Mean comparison of main effect of different types of cytokinin hormones on different traits.

Plant growth regulators (Cytokinins mg/L)	Percentage of callus formation	Percentage of embryogenesis callus	Percentage of embryo-formed calli	Percentage of callus regeneration
TDZ (0.5)	97.3333^{a}	60.3333^{bc}	38.6667^{ab}	16.6667^{b}
TDZ (1)	97.3333^{a}	52.6667^{c}	35.4778^{bc}	9.3333^{d}
TDZ (1.5)	100.0000^{a}	77.0000^{a}	41.6667^{a}	13.6667^{c}
BAP (0.5)	98.6667^{a}	73.3333^{a}	30.8889^{cd}	16.6667^{b}
BAP (1)	97.3333^{a}	42.1667^{d}	26.6667^{de}	9.3333^{d}
BAP (1.5)	92.0000^{a}	64.6667^{b}	38.5556^{ab}	33.3333^{a}
Kin (0.5)	96.0000^{a}	76.0000^{a}	43.3333^{a}	12.4444^{c}
Kin (1)	98.6667^{a}	33.3333^{e}	21.5556^{e}	0.0000^{e}
Kin (1.5)	97.3333^{a}	55.3333^{c}	38.6667^{ab}	0.0000^{e}

Means with the same letters are not significantly different at $p \leq 0.05$.

auxins, and then the rate of regeneration in the presence of 2,4-D and Pic, respectively. These two hormones had significant differences, and two auxin treatments, NAA and IAA, did not affecte regeneration induction.

Effect of cytokinin on different traits

Mean comparison of main effect for different types of cytokinins with different concentrations on callus formation percentage, showed that different concentrations had no significant differences, and the highest percentage was related to TDZ with 1.5 mg/L. (Table 3). Main comparison of the main effect of different types of cytokinins in different concentrations on the percentage of embryogenic callus exhibited that the highest percentage of embryogenic calli was observed in TDZ at 1.5 mg/L, which had no significant difference between BAP at 0.5 mg/L and Kin at 0.5 mg/L. The lowest percentage of embryogenic calli was also observed in the presence of BAP at 1 mg/L and Kin at 1 mg/L, and these two hormones had significant differences. Moreover, Table 3 shows that among different concentrations of TDZ, the highest percentage of embryogenic calli was observed at 1.5 mg/L and the lowest percentage of embryogenic calli was observed at 1 mg/L and a significant difference was observed between the highest and lowest values. In addition, among different concentrations of BAP, the highest percentage of embryogenic calli was observed at 0.5 mg/L, while the lowest percentage of embryogenic calli was observed at 1 mg/L, and there was a significant difference between the concentrations of hormones.

In different concentrations of Kin, the highest percentage of embryogenic calli was obtained at 0.5 mg/L and the lowest percentage of embryogenic calli was obtained at 1 mg/L, which showed a significant difference between the highest and the lowest percentage of embryogenic calli (Table 3). According to this Table, mean comparison on the effect of different concentrations of cytokinins on the percentage of embryogenic calli, exhibited that the highest percentage of embryogenic calli was obtained in the presence of TDZ at 1.5 mg/L, which had not a significant difference with Kin at 0.5 mg/L. The lowest percentage of embryo-formed calli was also observed in Kin at (1 mg/L). Also, according to Table 3, the highest percentage of embryogenic calli was observed at 1.5 mg/L and the lowest percentage of embryo-formed calli was observed at 1 mg/L between different concentrations of TDZ. While a significant difference was observed between the highest and the lowest values. Moreover, the highest percentage of embryogenic calli was obtained at 1.5 mg/L and the lowest percentage of embryogenic calli was obtained at 1 mg/L between different concentrations of BAP. There was a significant difference between the highest and the lowest percentages of embryogenic calli. Between different concentrations of Kin, the highest percentage of embryogenic calli was obtained at 0.5 mg/L and the lowest percentage of embryogenic calli was obtained at 1 mg/L, and there was a significant difference between the highest and the lowest embryogenic calli percentages.

