

OPTIMUM FACTORS FOR PATHENOCARPIC CUCUMBER OVARY CULTURE

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Abstract

Parthenocarpic cucumbers, which can bear fruit without pollination, produce seedless fruits that are highly desired by consumers, agricultural producers, and breeders. However, the seedless nature of these cucumbers poses challenges for breeding improvements, often requiring extended periods. Ovary culture or unpollinated ovule culture, a plant tissue culture technique, can expedite the production of inbred lines. This research aims to investigate the factors influencing ovules and ovaries culture in parthenocarpic cucumbers by examining the influence of three factors: genotype (Hom Bai Toey, Smile, Basimah and Cengel), incubation temperature (25°C and 33°C), and TDZ concentration (0.00, 0.04, 0.06, and 0.08 mg/L). The results showed that Smile with 0.06 mg/L TDZ induced the highest percentage of ELS at 90.0%, which was not significantly different from Smile with 0.08 mg/L TDZ and Cengel with 0.08 mg/L TDZ (83.3% and 70.0%, respectively). These cultures were then transferred to Murashige and Skoog (MS) medium without hormones to induce callus or ELS development into plants, resulting in the induction of five plants. Flow cytometer and ISSR marker analyses confirmed that 40% of the plants were double haploids and 60% were mixoploids. Additionally, ISSR analysis revealed that the double haploid plants derived from cucumber ovary culture exhibited genetic differences from all the mother plants.

Keywords: Cucumber (*Cucumis sativus* L.); Doubled haploid; Ovary culture; Parthenocarpic

Introduction

Cucumber (*Cucumis sativus* L.) is a globally important vegetable with diverse uses. It can be grown throughout all regions of Thailand year-round, is easy to cultivate, and can be harvested quickly (35-45 days after planting). In 2020, cucumbers were cultivated on a total area of

37,265 rai across 68 provinces, yielding a total production of 62,132 tons (Department of Agricultural Extension, 2020). The fruiting of cucumbers depends on environmental conditions for pollination; yields may decrease in the absence of pollinators or under unsuitable conditions such as

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lack of light, high humidity, or high temperatures. Growing parthenocarpic cucumbers can address fruiting issues caused by unsuitable environmental conditions (Gou *et al.*, 2022). Parthenocarpic cucumbers can produce fruit without pollination, resulting in seedless fruits, which are desired by consumers, producers, and breeders. Currently, there are very few parthenocarpic cucumber varieties suitable for hot and humid climates and commercial use. Therefore, breeding to produce inbred lines for use as parent plants in developing new F1 hybrid varieties is important. Conventional cucumber breeding generally requires self-pollination for at least 6-8 years to achieve high genetic stability. Most parthenocarpic cucumbers are gynoeious (producing only female flowers), necessitating sex transformation and careful management of each step, making the process complex, time-consuming, and costly. Ovary or unpollinated ovule culture is a biotechnological technique used to develop plants from female reproductive cells through the process of gynogenesis, which produces haploid and doubled haploid plants. Doubled haploid plants are directly useful due to their 100% genotype stability (Kaveeta, 1998)). This method can reduce the time required and produce true-breeding plants with diverse traits within 1-2 years, thereby saving costs related to space, cultivation, and maintenance, and shortening the breeding cycle.

The *in vitro* culture of unfertilized ovaries or ovules in cucurbit species is influenced by several key factors, including the growth stage of donor plants, the developmental stage of the ovary or ovule, pretreatment conditions, plant growth regulators, and additional supplements under specific environmental and culture conditions (Gémes-Juhász *et al.*, 2002; Diao *et al.*, 2009; Chen *et al.*, 2011; Sari and Solmaz, 2021; Nyirahabimana and Solmaz, 2024). Currently, several techniques have been developed to stimulate ovary development. For example, harvesting ovaries one day before anthesis has been shown to induce the nuclei of female gametophytic cells, which are at various floral developmental stages, to initiate embryogenesis and develop into plantlets (Li *et al.*, 2013; Ozsán *et al.*, 2017). Pretreatment or post-treatment of unfertilized ovaries or ovules at high (33-35°C) or low (4°C) temperatures has a significant effect on the gynogenetic process (Svirshchevskaya and Bormotov, 1994; Pazuki *et al.*, 2018; Deng *et al.*, 2020). In cucumber ovary cultures, thidiazuron (TDZ) at concentrations ranging from 0.02 to 0.06 mg/L has been successfully used for gynogenic induction and embryo regeneration (Gémes-Juhász *et al.*, 2002; Diao *et al.*, 2009; Deng *et al.*, 2020).

