

Callus and Embryo Induction from Anther Culture of *Cucumis melo* L. cv. Glamour

Fadzlin Qistina Fauzan^{1,2}, Arifullah Mohammed³, Ahmed Mahmood Ibrahim^{4*}, Raimi Mohamed Redwan¹, Suhana Zakaria¹, Dwi Susanto⁵, Mohd Razik Midin⁶, and Saleh Altuwaijri⁷

¹Faculty of Agro-Based Industry, Universiti Malaysia Kelantan, 17600 Jeli, Kelantan, Malaysia

²Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia

³Department of Biotechnology, Koneru Lakshmaiah University, 522 302 Vaddeswaram, Guntur, Andhra Pradesh, India

⁴Center for Biotechnology and Environmental Technology, University of Fallujah, 31002 Fallujah, Anbar, Iraq

⁵Faculty of Science and Technology, Universitas Halim Sanusi, Jl. Taman Siswa No. 4, Malabar, Kec. Lengkong, 40116 Bandung, West Java, Indonesia

⁶Department of Plant Science, Kulliyah of Science, International Islamic University Malaysia (IIUM), Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

⁷College of Veterinary Medicine, Qassim University, 52571 Buraidah, Al-Qassim, Saudi Arabia

ABSTRACT

To establish an efficient haploid production system for melon breeding, this study was conducted at the Laboratory of Universiti Malaysia Kelantan, Kelantan, Malaysia, in 2023, to evaluate the effects of cold pretreatment, surface sterilisation duration, and plant growth hormone concentrations on callus induction and embryogenesis in anther culture of *Cucumis melo* cv. Glamour, an important commercial melon cultivar. Early morning, unpollinated male flowers were harvested from 35

to 45-day-old plants. Sodium hypochlorite (NaOCl) containing Tween-20 was used as a surface sterilisation solution for anthers at 4, 7, and 4°C after cold pretreatment for 10, 20, and 30 minutes. Cultures were established on Murashige and Skoog (MS) basal medium supplemented with varying concentrations of 2, 4-D and BAP. Incubation was carried out at 25±2 °C under dark conditions. A ruler and graph paper were used to measure average callus size. Embryogenic structures were morphologically screened and validated through flow cytometry. All treatments resulted in 100% callus induction, but callus size and quality varied with treatment

ARTICLE INFO

Article history:

Received: 02 August 2025

Accepted: 03 June 2026

Published: 26 June 2026

DOI: <https://doi.org/10.47836/pjtas.49.3.18>

E-mail addresses:

fadzlinqistina279@gmail.com (Fadzlin Qistina Fauzan)

arifullahmd@gmail.com (Arifullah Mohammed)

ahmed.m.ibraheem@uofallujah.edu.iq (Ahmed Mahmood Ibrahim)

raimi@umk.edu.my (Raimi Mohamed Redwan)

suhana@umk.edu.my (Suhana Zakaria)

dwisusanto@uhs.ac.id (Dwi Susanto)

mohdrazik@iium.edu.my (Mohd Razik Midin)

stojery@qu.edu.sa (Saleh Altuwaijri)

* Corresponding author

conditions. The 3-day cold pretreatment significantly enhanced callus formation, while 20-minute sterilisation was optimal for healthy, viable callus. The medium containing 2, 4-D (5.0 mg/L) and BAP (0.5 mg/L) produced the largest callus and highest proliferation rate, whereas BAP (4.0 mg/L) combined with NAA (0.05 mg/L) was most effective for inducing embryogenesis. A flow cytometry analysis revealed that most calli were mixoploid. The results contribute to the development of a new protocol for anther culture in *C. melo*, which may be useful in improving the melon crop.

