International Journal of Agricultural and Natural Sciences
E-ISSN: 2651-3617 16(1): 90-106, 2023
doi: 10.5281/zenodo.8373169
Research Article

ROLE OF DIFFERENT PARAMETERS IN THE FORMATION OF EMBRYO-LIKE STRUCTURE AND CALLUS THROUGH OVARY CULTURE IN CUCUMBER (*Cucumis sativus* L.)

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(Received 7th January 2023; accepted 06th April 2023)

ABSTRACT. An instrumental aspect of modern plant breeding revolves around the production of haploid plants and the subsequent generation of double haploid (DH) plants. This innovation accelerates the process of achieving homozygosity through self-pollination, significantly truncating the timeline. In the present study, flowers of two cucumber varieties ('Kros' and 'Silah') were collected at three different stages: 12 hours before anthesis, at the time of anthesis, and 12 hours after anthesis following the sterilisation and culturing of ovaries in different induction mediums having Thidizuron (TDZ), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Kinetin (Kn) were used as plant growth regulators (PGRs). Ovaries were kept at 25°C for four weeks or at 35°C and 4°C for three days and then at 25°C for the remaining days for callus induction. Callus was transferred to a maturation medium having Naphthaleneacetic acid (NAA) and benzylaminopurine (BAP) as PGRs for the development of embryo-like structure (ELS). It was concluded that TDZ and the floral stage of 12 hours before anthesis showed the best results in inducing callus and ELS through ovary culture.

Keywords: *Cucumis sativus*, ovary culture, ELS, haploid

INTRODUCTION

The Cucurbitaceae family, comprising approximately 118 genera and 825 species, exhibits remarkable adaptability to diverse regions. Among the commercially cultivated species within this family, prominent members include cucumbers, watermelons, melons, and zucchinis. Cucumber, with a history of human consumption spanning over 3000 years, holds significant importance not only in the realm of nutrition but also in various industries, such as cosmetics, pharmaceuticals, and canning, particularly in the production of pickles [1, 2, 3, 4].

Cucumber production is carried out in 3,541,521 ha area in the world. In 2020, the global cucumber production reached a staggering 164 million tons, making it a vital agricultural commodity. China is leading the pack in cucumber production, boasting an impressive output of approximately 72 million tons. Turkey secures second in this ranking, producing 1.92 million tons of cucumbers. Russia follows closely as the third-largest cucumber producer, with Iran and Mexico also contributing significantly to the global production figures [5].

Cucumbers, ubiquitous in diets worldwide, hold considerable importance in human nutrition and well-being. These vegetables are shallow in calories, with just 12 calories

per 100 grams. Their alkaline nature is known to counteract the acidity of meat and dairy products, making them a valuable addition to diets promoting heart and vascular health. Cucumbers are 96% water, with 0.6 grams of protein, 0.1 grams of fat, and 2.2 grams of carbohydrates per 100 grams. While their protein, fat, and carbohydrate content may be modest, cucumbers compensate with a rich array of vitamins, enzymes, and minerals. Within that 100-gram serving, 45 IU of vitamin A, 0.03 mg of vitamin B1, 0.02 mg of vitamin B2, 0.3 mg of Niacin, 12 mg of vitamin C, 12 mg of calcium, 0.3 mg of iron, 15 mg of magnesium, and 24 mg of phosphorus [6].

Hybrid seed technology is a monumental achievement in plant genetic research, particularly for its profound impact on enhancing crop yields. The pursuit of hybrid seed breeding involves combining classical and modern breeding methods. The process can extend over numerous years when reliant solely on classical breeding approaches. The advent of modern breeding techniques, however, not only expedites the introduction of newly developed varieties to the market but also yields cost-saving advantages for breeding companies [7]. An instrumental aspect of modern plant breeding revolves around the production of haploid plants and the subsequent generation of double haploid (DH) plants. This innovation accelerates the process of achieving homozygosity through self-pollination, significantly truncating the timeline. Consequently, the production of haploid embryo-like structures (ELS) and the generation of DH plants have garnered substantial attention in both horticultural and field crop sectors in recent years. Developing precise protocols tailored to various plant species has proven invaluable to commercial entities operating in this domain, facilitating the streamlined creation of commercially viable varieties.

Various techniques have been employed in pursuing haploid plants, including gynogenesis, androgenesis, and parthenogenesis. These methodologies represent the forefront of research aimed at harnessing the potential of haploid plants for crop improvement and genetic studies. In cucumber gynogenesis, studies for haploid production are reported by Sauton [8], Niemirowicz-Szczytt and Dumas de Vaulx [9], Przyborowskiand Niemirowicz-Szczytt [10] and Gemes Juhasz et al [11]. Gynogenesis is called gynogenesis because it uses female gametes to be cultured in the appropriate inducing environment under *in vitro* conditions and to obtain haploid ELSs and plants. This technique directly cultures the ovarium containing the ovule [11].

Haploid studies in cucumber have been the focus of several research studies. One study by Shen et al. [12] investigated the diallel crossing among doubled haploids of cucumber to understand the significant reciprocal-cross differences. The researchers produced doubled haploids from divergent cucumber populations and generated reciprocal hybrids in a diallel crossing scheme. They measured plants' fresh and dry weights and estimated combining abilities and heterosis for early plant growth. The study found performance differences between reciprocal hybrids with identical nuclear genotypes. Another study by Przyborowski and Nlemirowicz-Szgzytt [10] explored the main factors affecting cucumber haploid embryo development and haploid plant characteristics. The researchers improved the method of haploid production in cucumbers by using suitable genotypes and optimising the irradiation dose. However, the preliminary experiments on cucumber haploid production yielded only a few plants. Claveria et al. [13] focused on optimising cucumber doubled haploid line production using *in vitro* rescue of *in vivo* induced parthenogenic embryos. The researchers aimed to accelerate breeding for resistant varieties by obtaining homozygous doubled haploid lines from new cucumber accessions. Gałazka and Niemirowicz-Szczytt [14] reviewed the research on haploid production in