However, the culture of parthenocarpic cucumber ovaries is still limited and not very successful, as ovaries rarely develop into diploid plants, depending on the genetics and conditions used in the culture (Raveerajvipul and Koonwong, 2010). Therefore, it is necessary to develop methods for culturing parthenocarpic cucumber ovaries. This study will investigate various factors affecting the culture of ovules and ovaries in parthenocarpic cucumbers. The techniques developed will be beneficial for the breeding of cucumbers or other parthenocarpic plants in the future.

Materials and Methods

Plant Material

Four cucumber genotypes were cultivated: one commercial genotype, Hombaitoey and three parthenocarpic genotypes, Smile, Basimah, and Cengel. The cultivation took place in a controlled environment greenhouse at the Scientific and Technological Equipment Center, Suranaree University of Technology (Nakhon Ratchasima, Thailand). The study area is located at a latitude of 14.881474 and a longitude of 102.015555. When the plants began to flower (25-30 days after transplantation), female flowers were selected one day before blooming, ensuring they were of similar size. These flowers were subjected to pre-treatment at 4°C for 4 days.

Ovary Culture Process

The cucumber flowers were surface-sterilized using 70% (v/v) ethanol for 1 min, followed by immersion in 20% (v/v) sodium hypochlorite for 20 min, then 10 min in 10% sodium hypochlorite, and finally washed with sterile distilled water three times. The tissues were then immersed in a 3% (v/v) H₂O₂ solution for 30 sec (adapted from Tantasawat *et al.*, 2011). The ovaries were sliced into 1-2 mm cross-sections under sterile conditions and placed on modified MS (Murashige and Skoog) medium supplemented with TDZ (Thidiazuron) hormones at concentrations of 0.00, 0.04, 0.06, and 0.08 mg/L. They were incubated at 25°C and 33°C in the dark for 2 days to stimulate initial development, then transferred to 25°C conditions with a 16/8 h (light/dark) photoperiod (80-100 µmol/m²/s) for 10 weeks. They were then transferred to hormone-free MS medium for 12-16 weeks. The results were recorded by recording the percentage of callus formation and ELS (embryo-like structure).

Statistical Analysis

To compare the effects of genotype, incubation temperature, and TDZ concentration on

the optimal conditions for inducing ELS and/or callus, a Factorial in Completely Randomized Design (CRD) with 5 replications will be used. The experiment includes 3 factors: (A) 4 cucumber genotypes: Hom Bai Toey (commercial cultivar), Smile, Basimah and Cengel; (B) 2 incubation temperatures: 25°C and 33°C; (C) 4 TDZ concentrations: 0.00, 0.04, 0.06 and 0.08 mg/L. Analyze the statistical variance of the percentage of callus and/or ELS formation. Compare the means using Duncan's New Multiple Range Test (DMRT) to evaluate the effects of genotype, incubation temperature, and TDZ concentration, as well as their interactions, on the induction of callus and/or ELS under optimal conditions. This will be done using the statistical software SPSS version 14.0 (Levesque and SPSS Inc., 2006).

Ploidy Determination by Flow Cytometer

Flow cytometry can be used to determine the DNA content of regenerated lines both before and after chromosome doubling (Baktemur *et al.*, 2022; Salehian *et al.*, 2023). First, add 500 µL of Quantum Stain NA UV 2 (A) to young cucumber leaves, finely chop, add about 0.05 g of Polyvinyl-Pyrrolidone (PVP) (to help remove waste), then suck the suspension and filter through a 30 µm nylon filter, add 500 µL of Quantum Stain NA UV 2 (B), shake the tube to mix the mixture, and measure the sample with a UV LED Flow cytometer, brand Quantum Analysis.