According to Table 3, the effect of different cytokinins with different concentrations on regeneration percentage, it was observed that the highest regeneration percentage was observed in the presence of BAP at 1.5 mg/L and the lowest regeneration percentage was observed in the presence of TDZ at 1 mg/L and BAP at 1 mg/L and there was a significant difference between the highest and lowest values. The highest percentage of regeneration was obtained at 0.5 mg/L TDZ and the lowest percentage of regeneration was obtained at 1 mg/L TDZ, and a significant difference was observed between the highest and lowest values (Table 3). In addition, between different concentrations

Table 4. Mean comparison of interaction effects of auxin and cytokinin hormones on callus formation percentage.

Cytokinins (mg/L)	Auxins (mg/L)				
	IBA (0.05)	NAA (0.05)	2,4-D (0.05)	IAA (0.05)	PIC (0.05)
TDZ (0.5)	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	86.66±6.66^{ab}
TDZ (1)	100±0^{a}	86.66±6.66^{ab}	100±0^{a}	100±0^{a}	100±0^{a}
TDZ (1.5)	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}
BAP (0.5)	100±0^{a}	93.33±6.66^{a}	100±0^{a}	100±0^{a}	100±0^{a}
BAP (1)	100±0^{a}	100±0^{a}	86.66±6.66^{ab}	100±0^{a}	100±0^{a}
BAP (1.5)	73.33±6.66^{b}	86.66±6.66^{ab}	100±0^{a}	100±0^{a}	100±0^{a}
Kin (0.5)	100±0^{a}	80±11.54^{ab}	100±0^{a}	100±0^{a}	100±0^{a}
Kin (1)	93.33±6.66^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}
Kin (1.5)	86.66±6.66^{ab}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}

Means with the same letters are not significantly different at $p \leq 0.05$.

Table 5. Mean comparison of auxin and cytokinin interaction effects on percentage of embryogenesis callus.

Cytokinins (mg/L)	Auxins (mg/L)				
	IBA (0.05)	NAA (0.05)	2,4-D (0.05)	IAA (0.05)	PIC (0.05)
TDZ (0.5)	86.66±6.66^{abc}	53.33±6.66^{f-l}	73.33±6.66^{a-f}	18.33±1.66^{o}	70±5.77^{b-g}
TDZ (1)	56.66±3.33^{e-k}	63.33±8.81^{d-i}	70±5.77^{b-g}	36.66±3.33^{j-o}	36.66±3.33^{j-o}
TDZ (1.5)	80±11.54^{a-d}	86.66±6.66^{a-c}	80±11.54^{a-d}	45±5.77^{h-n}	93.33±6.66^{a}
BAP (0.5)	80±11.54^{a-d}	78.33±1.66^{a-e}	91.66±8.33^{ab}	43.33±3.33^{i-n}	73.33±8.81^{a-f}
BAP (1)	36.66±3.33^{j-o}	66.66±6.66^{c-h}	57.50±10.10^{e-j}	30±5.77^{m-o}	20±0^{o}
BAP (1.5)	80±5.77^{a-d}	71.66±7.26^{a-g}	75±5^{a-f}	43.33±3.33^{i-n}	53.33±3.33^{g-m}
Kin (0.5)	83.33±8.81^{a-d}	50±5.77^{g-m}	63.33±8.81^{d-i}	93.33±6.66^{a}	90±5.77^{ab}
Kin (1)	35±2.88^{k-o}	26.66±3.33^{no}	4.40±26.66^{no}	31.66±4.40^{l-o}	46.66±7.26^{h-n}
Kin (1.5)	90±5.77^{ab}	46.66±6.66^{h-n}	3.33±43.33^{i-n}	46.66±8.81^{h-n}	50±5.77^{g-m}

Means with the same letters are not significantly different at $p \leq 0.05$.

of BAP, the highest percentage of regeneration was observed at 1.5 mg/L and the lowest percentage of regeneration was observed at 1 mg/L, and there was a significant difference between them. Between different concentrations of Kin, regeneration was observed only at 0.5 mg/L, and there was no regeneration in 1 and 1.5 mg/L concentrations.

Mean comparison of auxin and cytokinin interactions on callus formation percentage

According to Table 4, mean comparison of effects of auxin and cytokinin on the percentage of callus formation showed that except 1.5 mg/L BAP in the presence of 0.05 mg/L IBA, other treatments did not show any significant difference.