Keywords: Anther culture, callus, embryo, haploid, plant growth regulators

INTRODUCTION

Plant tissue culture is a foundational tool in modern plant biotechnology, enabling the regeneration of whole plants from isolated cells or tissues under controlled and sterile conditions. The concept of cellular totipotency, first proposed by Gottlieb Haberlandt in 1902 and later validated by Knudson's successful *in vitro* germination of orchid seeds in 1922, laid the foundation for what is now an essential technique in plant propagation and breeding (Bridgen et al., 2018; Thorpe, 2007). Among various *in vitro* approaches, androgenesis (anther culture) and gynogenesis (ovary culture) have emerged as powerful strategies for producing haploid and doubled haploid (DH) lines, which significantly accelerate the development of homozygous lines for hybrid seed production.

Tissue culture success relies on multiple factors, including the type of explant, culture medium composition, hormone balance, and pre-treatments applied to enhance response. The MS medium is popular for being a full supplement medium; however, it usually needs optimisation to meet the species requirements (Phillips & Garda, 2019). Hormones involved in the manipulation of *in vitro* morphogenesis, particularly in the induction of callus and somatic embryogenesis and shoot regeneration effects, are the plant growth regulators (2, 4-D and NAA as auxins and BAP and TDZ for cytokinins). Other medium constituents, such as sucrose, vitamins, and jellifying agents, also promote the *in vitro* plant growth.

Anther culture has been successfully applied in several cucurbit species, such as cucumber (*Cucumis sativus*), squash (*Cucurbita pepo*) and bitter melon (*Momordica charantia*) to induce callus and haploids (Asadi et al., 2018; Habiba, 2016; Nguyen et al., 2019). Nevertheless, species and cultivar-specific responses are well known, and protocols often need to be fine-tuned. Likewise, ovary culture is an effective alternative for haploid production, although they are less efficient than anther culture, which is useful in situation for anther culture has very low efficiency or is impossible (Deng et al., 2020; Ozsan et al., 2017). Especially, the fertility of both methods predominates upon pretreatments like cold and heat shocks affecting microspore reprogramming and embryogenesis initiation (Kurtar et al., 2016; Rakha et al., 2012).

Although the *in vitro* culture of the plant is well established, *C. melo* cv. Glamour has not yet been fully exploited for the stable protocols of haploid and double haploid plants production. Even though both androgenesis and gynogenesis have been investigated in other cucurbits, there are no optimised and reliable procedures established in summer squash (SS), hampering its use in breeding programs that need homozygous lines as parents. Furthermore, although cold preconditioning is widely used to increase the induction of callus, its impact on the reproductive tissues of *C. melo* cv. Glamour has not been completely explained. However, its role in anther culture is still unclear and deserves investigation. Some studies found that the cold treatment could suppress gynogenesis in the cucurbits; hence, the conflicting reports regarding the impact of cold exposure on anthers remain highly controversial (Ibrahim et al., 2023a).

Furthermore, the hormone-mediated impact on callus and embryo formation was not investigated systematically in this cultivar. Overall, most comparative studies looked at select numbers of hormone combinations, usually with no statistical resolution. Understanding how auxins and cytokinins interact across a wider range of concentrations is essential for controlling regeneration potential and embryogenic response. Finally, although tissue culture-induced ploidy variations, including mixoploidy and polyploidy, are well documented, there have been only a limited number of reports describing the use of flow cytometric examination of genetic stability in *C. melo* regenerants or clones obtained from anther or ovary cultures.

This study aimed to address these gaps by examining the effects of cold pretreatment, sterilisation duration, and hormone concentration on the induction of callus and embryo in anther culture of *C. melo* cv. Glamour. In addition to morphological evaluation, flow cytometry was employed to assess ploidy level, thereby contributing to the development of a robust *in vitro* protocol for future melon breeding and genetic improvement efforts.