ISSR Analysis

Extract DNA from fresh young leaf tissue using the FavorPrep™ Plant Genomic DNA Extraction Kit (Taiwan) according to the manufacturer's instructions. Measure the DNA concentration with a Nanodrop 2000 Spectrophotometer at 260 and 280 nm. Then, amplify the DNA using ISSR-PCR with four primers: ISSR 808 ((AG)8C), ISSR 809 ((AG)8G), ISSR 811 ((GA)8C), and ISSR 834 ((AG)8YT (Levi *et al.*, 2005; Parvathaneni *et al.*, 2011; Sorntip *et al.*, 2017). Prepare a 50 microliter reaction mixture consisting of 25 µl of 2X master mix PCR reaction, 2.0 µl of each primer (at 400 nM), and 2 µl of template DNA. Adjust the volume to 50 µl with water. Perform PCR with the following conditions: 95°C for 1 min; 95°C for 15 sec; 52°C for 30 sec; 72°C for 30 sec. Separate the DNA products from the PCR reaction by size on a polyacrylamide gel using electrophoresis. Stain the gel with silver nitrate to visualize and compare the size and number of DNA bands in each sample. Record the results and compare the similarities and differences in DNA band patterns between pure line

plants (doubled haploids) and hybrid plants (donor plants)

Table 1. Effect of genotype on the percentage of callus and ELS formation

Genotype	Callus (%)	ELS (%)
Hom Bai Toey	0.83±0.83b ^{1/}	27.50±6.30b
Smile	16.67±4.13a	57.50±6.96a
Basimah	4.17±2.44b	12.50±3.90c
Cengel	0.83±0.83b	40.00±6.11b
F-test	**	**

^{1/} Data are presented as mean ± SE. ** = statistically different at the 0.01 level.

Results and Discussion

The study on the influence of three factors genotype, incubation temperature, and TDZ concentration on the percentage of callus and ELS formation revealed significant differences in callus percentage among different cucumber genotypes ($P<0.01$; Table 1). The cucumber 'Smile' had the highest rates of callus and ELS formation (16.67% and 57.50%, respectively). Upon examining the characteristics of callus and ELS, it was found that the ovary of the 'Smile' developed into callus and ELS better than other cultivars. The ovary appeared greenish-yellow with a high amount of ELS, forming green or greenish-white clumps ready to develop into various parts of the cucumber plant. The callus appeared as greenish-yellow clumps ready to develop and increase in size. This is consistent with the findings of Gémes-Juhász *et al.* (2002), who cultured ovaries of six cucumber varieties (Perez F1, 7D/4C, KS10C, D20F2A5, E10D14, and Perez ML) on an induction medium modified CBM at temperatures of 18-35°C for 1-5 days. They found that different cucumber varieties responded differently to the culture medium, with the Perez F1 and 7D/4C varieties showing the highest embryo formation rates of 18.0% and 18.4%, respectively, and the highest development into plants of 4.1% and 7.1%, respectively. Diao *et al.* (2009) cultured ovaries of six F1 hybrid cucumber varieties (Jinlv, EC5, Biyu, Caoyou No. 3, Yamei No. 1, and Jinchun) on a modified MS medium and found that ovary tissues from different varieties responded differently to the culture medium, with the Jinchun variety showing the highest embryo formation rate (72.7%). Similarly, Tantasawat *et al.* (2011), Sorntip *et al.* (2017), and Nittawatanakul (2018) found that ovary tissues from different cultivars exhibited varying responses in terms of ELS and callus formation.

Table 2. Effect of genotype on the percentage of callus and ELS formation

TDZ (mg/L)	Callus (%)	ELS (%)
0.00	0.00±0.00b ^{1/}	0.00±0.00c
0.04	5.00±3.05ab	35.00±6.53b
0.06	10.00±3.42a	47.50±6.63ab
0.08	7.50±2.53a	55.00±6.03a
F-test	*	**

^{1/} Data are presented as mean ± SE. * = statistically significant at the 0.05 level; ** = statistically significant at the 0.01 level.