Mean comparison of auxin and cytokinin interactions on percentage of embryogenic callus

According to Table 5, mean comparison of auxin and cytokinin interactions on the percentage of embryogenic calli showed that the highest percentage of embryogenic calli was obtained in the presence of IAA 0.05 mg/L and Kin 0.5 mg/L. Also, the lowest percentage of embryogenic calli were obtained in the presence of IAA 0.05 mg/L, and TDZ 0.5 mg/L, and Pic 0.05 mg/L with BAP 1 mg/L, and a significant difference was observed between the highest and the lowest values.

Mean comparison of auxin and cytokinin interactions on percentage of embryogenic callus

According to Table 6, results of mean comparison of auxin and cytokinin interactions on the percentage of embryogenic calli revealed that the highest percentage of embryo calli was obtained in the presence of IBA (0.05 mg/L) and Kin (0.5 mg/L). Also, the lowest percentage of embryo calli was obtained with presence of 2,4-D (0.05 mg/L) and Kin (0.5 mg/L), and a significant difference was observed between the highest and the lowest values.

Mean comparison of auxin and cytokinin interactions on regeneration percentage

According to Table 7, mean comparison of auxin and

Table 6. Mean comparison of auxin and cytokinin interaction effects on the percentage of embryo-forming calli.

Cytokinins (mg/L)	Auxins (mg/L)				
	IBA (0.05)	NAA (0.05)	2,4-D (0.05)	IAA (0.05)	PIC (0.05)
TDZ (0.5)	66.66±4.40^{b-d}	43.33±3.33^{f-j}	58.88±4.84^{c-e}	0±0^{n}	24.44±4.44^{k-m}
TDZ (1)	76±3.46^{ab}	76.94±4.36^{ab}	24.44±4.44^{k-m}	0±0^{n}	0±0^{n}
TDZ (1.5)	70±5.77^{a-c}	52.22±7.77^{e-g}	36.11±2^{i-k}	0±0^{n}	50±5.77^{e-h}
BAP (0.5)	50±5.77^{e-h}	34.44±2.93^{i-l}	40±5.77^{g-j}	0±0^{n}	30±5.77^{j-m}
BAP (1)	45±2.88^{f-i}	32.77±1.46^{i-l}	55.55±5.55^{d-f}	0±0^{n}	0±0^{n}
BAP (1.5)	33.33±3.33^{i-l}	60±5.77^{c-e}	21.66±1.66^{lm}	43.33±3.33^{f-j}	34.44±2.93^{i-l}
Kin (0.5)	81.66±6^{a}	50±5.77^{e-h}	16.66±1.66^{m}	16.66±1.66^{m}	51.66±7.26^{e-g}
Kin (1)	53.33±3.33^{e-g}	30±5.77^{j-m}	0±0^{n}	0±0^{n}	24.44±4.44^{k-m}
Kin (1.5)	36.66±3.33^{h-k}	40±0^{g-j}	43.33±3.33^{f-j}	0±0^{n}	73.33±8.81^{ab}

Means with the same letters are not significantly different at $p \leq 0.05$.

Table 7. Mean comparison of auxin and cytokinin interaction effects on the percentage of callus regeneration.

Cytokinins (mg/L)	Auxins (mg/L)				
	IBA (0.05)	NAA (0.05)	2,4-D (0.05)	IAA (0.05)	PIC (0.05)
TDZ (0.5)	83.33±3.33^{a}	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}
TDZ (1)	46.66±1.66^{d}	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}
TDZ (1.5)	68.33±3.33^{b}	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}
BAP (0.5)	83.33±3.33^{a}	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}
BAP (1)	0±0^{e}	0±0^{e}	46.66±1.66^{d}	0±0^{e}	0±0^{e}
BAP (1.5)	0±0^{e}	0±0^{e}	83.33±3.33^{a}	0±0^{e}	83.33±3.33^{a}
Kin (0.5)	62.22±2.22^{c}	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}
Kin (1)	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}
Kin (1.5)	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}

Means with the same letters are not significantly different at $p \leq 0.05$.

cytokinin interaction effects on the percentage of regeneration showed that the highest percentage of regeneration was obtained in the presence of IBA 0.05 mg/L and TDZ 0.5 mg/L, IBA 0.05 mg/L along with BAP 0.5 mg/L, 2,4-D 0.05 mg/L and BAP 1.5 mg/L liter and Pic at 0.05 mg/L and BAP at 1.5 mg/L. Also, the lowest percentage of regeneration was obtained in the presence of IBA 0.05 mg/L and TDZ 1 mg/L and 2,4-D at 0.05 mg/L and BAP 1 mg/L, and a significant difference was observed between the highest and the lowest values.