cucumber and other cucurbits. They found that *in vitro* ovule and ovary culture is a low-efficiency method of cucurbit haploid induction, requiring further optimisation. Seguí-Simarro et al. [15] provided an overview of doubled haploid technologies *in vitro and in vivo*. They mentioned that haploidisation via pollen treatments has been reported in cucumber, but it works ineffectively with a low haploid induction rate. Haploid inducer lines are seen as an exception and are used when no alternative efficient methods are available. Hooghvorst and Nogués [16] discussed the opportunities and challenges in doubled haploids and haploid inducer-mediated genome-editing systems in cucurbits. They highlighted that in situ parthenogenesis via irradiated pollen is the preferred technique to obtain haploid plantlets in cucurbits such as cucumber. However, there are limiting factors that impede the efficient production of haploids. In conclusion, the studies on haploid studies in cucumber have focused on diallel crossing, factors affecting haploid embryo development, optimization of doubled haploid line production, and the challenges in haploid induction. These studies provide valuable insights into the methods and factors influencing haploid production in cucumbers.

Numerous studies have been conducted to induce haploid plants in various cucurbit species. For instance, Sari and Yetişir [17] employed the irradiated pollen method in melons to generate double haploid lines. Their research demonstrated successful haploid development without encountering inbreeding-related issues. Similarly, Solmaz [18] explored using gamma-ray radiation technology to induce haploidy in watermelons. However, Solmaz's findings indicated that the irradiated pollen technique still lacked reliability in consistently inducing haploidy, as it yielded a limited number of haploid plants.

Several factors influence anther culture, a pivotal aspect of haploid plant production. These factors encompass the plant's genotype, the growing conditions of donor plants, the microspore's developmental stage, the pre-treatment of flower buds, the composition of the culture media, and the conditions under which cultures are maintained [19]. Factors like genotype, flower stage, plant age, temperature, and the combination of growth hormones play a significant role in this process [20]. The choice of plant growth regulators (PGRs) and culture medium also influences the formation of embryogenic cell suspensions (ELS), callus formation, and subsequent plant regeneration. Sugars, in particular, are essential, providing energy and acting as osmotic regulators in the induction medium [21]. These considerations collectively shape the success and efficiency of haploid plant production in cucurbit species.

In light of the increasing importance of plant tissue culture techniques in crop improvement and breeding programs, this study investigates the pivotal role of various parameters in forming embryo-like structures (ELS) and callus through ovary culture in cucumber (*C. sativus* L.). Understanding the factors that influence these critical stages of plant development can contribute significantly to advancing cucumber breeding and generating haploid plants—a valuable resource for genetic studies and crop improvement efforts.

MATERIALS AND METHODS

Plant Materials

The investigation into embryo-like structures (ELS) formation through ovary culture in cucumber was conducted at the open-field agricultural research facilities within the Faculty of Agriculture, Erciyes University. The

laboratory-based work was carried out at the Tissue Culture Laboratory located at the Betül Ziya Erden Genome and Stem Cell Research Center, also affiliated with Erciyes University in Kayseri, Turkey. For this study, two distinct cucumber genotypes were employed, with seedlings procured from an agricultural company based in Antalya, Turkey. The cucumber varieties under examination were 'Kros' and 'Silah'. Donor parents for collecting female flowers designated for ovary culture were carefully selected from cucumber plants aged between 32 and 37 days. Flower samples were harvested once the second female flower became observable, marking a critical stage in the experiment's methodology.

Collecting Female Flowers

In this study, flower samples were meticulously gathered at three specific developmental stages (Fig. 1). The first stage involved collecting flowers precisely 12 hours before anthesis. The flowers remained closed at this point, eliminating concerns about inadvertent pollination. The second stage of sampling occurred at the time of anthesis. This was a critical juncture when flowers were either beginning to open or were fully open. To ensure accurate control over this stage, we took the precaution of closing the flowers one day before the anthesis. We collected them precisely when they reached this stage to prevent unintended pollination. The third and final stage involved collecting flowers 12 hours after anthesis. By this time, the flowers had undergone natural pollination. However, we employed clips to close the flowers, restricting any unnecessary pollination. Each of these carefully chosen stages played a crucial role in our research, facilitating the investigation of embryo-like structure (ELS) formation and the production of haploid plants through ovary culture in cucumbers.

Fig. 1. *Flower stages,* A)12 hours before anthesis, B) at the time of anthesis, C) 12 hours after anthesis

Preparation of Growth Medium

In tissue culture studies, the pivotal consideration lies in selecting the appropriate hormones, their concentrations, and the nutrient medium. To achieve this, various inducing and maturation hormones were meticulously combined in specific ratios, as detailed in Table 1. The fundamental components of the medium employed in the

experiment included MS (Murashige and Skoog), agar, and sugar. During the medium preparation process, careful attention was paid to adjusting the pH within the range of 5.7 to 5.8. Following the preparation, the medium underwent autoclaving at 121°C for 21 minutes to ensure sterility.

Sterilisation of Ovaries

The sterilisation of ovaries followed a stringent procedure to maintain aseptic conditions. Initially, the ovaries were rinsed thoroughly with tap water. Subsequently, within a sterile cabinet, they were immersed in a solution containing 75% ethanol for 5 minutes. Further sterilisation involved the addition of 1-2 drops of Tween-20 into a 20% sodium hypochlorite (NaClO) solution, where the ovaries were immersed for 15 minutes. Following this chemical treatment, the ovaries underwent an extensive rinsing process with sterile distilled water, repeated three times to ensure complete removal of any residual chemicals and contaminants.