MATERIALS AND METHODS

Anther Culture

The experiments were conducted in 2023 at the Laboratory of Universiti Malaysia Kelantan, Kelantan, Malaysia. Male flower buds of *C. melo* were collected from 35 to 45-day-old plants early in the morning when the temperature was low, and the plant water content was high, as suggested by Hatfield and Prueger (2015). Buds were pre-treated at 4-7 °C for 0, 1, 2, or 3 days. Surface sterilisation involved immersion in 70% ethanol for one minute, rinsing with autoclaved distilled water, shaking in a commercial stock solution of 5.25% sodium hypochlorite (NaOCl) supplemented with Tween-20 for 10, 20, or 30 minutes, followed by three final rinses with autoclaved distilled water. Sterilised flower buds were placed on filter paper to remove residual sterilant and water.

Anthers were excised from filaments and petals, then separated into segments and cultured on MS medium supplemented with plant growth regulators (Figure 1). Cultures were maintained at 25 ± 2 °C under dark conditions for callus induction and embryo initiation. Ploidy level of the calli was assessed using flow cytometry.

To prepare one litre of culture medium, 4.33 g MS basal powder, 30 g sucrose, 5.5 g agar, and 0-5.0 mg/L of plant growth regulators were used. The pH was adjusted to 5.6-5.8 to provide optimal conditions for explant growth. Autoclaving was conducted at 121 °C for 15 minutes as a sterilisation step (Ahmed et al., 2016 & Ibrahim et al., 2023b).

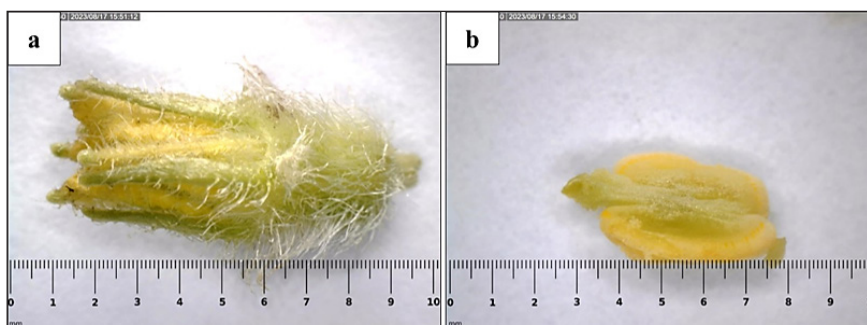


Figure 1. The condition and approximate size of (a) a closed *C. melo* male flower and (b) anther used in this study

Flow Cytometry

To determine the samples' ploidy levels using flow cytometry, each sample was chopped into small pieces and placed in separate petri dishes. Nuclei were extracted by manually chopping the tissues in lysis buffer. Among the three tested buffers (LBO1, Tris-MgCl₂, and Otto), LBO1 was chosen as it gave the clearest histogram peaks as suggested by Midin et al. (2017) and Tomaszewska et al. (2021).

The nuclei suspensions were filtered through a 50 µm nylon mesh, then treated with RNase A and propidium iodide (PI). PI was used as it binds to DNA and produces accurate fluorescence peaks with low variation (Doležel and Bartos, 2005; Doležel et al., 2007; Midin et al., 2017).

Samples were incubated at 4 °C for 5 minutes before analysis through a FACSCalibur flow cytometer (Becton Dickinson, USA) with a 488 nm laser. Each run recorded 5000 events across 1024 channels. Young leaves or cotyledons from non-tissue culture plants were used as ploidy controls. Peak data were analysed using CellQuest3Q software, following the method by Midin et al. (2017).

Experimental Design and Statistical Analyses

All the experiments were arranged in a Completely Randomised Design (CRD) with five replications per treatment. The one-way ANOVA was used to analyse the statistical

data of cold-pre-treated durations, surface sterilisation exposure time and plant growth regulator combinations. Data was analysed and segregated using Duncan Multiple Range Test (DMRT) at a significance level of $P \leq 0.05$. SPSS software (Version 25.0) was used for the statistical analyses.