DISCUSSION

Different species of Cucurbitaceae show different responses to micropropagation. These differences may be caused by many factors such as genotype, composition of culture medium, physical growth factors such as light, temperature, humidity, etc. (Jesmin and Mian, 2016). Basal MS based media supplemented with 5.0 mg/L Zeatin and 0.2 mg/L NAA was the most effective medium for the induction of large number of embryo like structures (ELS) (Pradeepkumara *et al*., 2023).

Results of this research showed that the percentage of callus formation in the presence of different hormones did not show a significant difference and most of the treatments showed 100% callus formation. Differences in the percentage of callus formation observed in this study with other studies can be due to the difference in the type of variety used, the season of the year when the explants were separated from the mother plant, the difference in the place of plant growth in the greenhouse or field and etc.

Results showed that the highest percentage of embryogenic calli (93.33) was observed in the presence of IAA and Kin each at 0.05 mg/L. The results of the present research were somewhat consistent with the results of Sorentip *et al*. (2017), although differences

were observed due to the change of the cultivation environment, the type of variety and the concentration of growth regulators. They reported that the highest percentage of embryogenic calli was obtained at 1.5 mg/L and the lowest percentage of embryogenic calli (59.89%) was obtained when the ovaries were cultured on the culture medium containing 0.5 mg/L NAA, 0.2 mg/L Benzylaminopurine, 0.2 mg/L TRIA, 100 mg/L proline, 20 mg/L ascorbic acid, and 0.20 mg/L silver nitrate. They also reported in their research that the type of genotype had no significant effect on the percentage of embryogenic calli.

Diao *et al*. (2009) reported 65.7% callus forming embryos in the medium containing 0.02 mg/L of TDZ. In the present study, the percentage of formation of embryogenic calli was reported to be 77% at 1.5 mg/L, which is consistent with the results mentioned above, and the small difference observed can be attributed to the difference in the concentration of used TDZ. Therefore, it can be stated that with increasing TDZ concentration in the culture medium, the percentage of formation of embryogenesis calli also increases.

Results of mean comparison of auxin and cytokinin interaction effects on regeneration percentage showed that the highest regeneration percentage was 88.33% in the presence of IBA at 0.05 mg/L and TDZ at 0.5 mg/L. In a similar research, interaction effect of TDZ, cold pretreatment and genotype was investigated on the percentage of ovary regeneration in cucumber. Results of Deng *et al*. (2020) showed that cold pretreatment for 4 days, TDZ at 0.6 mg/L and their interaction with genotype could be an effective method to improve gynogenesis efficiency. The highest percentage of regeneration induction was reported to be 79.3%. In addition, results of Deng *et al*. (2020) showed that the effect of TDZ concentration on the percentage of plant regeneration was significant and with increasing TDZ concentration, plant regeneration rate increased. The highest percentage (58.3%) of plant regeneration was observed in the presence of 0.06 mg/L (Deng *et al*., 2020).

While in the present study, with increasing TDZ concentration, the percentage of plant regeneration decreased from 83.33% to 68.33%, which could be interpreted as the interaction between TDZ and other auxins. In addition, in another study, the percentage of regeneration in the medium containing 0.02 mg/L of TDZ was observed about 16.92% (Ye *et al*., 2015).

Researchers investigated various factors such as heat shock pretreatment at temperatures between 8 and 35 °C, TDZ concentration and silver nitrate on the formation of embryos from cucumber ovary culture in different species (Diao *et al*., 2009). Their results showed that heat shock for 3 days at 35 °C at the beginning of cultivation led to the highest frequency of embryo formation (89.4%) compared to 2 and 4 days. In addition, they reported that the use of TDZ had a positive effect on the formation of embryos, so that the highest percentage of embryogenesis (72.7) was observed in the induction medium containing 0.04 mg/L of TDZ. Also, their results showed that the addition of AgNO3 to the induction medium had no significant effect on the percentage of embryo formation, but it reduced the regeneration period and improved the number of embryos formed in each section of the ovary. In the present study, using TDZ led to an increase in the percentage of embryo formation (77%) among all added cytokinins, which is consistent with the results of Diao *et al*. (2009). The observed differences in the percentage of embryo formation in the present study could be related to the difference in the type of used cultivars and TDZ concentration.