Table 1. *Combinations of plant growth regulators*

	Medium	**Agar (g/L)**	**2, 4-D (mg/L)**	**Kn (mg/L)**	**TDZ (mg/L)**	**NAA (mg/L)**	**BAP (mg/L)**
Induction Medium	I1	7	0	0	-	-	-
	I2	7	0.5	1	-	-	-
	I3	7	1.0	5	-	-	-
	I4	7	1.5	10	-	-	-
	I5	7	-	-	0.06	-	-
	I6	7	-	-	0.12	-	-
Maturation Medium	M1	7	-	-	-	0.5	2
	M2	7	-	-	-	1	3
	M3	7	-	-	-	1	4
	M4	7	-	-	-	0.05	-

Culture of Ovaries

In the context of ovary tissue culture, meticulous care was taken to ensure the sterility of the ovary samples. The outer layers of the ovaries were delicately excised using sterile forceps and scalpels. This process was carried out precisely to minimise potential harm to the ovules. Additionally, any sections of the ovaries that may have sustained damage during the sterilisation procedure were thoughtfully removed. This careful approach aimed to optimise the utilisation of the nutrient medium provided for the culture. Subsequently, ovaries containing ovules were dissected into slices measuring approximately 2-3mm in thickness. These ovary slices were then carefully placed in 60mm diameter Petri dishes. Now prepared and sectioned, the ovaries were introduced into a callus induction medium for 4-6 weeks. Following this initial phase, the same protocol was followed as the ovaries were transferred to an ELS (Embryogenic-Like Structures) maturation medium.

Preliminary Heat and Cold Stress and In Vitro Culture Conditions

To assess the effects of environmental stressors, the ovary tissue underwent a regimen of both heat and cold stress for three days. For each unique combination of plant growth regulators (PGR), 15 Petri plates were

meticulously prepared. Within these 15 plates, five were exposed to a temperature of 35°C, another five were subjected to a chilling 4°C, and the remaining five were maintained at a standard temperature of 25°C. Following the 3-day stress period, all ten plates were transferred to a controlled growth chamber set at 25°C, with a light and dark photoperiod cycle of 16 hours of light and 8 hours of darkness.

Statistical Analysis

For the analysis of the obtained data, it was presented as percentages and angle transformation was applied to each sample. The Least Significant Difference (LSD) between different factors was assessed using a Student's t-test. The statistical analyses were executed using specialised statistical software, namely JMP® software (SAS Institute, Cary, NC) version 8.00. Data was analysed based on different parameters like flower stage, temperature, hormonal combination, flower stage*temperature, flower stage*hormones, temperature*hormones, temperature*flower stage*PGRs.

RESULT AND DISCUSSION

Analysis of Callus Formation in Genotype 'Kros'

The study analysed callus formation in the 'Kros' genotype, focusing on various parameters, including hormone type, hormone*flower stage interaction, and hormone*flower stage*temperature interaction. The results are presented in Table 2, which displays callus formation percentages for different combinations. Among the different hormonal combinations, the highest callus formation, 80%, was observed for the fifth combination involving 0.06mg/L TDZ. Conversely, the lowest callus formation, 46%, was recorded for the 1.5mg/L 2,4D + 10mg/L Kn combination. In the context of the hormone*flower stage interaction, the lowest value of 40% was obtained for the combination involving 1.5mg/L 2,4D+10mg/L Kn+MS with a flower stage of 12 hours before anthesis.

On the other hand, the highest callus formation values of 86% were observed in three cases: 0.06mg/L TDZ at the time of anthesis, 0.12mg/L TDZ at the time of anthesis, and 0.12mg/L TDZ 12 hours before anthesis. For the parameter combining hormone, temperature, and flower stage, the highest callus formation values were observed in three combinations: 0.06mg/L TDZ at the time of anthesis at 25°C, 0.06mg/L TDZ at the time of anthesis at 35°C, and 0.12mg/L TDZ 12 hours before anthesis at 35°C. From the analysis presented in Table 2, it can be concluded that TDZ hormone was the most effective in inducing callus in cucumber ovaries. TDZ was used in two concentrations, 0.06mg/L and 0.12mg/L, yielding similar callus formation percentages and angle transformation results. The low LSD value of 5.6794 further confirms the significance of hormonal application in callus induction.

Table 2. *% of Callus formation in genotype 'Kros'*

	Application			Callus formation (%)		
	Medium	Flower Stage	Temperature (℃)	Hormone*Stage* Temperature	Hormone*Stage	Hormone
1	I1	12 hours before anthesis	25	40 (39)	48efg (44,33)	51c (45,66)
			35	60 (51)		
			4	46 (43)		
		At the time of anthesis	25	53 (47)	51efg (45,66)	
			35	46 (43)		
			4	53 (47)		
		12 hours after anthesis	25	53 (47)	53def (47)	
			35	53 (47)		
			4	53 (47)		
2	I2	12 hours before anthesis	25	60 (51)	60cde (51)	60b (51,44)
			35	60 (51)		
			4	60 (51)		
		At the time of anthesis	25	66 (55)	68bc (56,3)	
			35	66 (55)		
			4	73 (59)		
		12 hours after anthesis	25	60 (51)	53def (47)	
			35	60 (51)		
			4	40 (39)		
3	I3	12 hour before anthesis	25	53 (47)	60cde (51)	53c (47)
			35	60 (51)		
			4	66 (55)		
		At the time of anthesis	25	46 (43)	53def (47)	
			35	60 (51)		
			4	53 (47)		
		12 hour after anthesis	25	40 (39)	46fg (43)	
			35	53 (47)		
			4	46 (43)		
4	I4	12 hour before anthesis	25	40 (39)	40g (39)	46c (43,00)
			35	40 (39)		
			4	40 (39)		
		At the time of anthesis	25	53 (47)	55def (48,3)	
			35	60 (51)		
			4	53 (47)		
		12 hour after anthesis	25	46 (43)	44fg (41.66)	
			35	53 (47)		
			4	30 (35)		
5	I5	12 hour before anthesis	25	86 (72)	77ab (63.3)	80a (67,33)
			35	73 (59)		
			4	73 (59)		
		At the time of anthesis	25	93 (81)	86a (75.3)	
			35	93 (81)		
			4	73 (64)		
		12 hour after anthesis	25	80 (63)	77ab (63.3)	
			35	80 (68)		
			4	73 (59)		
6	I6	12 hour before anthesis	25	80 (68)	86a (73.66)	79a (66,44)
			35	93 (81)		
			4	86 (72)		
		At the time of anthesis	25	86 (72)	86a (72)	
			35	86 (72)		
			4	86 (72)		
		12 hour after anthesis	25	66 (55)	64cd (53.66)	
			35	53 (47)		
			4	73 (59)		