In terms of TDZ concentration, it was found that 0.08 mg/L resulted in the highest percentages of callus and ELS formation (7.50% and 55.00%, respectively); however, these values were not significantly different from those obtained with 0.06 mg/L TDZ (10.00% and 47.50%, respectively) (Table 2). These results are consistent with those reported by Deng *et al.* (2020), who demonstrated that unpollinated cucumber ovaries cultured on MS medium supplemented with 0.06 mg/L TDZ achieved up to 79.3% plant regeneration. Similarly, Baktemur *et al.* (2022) found that TDZ at 0.04 mg/L in MS medium was more effective in promoting regeneration compared to media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) combined with kinetin and 6-benzylaminopurine (BAP). In another study, Li *et al.* (2013) used TDZ in combination with silver nitrate (AgNO₃) in CBM medium to induce gynogenesis from cucumber ovules, reporting that TDZ concentrations ranging from 0.03 to 0.07 mg/L resulted in gynogenic frequencies between 7.85% and 12.14%. Additionally, Diao *et al.* (2009) cultured cucumber ovaries on MS medium supplemented with various TDZ concentrations (0.00, 0.01, 0.02, and 0.04 mg/L) and found that 0.04 mg·L⁻¹ was the most effective in promoting embryo development. Notably, previous studies have confirmed that TDZ alone can replace the requirement for both auxins and cytokinins in the induction of somatic embryogenesis in several plant species (Saxena *et al.*, 1992; Visser *et al.*, 1992; Pai and Desai, 2018).

Regarding incubation temperature, culturing in the dark for 2 days at 25°C resulted in a higher callus formation rate (8.33%) compared to 33°C (2.92%) (Table 3). This finding aligns with Tantasawat *et al.* (2015) and Nittawatanakul (2018), who reported no significant difference in callus formation rates between 25°C and 35°C. For ELS formation, temperature did not significantly affect the percentage of ELS, with both temperatures yielding high ELS rates. This contrasts with Gémes-Juhász *et al.* (2002), who reported that cucumber culture at 35°C for 2-5 days resulted in the highest embryo formation

rates compared to 24°C and 28°C. Tantasawat *et al.* (2015) reported that 25°C was more effective for ELS formation than 35°C (46.27% vs. 35.22%). Deng *et al.* (2020) also found that cucumber culture at 33°C for 2 days was suitable for ovary development. However, the suitability of incubation temperature for ovary tissue culture appears to depend on the genotype of the cucumber being cultured (Sornthip *et al.*, 2017), and many plants may not respond well to high or low temperatures (Gusa *et al.*, 2006).

The study on the interactions between two factors revealed that the combination of cucumber varieties and TDZ concentration significantly influenced the percentage of ELS formation. Specifically, the ‘Smile’ with 0.06 mg/L TDZ induced the highest percentage of ELS, although this was not significantly different from ‘Smile’ with 0.08 mg/L TDZ and ‘Cengel’ with 0.08 mg/L TDZ. However, no significant interaction was observed between the variety and incubation temperature, nor between TDZ concentration and incubation temperature, on callus and ELS formation percentages. Additionally, the study of the combined effects of three factors genotype, TDZ concentration, and incubation temperature on callus and ELS formation revealed no significant interaction among these three factors regarding callus and ELS percentages.

After culturing the ovaries of four cucumber genotypes on MS medium without hormones for 12 weeks, it was found that a total of 5 plants were successfully regenerated. These included one plant from the ‘Hom Bai Toey’ (MS + TDZ 0.04 mg/L + 25°C), one from the ‘Smile’ (MS + TDZ 0.08 mg/L + 33°C), and three from the ‘Basimah’ (MS + TDZ 0.04 mg/L + 25°C, MS + TDZ 0.06 mg/L + 25°C, and MS + TDZ 0.08 mg/L + 33°C) (Table 4; Figure 1). This is consistent with the findings of Tantasawat *et al.* (2011), who reported that after culturing ovaries for 1-3 months, transferring them to hormone-free MS medium could induce the development of complete plants and roots.