RESULTS

Preliminary Study

In the preliminary study, anther culture successfully induced callus formation under a 0-day cold pretreatment and 10-minute 5.25% NaOCl + 3 drops of Tween-20 surface sterilisation, following the methods by Song et al. (2007) and Golabadi et al. (2017). Among the hormone combinations tested, 2, 4-D (1.5 mg/L) and BAP (0.75 mg/L) produced the highest amount of callus and the largest callus size. Therefore, these three conditions, which are cold pretreatment duration, sterilisation time, and hormonal concentrations, were selected for further optimisation. The use of 2, 4-D and BAP in the induction medium was followed by Kurtar et al. (2016).

The Effect of Cold Pretreatment

Anther-derived callus induction and proliferation were periodically examined during the cold pretreatment, and the callused structures were evaluated at eight to twelve weeks of culture. Surface sterilisation was standardised at 10 minutes of 5.25% NaOCl along with Tween-20 treatment, and a hormonal regime of 2, 4-D (1.5 mg/L) + BAP (0.75 mg/L) was uniform in all treatments. In the current study, formation of indicative callus was observed in all treatment groups irrespective of duration of cold pretreatment (0, 1, 2, 3 days at 4-7 °C) or surface sterilisation time period (10, 20, 30 minutes in 5.25% NaOCl along with Tween-20) and each treatment was found with 100% callus induction as recognized in some research (Ahmed et al., 2015). However, no callus was observed in hormone-free MS0 media, indicating that callus formation was strictly dependent on the presence of plant growth hormones. Induction rates were calculated by dividing the number of replicates producing calli by the total number of replicates and expressing the result as a percentage (Naïtchéde et al., 2023).

As shown in Figure 2 (2A-2D) and Table 1, all cold pretreatment durations were capable of triggering callus induction in less than four weeks. Although 100% induction was recorded across all treatments, the proliferation and the callus size varied. The largest callus was observed in media 2D (3-day cold pretreatment), followed by media 2B, 2C, and 2A. A one-way ANOVA was conducted to statistically assess whether the mean callus sizes differed significantly among the cold pretreatment groups. The analysis yielded $F(3, 16) = 3.297$, $p = 0.05$, indicating that differences among group means were not statistically significant at the conventional level, thus supporting the null hypothesis.

However, post hoc analysis revealed significant pairwise differences between certain treatment groups. This highlights that while overall group variation was not statistically confirmed, specific comparisons reveal meaningful differences. In Table 1, treatment means followed by different letters denote statistically significant differences.

The Effect of Surface Sterilisation (Exposure Time)

In assessing the effect of surface sterilisation duration on callus induction and proliferation, anther cultures were monitored periodically, and results were also analysed after eight to twelve weeks of culture. Based on preliminary callus induction results, cold pretreatment was fixed at 0-day duration, and the hormonal combination of 2, 4-D + BAP was maintained across all

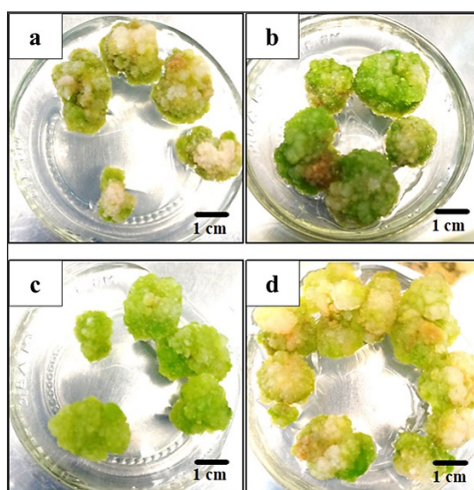


Figure 2. Callus proliferation after 12 weeks, under different durations of cold pretreatments (CPT), (a): 0-day CPT, (b): 1-day CPT, (c): 2-day CPT, and (d): 3-day CPT

Table 1

The effect of different durations of cold pretreatment on callus induction and proliferation