$LSD_{Horrmone}$: 5.6794, $LSD_{Hormone*Stage}$: 9.8371, $LSD_{Hormone*Stage*Temprature}$: N.S., $LSD_{Temprature}$: N.S., LSD_{Stage}: 4.0159, $LSD_{stage*temprature}$: N.S., $LSD_{hormone*temprature}$: N.S.

In Fig. 2, we are presented with a graph illustrating the percentage of callus formation in the 'Kros' genotype across different flower stages. The data reveals that callus formation percentages vary significantly concerning flower stages. At the time of anthesis, we observe the highest percentage, 67%. This signifies that collecting flower samples precisely at the moment of anthesis yields the most favourable conditions for

robust callus growth. In contrast, callus formation percentages dip slightly to 62% when flowers are sampled 12 hours before anthesis and decrease to 57% when sampled 12 hours after anthesis. Of particular note is the peak observed at the second stage, which corresponds to the time of anthesis. However, it is essential to highlight the importance of ensuring that flowers remain closed before the onset of anthesis to prevent inadvertent pollination. Any pollination at this stage could potentially disrupt the entire experiment. In summary, the data underscores the critical role of the flowering stage in influencing callus formation, with the highest callus yield achieved when flowers are sampled precisely at the moment of anthesis. These findings provide valuable insights for optimising callus induction procedures in the 'Kros' genotype.

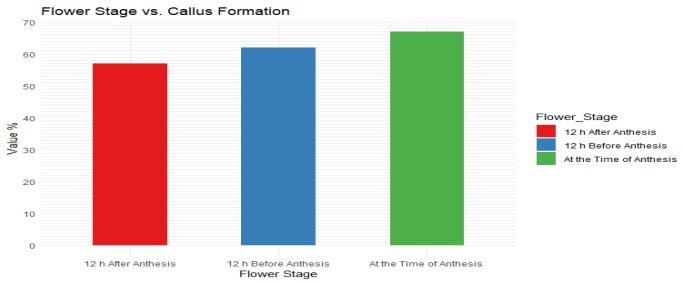

Fig. 2. *Estimation of callus formation by flower stage in 'Kros'*

In Fig. 3, the graph illustrates the percentage of callus formation in the 'Kros' genotype as a function of temperature. Specifically, the experiment examines the impact of three primary temperatures: 25°C, 35°C, and 4°C. Additionally, ovaries were exposed to two different temperature conditions as part of the preliminary stress treatment, the effectiveness of which is yet to be determined. The data reveals that callus formation percentages vary slightly across the three temperature conditions. At 35°C, a callus formation rate of 64% is observed.

Meanwhile, 25°C, callus formation is recorded at 61%, and 4°C, 60%. The similarity in callus percentages across all three temperature conditions suggests that callus formation might occur with similar outcomes, whether temperature is considered a factor. The data suggests that temperature variations within the tested range do not significantly affect callus formation in the 'Kros' genotype.

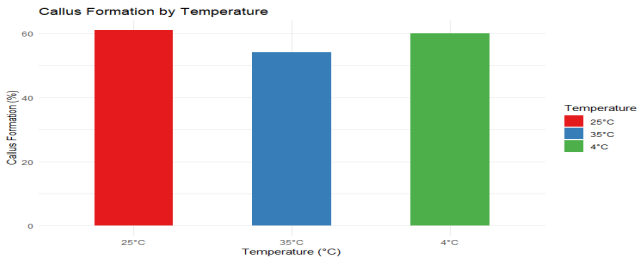

Fig. 3. *Estimation of callus formation by temperature in 'Kros'*

Fig. 4 presents a graph depicting the relationship between callus formation percentages and the combined factors of flower stage and temperature in the 'Kros' genotype. Notably, the highest callus formation percentage, reaching 69%, is observed for the condition "at the time of anthesis + 35°C," while the lowest percentage, at 53%, is recorded for "12 hours after anthesis + 4°C." In summary, the data indicates that the combined influence of flower stage and temperature was not an efficient parameter for

enhancing callus formation in the 'Kros' genotype. The lack of statistical significance underscores that this particular factor combination does not significantly impact the outcome of callus induction in this experimental context.