Ploidy determination by flow cytometer

From the culture of cucumber ovaries of 4 genotype in different culture media, a total of

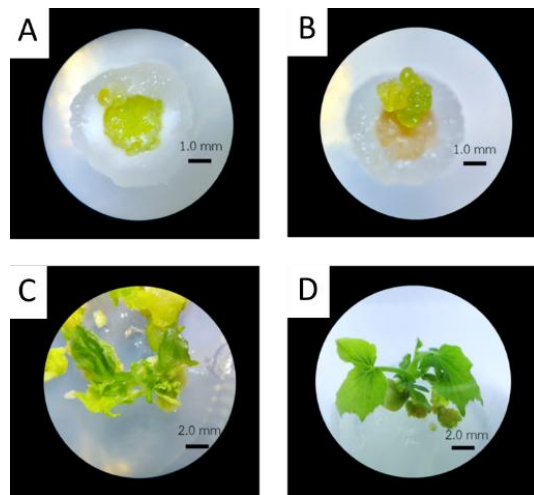
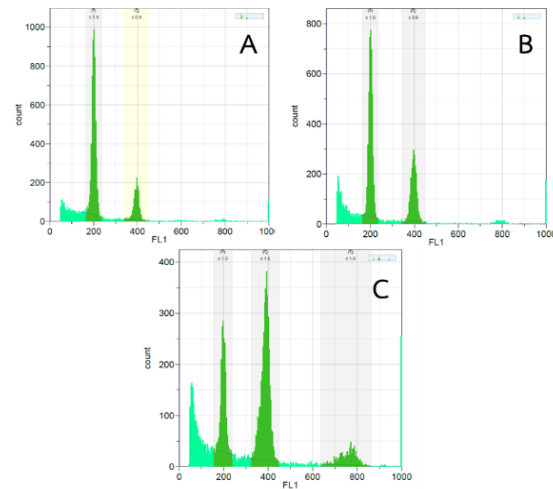
Table 3. Effect of incubation temperature on the percentage of callus and ELS formation

Temperature (°C)	Callus (%)	ELS (%)
25	8.33±2.41a ^{1/}	38.33±4.86
33	2.92±1.06b	30.42±4.20
F-test	*	ns

^{1/} Data are presented as mean ± SE. ns = not significantly different; * = statistically significant at the 0.05 level.

Table 4. Ploidy level in plants regenerated through induction

Genotype	TDZ (mg/L)	Temperature (°C)	Plant number	Double haploid	Mixoploid
Hom Bai Toey	0.04	25	1	1	
Smile	0.08	33	1		1
Basimah	0.04	25	1	1	
Basimah	0.06	25	1		1
Basimah	0.08	33	1		1

**Figure 1. Plant Regeneration from Cucumber Ovary Culture. (A) Embryo germination (B) Cotyledonary embryos (C) Shoot-like organogenesis (D) Plant regeneration****Figure 2. Chromosome number analysis of cucumber using a flow cytometer. (A) haploid (control plants) (B) double haploid (C) mixoploid**

5 plants were successfully induced. These plants were assessed for true breeding using a flow cytometer. Compared to the control plants, the results showed that 2 plants (40%) were diploid and 3 plants (60%) were mixoploid (Figure 2). However, since flow cytometry assesses DNA content at the chromosomal level, the presence of diploid and mixoploid plants derived from female gametophyte cultures may be attributed to spontaneous chromosome doubling during gynogenesis induction. This phenomenon typically occurs during mitotic division, where incomplete chromosome segregation or spindle apparatus failure leads to the formation of cells with doubled (diploid) or even higher ploidy levels. This is consistent with the findings of Tantasawat *et al.* (2011), who reported that natural double haploid plants of cucumber have a relatively high proportion, thus negating the need for chemicals such as colchicine to induce chromosomal doubling. Therefore, molecular markers are necessary to distinguish between homozygous dihaploids resulting from natural chromosomal doubling and diploids resulting from somatic tissue development (Song *et al.*, 2007).