In a study conducted by Golabadi *et al*. (2016), results of mean comparison between different hormonal combinations showed that the combination of 2,4-D+BAP (1.5+4 mg/L) had the highest percentage of embryogenesis (37.5%). This result is consistent with the findings of the present research. According to the results of Baktemur *et al*. (2022) 2,4-D added to the nutrient media appeared to be successful in the induction of ovary culture in cucumber.

According to Table 5, interaction effect of 2, 4-D and BAP at 0.05 and 0.5 mg/L, respectively, caused a high percentage of embryogenesis (91.66 percent) in cucumber ovary, which did not show a significant difference with the highest percentage of embryogenic callus in the present study (93.33%). In addition, the highest percentage of callus induction in Golabadi *et al*. (2017) research, was obtained in the presence of IAA+KIN (1.5+1 mg/L) and 2,4-D+BAP (0.5+2 mg/L).

These results showed that the presence of auxin in the culture medium increased callus formation. In the present study, a high percentage of callus formation (100%) was observed in these treatments. Although the concentration of cytokinins used in the present study was similar to the research carried out by Gol-Abadi *et al*. (2017), but auxin concentration in this study was much lower and considering the high percentage of callus formation in this research it can demonstrate that in cucumber ovary cultivation, the lower amounts of auxin compared to its high amounts increased the

percentage of callus formation.

In some culture media, the presence of low levels of auxin is necessary to stimulate explants to form embryos. This result is consistent with the research findings on the culture of cucumber cotyledons (Ugandhar *et al*., 2011). They reported that the presence of 0.5 mg/L IAA and 3 mg/L BAP or 3 mg/L KIN increased the percentage of callus formation and also induced root formation. In the present study, the lowest percentage of embryogenesis (20%) was observed in the presence of PIC and BAP at 0.05 and 0.5 mg/L, respectively, while in study conducted by Golabadi *et al*. (2017), the lowest rate of embryogenesis in cucumber ovaries (1.65%) in presence of IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5 mg/L) was observed.

Jesmin and Mian (2016), investigated the effect of different types of auxin and cytokinin hormones on different explants such as leaves, stems and cotyledons of cucumber in MS culture medium. Their results showed that callus induction occurred in the MS culture medium containing different concentrations of growth regulators including 2,4-D, BAP, NAA. Their results show that the frequency of callus induction, callus growth and the nature of callus are affected by the type and concentration of plant growth regulators, which is in agreement with present study results. Results also showed that callus induction, callus fresh weight and also the percentage of embryogenesis were affected by the difference in concentration and type of growth regulators (Jesmin and Mian, 2016).

The results of this study showed that out of 10 regenerated plants, one plant (10%) was haploid and 9 plants (90%) were diploid. Similar results were reported in cucumber (Diao *et al*., 2009) and other species such as pumpkin (Metwally *et al*., 1998), cantaloupe (Ficcadenti *et al*., 1999) and onion (Alan *et al*., 2004). In a research on Chinese cucumber cultivars, Diao *et al*. (2009) reported that most of the regenerated seedlings (82.5) obtained from the ovary culture without application of colchicine treatment were diploid, and the percentage of diploid seedlings was similar to the percentage of diploid seedlings obtained in the present study (90%).

In another research on cucumber plant, Sorentip *et al*. (2017), investigated the ploidy level of 10 regenerated seedlings as well as mother plants "Chai Lai" and "Big C" by chromosome counting method. Their results showed that these 10 regenerated seedlings contain different levels of ploidy, including haploid, diploid and triploid plants. In their research, out of 6 regenerated seedlings from "Big C" variety, most seedlings (83.3) were diploid and only 16.7% of regenerated plants were haploid. Out of 4 seedlings examined in "Chai Lai" variety, two seedlings were haploid (50%), one was diploid (25%) and one was triploid (50%). These findings are similar to our results. The higher percentage of haploid seedlings in "Chai Lai" variety compared to the present study could be attributed to the differences in the varieties.