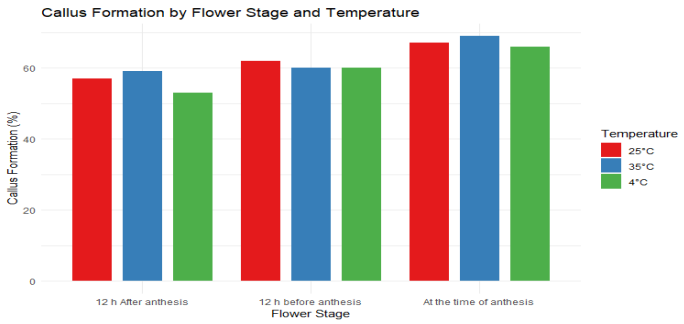

Fig. 4. *Estimation of callus formation by Stage*Temperature in 'Kros'*

Fig. 5 illustrates the percentage of callus formation in the 'Kros' genotype concerning the combined factors of hormone and temperature. The highest callus formation percentage, reaching 86%, is observed for the condition involving 0.06mg/L TDZ at 25°C. Conversely, the lowest percentage, at 42%, is recorded for the condition with 1.5mg/L 2,4D and 10mg/L Kn at 4°C. In summary, the data indicates that the combined influence of hormone type and temperature does not appear crucial in promoting or inhibiting callus formation in the 'Kros' genotype.

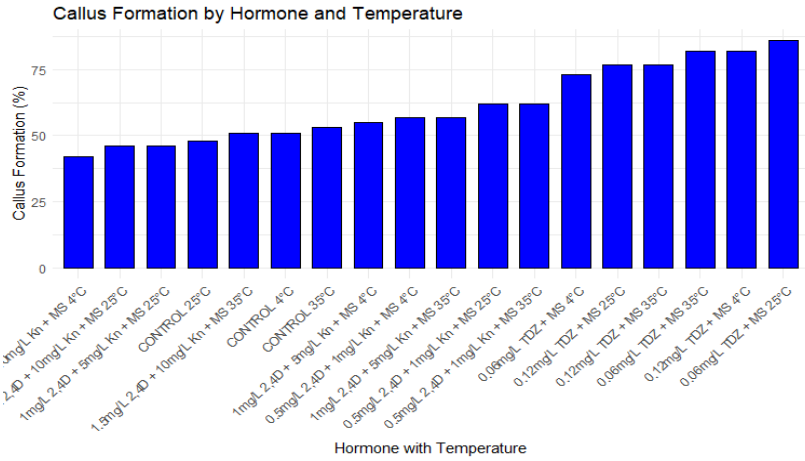

Fig. 5. *Estimation of callus formation by Hormone*Temperature by 'Kros'*

Analysis of Callus Formation in Genotype 'Silah'

Callus formation percentages were meticulously recorded in the 'Silah' genotype following 4-6 weeks of ovary culture, focusing on various parameters. Notably, the interaction between hormone, temperature, and flower stage did not significantly impact callus formation, as evidenced by non-significant LSD values. However, when considering the parameter of hormone and flower stage (Hormone*Stage), significant effects were observed, with the highest callus formation occurring under conditions such as "at the time of anthesis + 0.06mg/L TDZ" and "12 hours before anthesis + 0.12mg/L TDZ." Additionally, the parameter of hormone alone also yielded significant variations, with the highest callus formation percentage recorded for 0.12mg/L TDZ and the lowest for 1.5mg/L 2,4D + 10mg/L Kn. These findings underscore the nuanced influences of

different parameters on callus formation in the 'Silah' genotype, providing valuable insights for optimising callus induction procedures in this context.

Table 3. *% of callus formation in genotype 'Silah'*

	Applications			Callus formation %		
	Hormone	Flower stage	Temperature (℃)	Hormone*Stage* Temperature	Hormone* Stage	Hormone
1	I1	12 hours before anthesis	25	73.33(59.21)	62.22bcd (52.42)	62.96c (54.52)
			35	46.66 (43.07)		
			4	66.66 (54.99)		
		At the time of anthesis	25	60 (56.15)	57.77def (51.40)	
			35	60 (50.77)		
			4	53.33 (47.30)		
		12 hours after anthesis	25	53.33 (46.92)	68.88bc (59.74)	
			35	80 (68.07)		
			4	73.33 (64.22)		
2	I2	12 hours before anthesis	25	73.33 (59.21)	71.11b (57.80)	54.81cd (47.98)
			35	66.66 (54.99)		
			4	73.33 (59.21)		
		At the time of anthesis	25	53.33 (46.92)	48.88efg (44.35)	
			35	40 (39.23)		
			4	53.33 (46.92)		
		12 hours after anthesis	25	46.66 (43.07)	44.44fgh (41.79)	
			35	46.66 (43.07)		
			4	40 (39.23)		
3	I3	12 hours before anthesis	25	53.33 (46.92)	46.66fgh (43.07)	48.8de (44.44)
			35	46.66 (43.07)		
			4	40 (39.23)		
		At the time of anthesis	25	46.66 (43.07)	44.44fgh (41.79)	
			35	40 (39.23)		
			4	46.66 (43.07)		
		12 hours after anthesis	25	60(51.14)	55.55efg (48.45)	
			35	60(51.14)		
			4	46.66(43.07)		
4	I4	12 hours before anthesis	25	53.33(46.92)	53.33efg (46.92)	42.96e (40.73)
			35	53.33(46.92)		
			4	53.33(46.92)		
		At the time of anthesis	25	46.66(43.07)	33.33h (34.88)	
			35	26.66(30.79)		
			4	26.66(30.79)		
		12 hours after anthesis	25	46.66(43.07)	42.22gh (40.38)	
			35	46.66(43.07)		
			4	33.33(35.01)		
5	I5	12 hours before anthesis	25	53.33(47.30)	60cde (52.81)	73.3b (62.51)
			35	66.66(60)		
			4	60(51.14)		
		At the time of anthesis	25	93.33(81.14)	88.88a(75.24)	
			35	86.66(72.29)		
			4	86.66(72.29)		
		12 hours after anthesis	25	73.33(59.21)	71.11b(59.47)	
			35	60(51.14)		
			4	80(68.07)		
6	I6	12 hours before anthesis	25	80(68.07)	88.88a(76.78)	82.22a(71.10)
			35	93.33(81.14)		
			4	93.33(81.14)		
		At the time of anthesis	25	86.66(76.92)	86.66a(75.38)	
			35	86.66(76.92)		
			4	86.66(72.29)		
		12 hours after anthesis	25	73.33(63.84)	71.11b(61.14)	
			35	53.33(47.30)		
			4	86.66(72.29)		

LSD_{stage} :N.S., $LSD_{hormone}$:6.86, $LSD_{temperature}$:N.S., $LSD_{stage*hormone}$:12.27, $LSD_{stage*temperature}$:N.S., $LSD_{hormone*temperature}$:N.S., $LSD_{stage*hormone*temperature}$: NS.