Experiment	Media (Label)	Duration of Cold pretreatment (4 -7 °C)	Percentage of Callus Induction	Average Diameter of the Callus (mm) after 12 Weeks (5 Replicates)
Assessment of the duration of cold pretreatment	Figure 2a	0 day	100%	16.2 ± 0.97 ^b
	Figure 2b	1 day	100%	18.2 ± 1.86 ^b
	Figure 2c	2 days	100%	16.8 ± 1.39 ^{ab}
	Figure 2d	3 days	100%	22.4 ± 1.78 ^a
MS0	-	0 day	0%	-

Note. *Means with the same letter were not significantly different from each other (P<0.05 ANOVA followed by DMRT)

treatments. All surface sterilisation durations (10, 20, and 30 minutes of NaOCl + Tween-20) resulted in 100% callus induction (Table 2). A 100% induction rate was recorded when all replicates within a treatment successfully formed calli.

Callus induction was found to be strongly dependent on the presence of plant growth regulators. No callus formed in the hormone-free medium (MS0), regardless of sterilisation duration, emphasising that hormonal supplementation is critical for initiating callogenesis. While surface sterilisation duration was a controlled variable, its influence on callus formation was only evident in the presence of hormones. Positive callus induction was observed in anthers sterilised for 10, 20, and 30 minutes using NaOCl + Tween-20 in 2E-2G media (Figure 3). All treatments successfully induced calli, with variations in proliferation and callus size depending on the sterilisation duration.

A one-way ANOVA was conducted to compare the effects of different surface sterilisation durations (5.25% NaOCl + Tween-20) on callus size (mm). The analysis indicated no statistically significant differences among the groups $F(2, 12) = 3.764$, $p = 0.054$. The p -value of 0.054 slightly exceeds the 0.05 threshold, indicating that the differences in callus size across sterilisation durations were not statistically significant

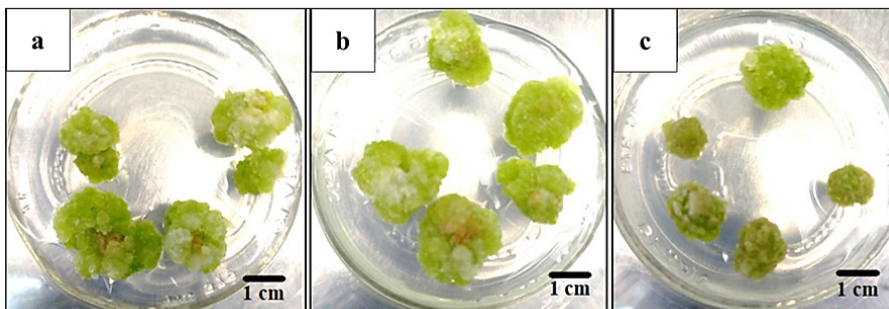


Figure 3. Callus proliferation after 12 weeks, under different durations of surface sterilisation (5.25% NaOCl + 3 drops of Tween-20), (a) 10-minute, (b) 20-minute, and (c) 30-minute

Table 2

The effect of different durations of surface sterilisation on callus induction and proliferation

Experiment	Media (Label)	Duration of surface sterilisation (10-30 min 5% NaOCl + Tween-20)	Percentage of Callus Induction	Average Diameter of the Callus (mm) after 12 Weeks (5 Replicates)
Assessment of the duration of surface sterilisation	Figure 3a	10 min	100%	15.00 ± 1.76^{ab}
	Figure 3b	20 min	100%	16.80 ± 0.37^a
	Figure 3c	30 min	100%	12.40 ± 0.81^b
MS0	-	-	0%	-

Note. *Means with the same letter were not significantly different from each other ($P < 0.05$ ANOVA followed by DMRT)

at the 5% level, thus supporting the null hypothesis of equal means. However, post hoc analysis revealed significant differences between specific pairs of groups, suggesting that pairwise differences existed despite the overall ANOVA result.