In the research carried out by Sorntip *et al*. (2017), the percentage of haploid plants obtained from ovary culture in two cultivars "Big C" and "Chai Lai" showed different percentages (16.7% and 50%, respectively) could be the result of type of parental plants as the major impact on the production of haploid plants in ovary culture. Diploid seedlings may be created as a result of spontaneity diploidization, also during formation of embryo-like structures in cultured zygote or as a result of the regeneration of somatic cells of parental plant (Sorntip *et al*., 2017).

CONCLUSION

Haploids could be obtained using ovary culture. Current study showed the induction of callus and embryo in cucumber using ovary as the explant. The results of this research showed that the percentage of callus formation in the presence of different hormones did not show a significant difference and most of the treatments showed 100% callus formation. In this experiment, it was observed that the highest percentage of embryogenic calli (93.33) was obtained in the presence of IAA and Kin at 0.05 mg/L. The highest regeneration percentage of 88.33% was obtained in the presence of IBA at 0.05 mg/L and TDZ at 0.5 mg/L. In present study, by increasing the TDZ concentration, the percentage of plant regeneration decreased from 83.33% to 68.33%, which could be due to the interaction of TDZ with other auxin hormones used in the study. Results of this study showed that out of 10 regenerated plants, one plant (10%) was haploid and 9 plants (90%) were diploid. It could be concluded that the type of parental plant could have a major impact on the production of haploid plants in ovary culture.

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REFERENCES

Alan A. R., Brants A., Cobb E., Goldschmied P. A., Mutschler M. A., and Earle, E. D. (2004). Fecund gynogenic lines from onion (*Allium cepa* L.) breeding materials. *Plant Science,* 167(5): 1055-1066.

Asadi A., and Seguí-Simarro J. M. (2021). Production of doubled haploid plants in cucumber (*Cucumis sativus* L.) through another culture. In: Doubled Haploid Technology, Springer, 71-85.

Baktemur G., Keleş D., Kara E., Yıldız S., and Taşkın H. (2022). Effects of genotype and nutrient medium on obtaining haploid plants through ovary culture in cucumber. *Molecular Biology Reports,* 49: 5451-5458

Basavaraju R. (2011). Plant tissue culture-agriculture and health of man. *Indian Journal of Science and Technology,* 4(3): 333-335.

Basu S. K., Datta M., Sharma M., and Kumar A. (2011). Haploid production technology in wheat and some selected higher plants. *Australian Journal of Crop Science,* 5(9): 1087-1093.

Blinkov A. O., Varlamova N. V., Kurenina L. V., and Khaliluev M. R. (2022). The Production of helianthus haploids: A review of its current status and future prospects. *Plants,* 11(21): 2919. DOI: https://doi.org/10.3390/plants11212919.

Burg S. P., and Burg E. A. (1966). The interaction between auxin and ethylene and its role in plant growth. *Proceedings of the National Academy of Sciences of the United States of America,* 55(2): 262.

Delaplane K. S., and Mayer D. F. (2000). Crop pollination by bees. Cambridge, U.K.: CABI, pp. 344.

Deng Y., Tang B., Zhou X., Fu W., Tao L., Zhang L., and Chen J. (2020). Direct regeneration of haploid or doubled haploid plantlets in cucumber (*Cucumis sativus* L.) through ovary culture. *Plant Cell, Tissue and Organ Culture (PCTOC),* 142(2): 253-268.

Diao W. P., Jia Y. Y., Song H., Zhang X. Q., Lou Q. F., and Chen J. F. (2009). Efficient embryo induction in cucumber ovary culture and homozygous identification of the regenetants using SSR markers. *Scientia Horticulturae,* 119(3): 246-251.

Faris N., Rakoczy-Trojanowska M., Malepszy S., and Niemirowicz-Szczytt K. (2000). Diploidization of cucumber (*Cucumis sativus* L.) haploids by in vitro culture of leaf explant. *Progress in Biotechnology*, 17: 49-54.

Farjaminezhad R., Asghari R., Zare N., and Farjaminezhad M. (2012). Study of karyological characteristics in several accessions of *Papaver bracteatum* Lindl. *Agricultural Biotechnology Journal*, 3(2): 47-58.

Ficcadenti N., Sestili S., Annibali S., Di Marco M., and Schiavi M. (1999). *In vitro* gynogenesis to induce haploid plants in melon *Cucumis melo* L. *Journal of Genetics and Breeding*, 53: 255-258.