Fig. 6 presents the results of callus formation percentages with flower stage as the parameter of interest. The highest callus formation percentage, reaching 63%, is observed for the condition "12 hours before anthesis," followed by 60% at "the time of anthesis," and 58% for "12 hours after anthesis." However, it is essential to note that the LSD (Least Significant Difference) test value for the flower stage parameter is found to be non-significant. This implies that the flowering stage alone cannot yield significantly different or virtuous results regarding callus formation. The data suggests that callus formation percentages vary slightly with different flower stages. Still, this parameter, on its own, does not significantly influence the outcome of callus induction in this experimental context.

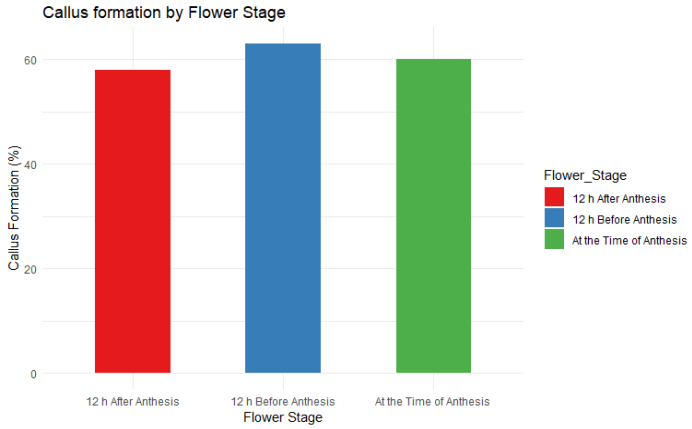

Fig. 6. *Estimation of callus formation by flower stage in 'Silah'*

Fig. 7 displays the relationship between temperature and callus formation percentages. It is evident from the graph that the values do not exhibit significant variation. The highest callus formation percentage, at 62%, is recorded at 25°C, while the lowest percentage, at 58%, is observed at 35°C. Crucially, the LSD test value for the temperature parameter is insignificant. This implies that temperature, in isolation, does not significantly impact the outcomes of callus induction. In summary, the data demonstrates that the different temperature conditions tested in the experiment do not yield markedly different results in terms of callus formation percentages. Therefore, temperature alone is not crucial in achieving noteworthy changes in callus formation

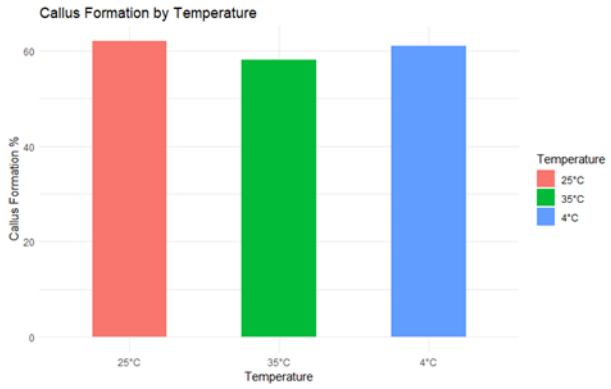

Fig. 7. *Estimation of callus formation by temperature in 'Silah'*

As a bar graph, Fig. 8 shows the relation between temperature*flower stage and callus formation %. The highest value of 64% is observed for the combination 1) 12 hours before

anthesis+4°C, 2) 12 hours before anthesis+ 25C, and 3) at the time of anthesis+ 25°C. lowest value is for the combination at the time of anthesis+35°C, which is 57%. The LSD test value is not significant, which means this parameter did not seem to be crucial in callus formation.

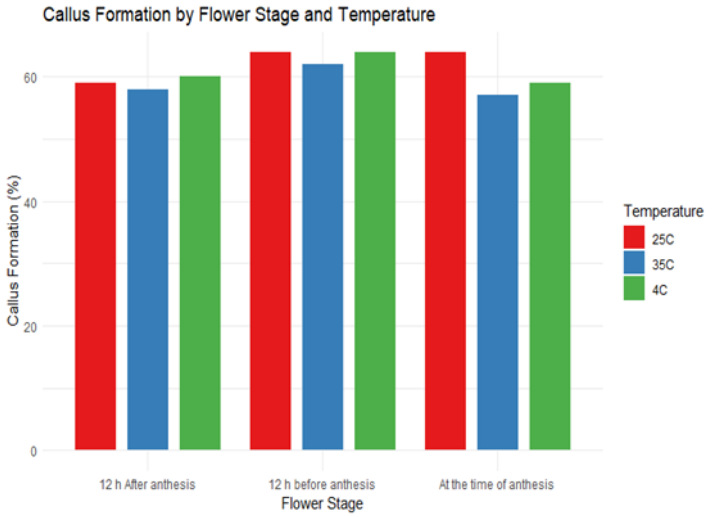

***Fig. 8**. Estimation of callus formation by Temperature* Stage in 'Silah'*

In Fig. 9, the graph demonstrates the relation between parameter temperature*hormone and callus formation. The highest value, 89%, is seen in combination with 0.12mg/L TDZ+4°C, and the lowest value, 38%, is observed in the case of 1.5mg/L 2,4D Kn. + 4°C. LSD test value for parameter temperature*hormone is insignificant, which means it is worthwhile to use as a single factor in callus formation.