ISSR Analysis

Four ISSR primers (ISSR 808, 809, 811, and 834), which have been reported to be effective in assessing homozygosity in cucumber (Singh *et al.*, 2016; Sorntip *et al.*, 2017), were employed to assess five cucumber plants developed from ovary culture. These plants included one from the 'Hom Bai Toey' (MS+TDZ 0.04 mg/L + 25°C) designated as H1, one from the 'Smile' (MS+TDZ 0.08 mg/L + 33°C) designated as S1, and three from the 'Basimah' (MS+TDZ 0.04 mg/L + 25°C, MS+TDZ 0.06 mg/L + 25°C, and MS+TDZ 0.08 mg/L + 33°C) designated as B1-B3, respectively. The results showed that all four primers produced polymorphic DNA bands, revealing genetic differences between the DNA extracted from the donor plants (the 'Hom Bai Toey', 'Smile', and 'Basimah' cultivars) and the plants derived from the ovary cultures of these same cultivars. Each primer generated a different number of ISSR bands: ISSR 808 yielded 2 bands (375 and 500 bp), ISSR 809 yielded 1 band (290 bp), ISSR 811 yielded 2 bands (500 and 550 bp), and ISSR 834 yielded 3 bands (175, 300, and 510 bp) (Table 5). The use of all four primers together enabled effective differentiation of genetic

Table 5. ISSR banding patterns of cucumber plants obtained from ovary culture

Plantlets	flow cytometer	ISSR (bp)							
		ISSR 808		ISSR 809		ISSR 811		ISSR 834	
		375	500	290	500	550	175	300	510
DH ^a	diploid	- ^b	-	+	-	+	+	+	+
H1	Double haploid	+	+	-	-	+	+	+	-
DS	diploid	-	-	+	-	+	-	+	-
S1	mixoploid	-	-	+	-	+	+	+	-
DB	diploid	-	-	+	-	-	-	+	-
B1	Double haploid	-	-	+	-	-	+	+	-
B2	mixoploid	-	-	+	+	+	+	-	-
B3	mixoploid	-	-	+	+	+	+	-	-

^a DH = Donor plant of the 'Hom Bai Toey', H1 = Plant derived from ovary culture of the 'Hom Bai Toey', DS = Donor plant of the 'Smile', S1 = Plant derived from ovary culture of the 'Smile', DB = Donor plant of the 'Basimah', B1-B3 = Plants derived from ovary culture of the 'Basimah' (Plants 1-3)

^b + = Presence of ISSR band, - = Absence of ISSR band

variations between double haploid plants and the donor plants, which have the same number of two chromosomal sets. Moreover, ISSR has also been successfully applied in other economically important crops to identify spontaneously occurring double haploid (DH) plants (Chen *et al.*, 1998; Hemaprabha *et al.*, 2013).

Conclusions

From the study of factors affecting the ovule and ovary culture of cucumbers, it was found that the cucumber cultivar 'Smile', when combined with TDZ at a concentration of 0.06 mg/L, achieved the highest ELS induction rate (90.0%). However, this was not significantly different from 'Smile' with TDZ at 0.08 mg/L and Cengel with TDZ at 0.08 mg/L (83.3% and 70.0%, respectively). Subsequently, the cultures were transferred to MS medium without hormones to induce the development of callus or ELS into plants. A total of 5 plants were successfully regenerated: 1 plant from cucumber cultivars 'Hom Bai Toey' (MS+TDZ 0.04 mg/L + 25°C), 1 plant from Smile (MS+TDZ 0.08 mg/L + 33°C), and 3 plants from Basimah (MS+TDZ 0.04 mg/L + 25°C, MS+TDZ 0.06 mg/L + 25°C, and MS+TDZ 0.08 mg/L + 33°C). When verified by flow cytometer, 40% of the plants were double haploid and 60% of the plants were mixoploid. Additionally, ISSR analysis showed that the double haploids obtained from the cucumber ovule cultures had distinct genetic profiles compared to the original donor plants.

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