The Effect of Hormone Concentrations and Combinations

In evaluating hormonal concentrations and combinations for *C. melo* anther-derived callus induction and proliferation, eight treatments containing different concentrations of 2, 4-D and BAP were assessed after four weeks. All treatments used a consistent cold pretreatment (0-day) and sterilisation duration (10 min NaOCl + Tween-20). The hormone concentrations were adapted from protocols by Nguyen et al. (2019) and Kurtar et al. (2016). Successful callus induction and proliferation (100%) was observed in most treatments (4d-4h), except for several treatments (4a, 4b, and 4c), which showed no response (Figure 4). In treatments with positive induction, green and healthy calli were observed, with size variation depending on the concentrations of 2, 4-D and BAP (Table 3).

Callus growth was assessed by measuring the area using graph paper under a dissection microscope. Media in Figures 4b and 4c showed no induction, with average callus sizes of $1.30 \pm 0.12 \text{ mm}^2$ and $0.95 \pm 0.17 \text{ mm}^2$, respectively. Both were smaller than the average anther size prior to culture ($1.55 \pm 0.55 \text{ mm}^2$), indicating necrosis. These media contained only high concentrations of 2, 4-D, suggesting that 2, 4-D alone at 2.0-5.0 mg/L was not conducive to anther-derived callus induction in *C. melo*.

The rate of callus proliferation was calculated as a relative change, where the absolute value of the change was divided by the mean of the values and multiplied by 100% (Brauen et al., 2021).

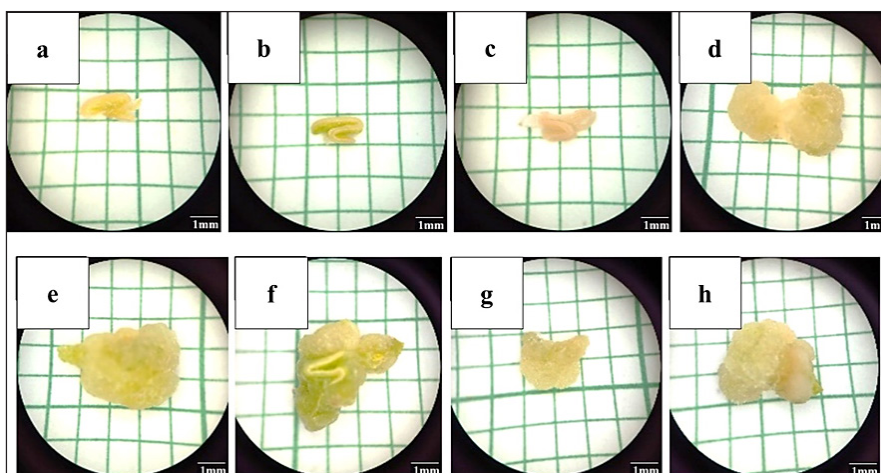


Figure 4. Callus proliferation after four weeks, under different hormonal concentrations and combinations, (a) MS0, (b) 2, 4-D (2.0 mg/L), (c) 2, 4-D (5.0 mg/L), (d) 2, 4-D (2.0 mg/L) + BAP (0.5 mg/L), (e) 2, 4-D (2.0 mg/L) + BAP (1.0 mg/L), (f) 2, 4-D (5.0 mg/L) + BAP (0.5 mg/L), (g) 2, 4-D (5.0 mg/L) + BAP (1.0 mg/L), and (h) 2, 4-D (1.5 mg/L) + BAP (0.75 mg/L)

Table 3

The effect of different durations of surface sterilisation on callus induction and proliferation