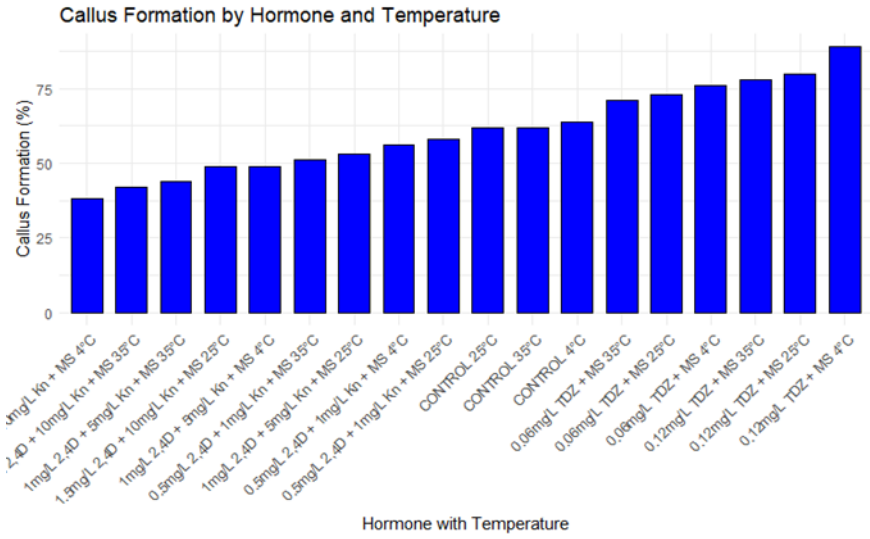

***Fig. 9**. Estimation of callus formation by PGRs*Temperature in 'Silah'*

After 4-6 weeks of callus culture, we carefully examined the formation of ELSs in the petri plates, focusing solely on the type of hormone used as the parameter of interest without considering other variables. We selected four different hormonal combinations, as detailed in Table 1, and cultured the callus under these conditions while maintaining a

constant temperature of 25°C in the growth incubator. Unfortunately, ELS formation was limited, and only a few ELS were observed, as depicted in Fig. 2. Statistical analysis was conducted separately for both genotypes, explicitly focusing on the single parameter "hormone." Surprisingly, the highest ELS formation was achieved in both genotypes with the same hormonal combination. Table 4 provides the ELS percentages for the 'Kros' genotype. According to the data, the highest percentage of ELS, at 15%, was obtained with the hormonal combination of 1mg/L NAA and 3mg/L BAP. The LSD test value was found to be non-significant.

Similarly, Table 5. presents ELS percentages for the 'Silah' genotype concerning hormonal combinations. The highest value, reaching 25%, was observed for the hormonal combination of 1mg/L NAA and 3mg/L BAP. Once again, the LSD test value was not deemed significant. NAA and BAP emerged as crucial factors in callus maturation and ELS formation genotypes. Interestingly, the 'Silah' genotype exhibited a slightly higher ELS than the 'Kros' genotype, although the difference was not notably substantial.

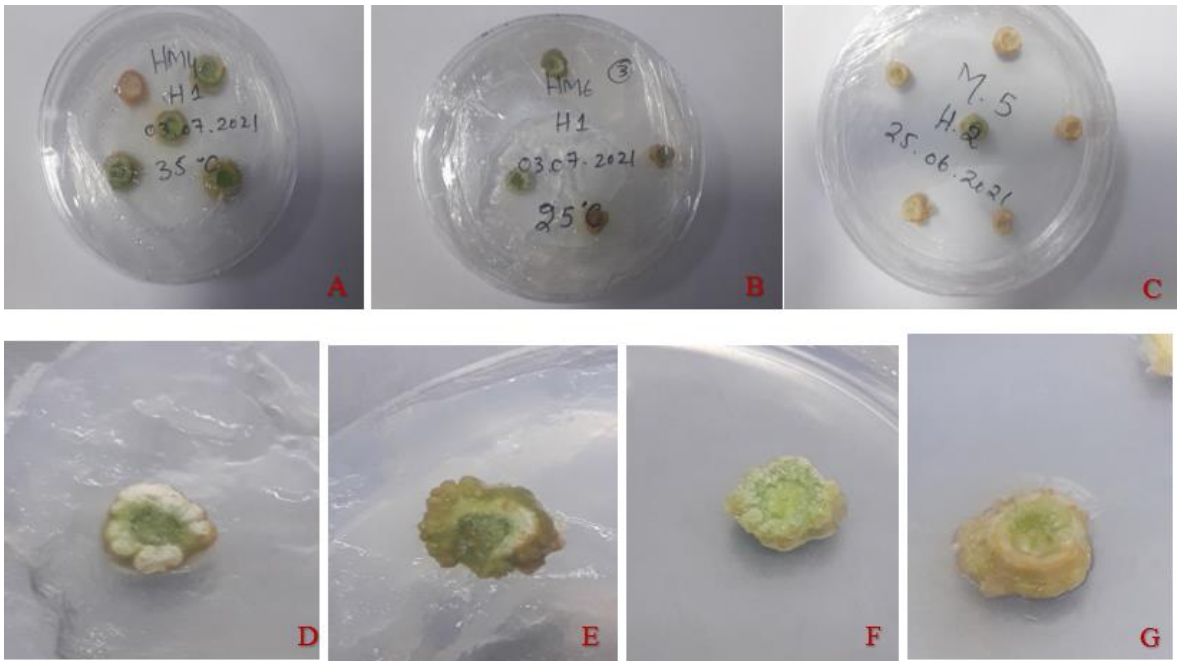

Fig.2. *Callus and ELS obtained from varieties' Silah' and 'Kros'*

Table 4. *% of ELS formation in genotypes' Kros' and 'Silah'*

Variety	% of ELS formation in Cucumber genotypes	
	Hormone	ELS formation
'Kros'	M1	0 (0.00)
	M2	15 (16.44)
	M3	10 (9.80)
	M4	0 (0.00)
'Silah'	M1	0 (0.00)
	M2	25 (29.73)
	M3	10 (9.80)
	M4	5 (6.64)