Gałązka J., and Niemirowicz-Szczytt K. (2013). Review of research on haploid production in cucumber and other cucurbits. *Folia Horticulturae*, 25(1): 67-78.

García-Lara S., Chuck-Hernandez C., and Serna-Saldivar S. O. (2019). Development and structure of the corn kernel. Corn (Third Edition), 147-163.

Golabadi M., Ghanbari Y., Keighobadi K., and Ercisli S. (2017). Embryo and callus induction by different factors in ovary culture of cucumber. *Journal of Applied Botany and Food Quality*, 90: 68-75.

Jesmin R., and Mian M. A. K. (2016). Callus induction and efficient plant regeneration in Cucumber (*Cucumis sativus* L.). *Journal of Bioscience and Agriculture Research*, 9(02): 796-803.

Kalloo G., and Bergh B. O. (1994). Genetic improvement of vegetable crops. Directorate of Vegetable Research, 1 Gandhinagar, Sunderpur, Varanasi, India, Pergamon Press.

Kumar N., and Reddy M. (2011). *In vitro* plant propagation: a review. *Journal of Forest and Environmental Science*, 27(2): 61-72.

Metwally E., Moustafa S., El-Sawy B., Haroun S., and Shalaby T. (1998). Production of haploid plants from in vitro culture of unpollinated ovules of *Cucurbita pepo*. *Plant Cell, Tissue and Organ Culture*, 52(3): 117-121.

Pradeepkumara N., Dey S. S., and Talukdar A. (2023). Cucumber F1 hybrid derived from two contrasting inbreds ensures high frequency gynogenesis for induction of haploids through a modified *in vitro* based protocol. *South African Journal of Botany*, 157: 314-324.

Sorntip A., Poolsawat O., Kativat C., and Tantasawat P. A. (2017). Gynogenesis and doubled haploid production from unpollinated ovary culture of cucumber (*Cucumis sativus* L.). *Canadian Journal of Plant Science*, 98(2): 353-361.

Stanghellini M. S., Schultheis J. R., and Ambrose J. T. (2002). Pollen mobilization in selected cucurbitaceae and the putative effects on pollinator abundance on pollen depletion rates. *Journal of American Society for Horticultural Science*, 127: 729-736.

Tantasawat P. A., Sorntip A., Poolsawat O., Chaowiset W., and Pornbungkerd P. (2015a). Evaluation of factors affecting embryo-like structure and callus formation in unpollinated ovary culture of cucumber (*Cucumis sativus*). *International Journal of Agriculture and Biology*, 17: 613-618.

Tantasawat P. A., Sorntip A., and Pornbungkerd P. (2015b). Effects of exogenous application of plant growth regulators on growth, yield, and *in vitro* gynogenesis in cucumber. *HortScience*, 50: 374-382.

Ugandhar T., Venkateshwarrlu M., Begum G., Srilatha T., and Jaganmohanreddy K. (2011). *In Vitro* plant regeneration of Cucumber (*Cucumis sativum* L.) from cotyledon and hypocotyl explants. *Science Research Reporter*, 1(3): 164-169.

Ye W., Xing-fang G., Sheng-ping Z., and Han M. (2015). Studies on haploid plant induction via *in vitro* unfertilized ovule culture# br# of cucumber. *Acta Horticulturae Sinica*, 42(11): 2174 -2182.

Yetisir H., and Sari N. (2003). A new method for haploid muskmelon (*Cucumis melo* L.) dihaploidization. *Scientia*

Horticulturae, 98(3): 277-283.

Zargar M., Zavarykina T., Voronov S., Pronina I., and Bayat M. (2022). The recent development in technologies for attaining doubled haploid plants *in vivo*. *Agriculture*, 12(10): 1595. DOI: https://doi.org/10.3390/agriculture12101595.

Zieliński H., Surma M., and Zielińska D. (2017). The naturally fermented sour pickled cucumbers. In: Fermented Foods in Health and Disease Prevention, Elsevier, 503-516.

Zou T., Song H., Chu X., Tong L., Liang S., Gong S., Yang H., and Sun X. (2020). Efficient induction of gynogenesis through unfertilized ovary culture with winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.). *Scientia Horticulturae*, 264: 109152.