Genotypes of donor plants significantly affected ELS and callus formation abilities among five Thai cucumber cultivars [22]. In our study, we also got different results in

both varieties. We got more ELS in 'Silah' than in 'Kros'. Temperature shock (high or low temperature) is preferred to improve gynogenesis by diverting normal gametophytic development into a sporophytic mode of production, resulting in callus and ELS formation [23, 24]. Recently, it has been shown that thermal shock pre-treatment of ovary slices at 35°C for 2–4 days significantly induced the ELS formation of six Chinese cucumber cultivars [25]. Gemes-Juhasz et al. [11] also reported that 35°C thermal shock pre-treatment was effective in the cucumber ovary culture of five parthenocarpic breeding lines and a hybrid variety. Using six cucumber hybrids, it has been reported that the highest proportion of ELS was obtained with three days of 35°C pre-treatment [26]. Similarly, pre-treatment of summer squash ovaries at 4 or 32°C for four days produced a better ELS response than untreated control [23]. Likewise, we got different results for heat and cold shock treatments in both varieties. In 'Kros', callus formation was observed highest at 35°C, while in 'Silah', the best results were seen at 25°C. Insensitivity to thermal and cold pre-treatment on several plants has also been reported [27, 28]. It was shown that squash ovules without cold pre-treatment at 4°C produced a better ELS response than the ones treated at 4°C for 2, 4, or 8 days [27]. Similarly, pre-treatment at 4°C for up to 9 days or at 32°C for one day did not improve the gynogenic development of pistil culture [28]. Our research also shows that temperature is not a significant parameter for callus formation. ELS and callus formation potentials varied significantly when using different plant growth regulators. Overall, M2 was the best maturation medium for ELS formation, while I5 and I6 were the best induction medium for callus formation in both varieties. Although some researchers were successful in obtaining haploid/doubled haploid plants from ovule-derived calli [29, 25, 30], others failed in obtaining haploids, like Lazarte and Sasser [31], who used cucumber to produce haploid plants through in vitro culture, reported that ELSs developed following callus development, but haploid plants could not be obtained. Similarly, we could not regenerate any plant from calli or ELS. Therefore, M2, which induced the highest ELS formation, was also considered the best maturation medium for efficient haploid/doubled haploid production. It is interesting to note that in both varieties, the highest ELS formation was induced by M2, which contains NAA and BAP and for callus induction, I5 and I6 performed best in both varieties containing TDZ either 0.06mg/L or 0.12mg/L with MS. TDZ has been frequently reported as the most efficient growth regulator for gynogenesis and calli induction in cucurbit crops [11, 32, 25, 33, 34]. The % of ELS formation obtained in Silah on M2, which contained 0.06 mg/L TDZ (38.4 to 64.6%), was comparable to TDZ is used by many researchers like it was reported by Diao et al. [25] using 0.02 mg/L TDZ with six Chinese cucumber cultivars (20 to 65.7%), confirming the TDZ efficiency of the ovary culture of cucumber. However, at higher TDZ concentrations (0.04 mg/L), they found up to 72.7% ELS formation. In melon, 0.04 mg/L TDZ could also induce ELS formation of a Chinese cultivar up to 76.6% when combined with 4°C pre-treatment for four days [33]. When using I5 and I6, which contained a high TDZ concentration (0.06mg/L and 0.12mg/L, respectively) together with MS, we were able to induce callus formation efficiency (up to 80% and 79% in 'Kros' while 72% and 83% in 'Silah'). This new induction medium will be useful for haploid/doubled haploid production. Tantasawat et al. [22] found that more ELSs developed after transferring to the differentiation medium having NAA and BAP. However, all differentiation media led to an equivalent % of ELS and callus formation. Our study used NAA and BAP as ELS maturating medium, and the highest ELS was obtained from M2 (1mg/L NAA and 3m/L BAP). The first study on "Ovule-Ovary culture" in the Cucurbitaceae family was the study of Chambonnet and Dumas de Vaulx

[35] on squash. In this study, the researchers reported that the ovules they isolated from the squash ovaries taken 1 or 2 days before the anthesis period gave the most successful results. In our research, we collected flowers at three different stages (12 hours before anthesis, at the time of anthesis, and 12 hours after anthesis); however, callus formation was higher only at 12 hours before anthesis in 'Silah' while in 'Kros' it was seen at 12 hours before and at the time of anthesis.

CONCLUSIONS

This study examined the effect of temperature, flower stage, PGRs, and genotype on callus and ELS formation in cucumber plants. Genotypes perform differently under the same growing conditions and play an efficient role in haploid formation. In our study, we got 25% ELS in 'Silah' and 15% in 'Kros'. ELS formation leads us to the bright side of a picture and gives us a different aspect of conditional analysis. Further improvements are still needed, and more protocols should be followed to get haploid embryos and plants. Biotechnology and plant tissue culture studies continue to gain momentum worldwide. With the advancement in this field, there has been significant progress in haploid plant production. In recent years, the CrisprCas9 technology, which has swept across the globe, has made it possible to obtain haploid plants [36, 37] . Consequently, combining classical and evolving modern technologies makes it possible to obtain haploid plants, which play a crucial role in plant breeding.

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