Experiment	Media (Label)	Hormonal Combination	Percentage of Callus Induction	Average Size of the Callus (mm ²) (5 Replicates)	Rate of Proliferation
Assessment of hormonal combination	Figure 4a	MS0	0%	Necrosis	0.00 ± 0.00 ^c
	Figure 4b	2, 4-D (2.0 mg/L) + BAP (0 mg/L)	0%	Necrosis	0.00 ± 0.00 ^c
	Figure 4c	2, 4-D (5.0 mg/L) + BAP (0 mg/L)	0%	Necrosis	0.00 ± 0.00 ^c
	Figure 4d	2, 4-D (2.0 mg/L) + BAP (0.5 mg/L)	100%	4.95 ± 0.86 ^{ab}	2.10 ± 0.50 ^b
	Figure 4e	2, 4-D (2.0 mg/L) + BAP (1.0 mg/L)	100%	4.50 ± 1.02 ^{bc}	1.90 ± 0.66 ^{bc}
	Figure 4f	2, 4-D (5.0 mg/L) + BAP (0.5 mg/L)	100%	6.45 ± 0.34 ^a	4.62 ± 1.3 ^{6a}
	Figure 4g	2, 4-D (5.0 mg/L) + BAP (1.0 mg/L)	100%	3.10 ± 0.23 ^{cd}	1.00 ± 0.15 ^{bc}
Positive control	Figure 4h	2, 4-D (1.5 mg/L) + BAP (0.75 mg/L)	100%	4.20 ± 0.82 ^{bc}	1.71 ± 0.53 ^{bc}

One-way ANOVA followed by Duncan's Multiple-Range Test (DMRT) was used to evaluate the significance of differences between treatments (Table 4). Among all treatments, media in Figure 4f produced the largest callus area ($6.45 \pm 0.34 \text{ mm}^2$) and the highest proliferation rate (4.62 ± 1.36), both significantly greater ($p < 0.05$) than other treatments. Media in Figure 4d ($4.95 \pm 0.86 \text{ mm}^2$) also yielded significantly larger callus sizes compared to the rest. Conversely, media in Figures 4b and 4c exhibited minimal growth and necrosis, with no proliferation rate recorded.

Statistical analysis confirmed that the combination of 5.0 mg/L 2, 4-D and 0.5 mg/L BAP (Figure 4f) was the most effective treatment overall. Media 4d and 4e also showed significant performance, with relatively large callus areas ($4.95 \pm 0.86 \text{ mm}^2$ and $4.50 \pm 1.02 \text{ mm}^2$, respectively). A significant correlation between media composition and callus size emphasises the critical role of hormonal balance in supporting callus induction and proliferation in *C. melo*. In contrast, treatments in Figures 4a-4c were found unsuitable, likely due to necrosis resulting from hormonal imbalance or absence.

Embryogenic Callus Initiation from *C. melo* Anther Culture

The optimised culture conditions (3 days cold pretreatment, surface sterilisation with a 20 min 5.25% NaOCl + 3 drops of Tween-20 wash and a 5.0 mg/L 2, 4-D + 0.5 mg/L BAP hormonal treatment) were then used to culture fresh anthers to achieve maximum calli

induction. The developed calli were transferred regularly in a fresh medium in order to induce maturation, in each sub-culturing plan, the same sub-culturing method was used as that of sub-culturing based on Asadi et al. (2018), reporting that calli sub-cultured every 21 days remarkably enhanced growth of callus and morphogenic competence in cucumber (*Cucumis sativus*).

The callus of the maturation culture was sub-cultured to fresh medium supplemented with NAA (0.05 mg/L) + BAP (4.0 mg/L) after 12 weeks of incubation, as described by Kurtar et al. (2016) previously, for embryogenic callus initiation. The objective of this experiment was to induce embryogenic callus or shoots. The concentration of cytokinin BAP was raised in the embryogenic callus initiation over the callus induction and maturation medium. Cytokinin was applied at a higher rate than auxin, resulting in an 80:1 cytokinin-auxin ratio (4.0 mg/L BAP versus 0.05 mg/L NAA). It was decided to use this specific ratio since intermediate hormone ratios promote unorganised callus growth, while an extreme shift toward high levels of cytokinins and minimal auxin concentrations is crucial for suppressing auxins' inhibitory effects on somatic embryogenesis, resulting in the differentiation of embryogenic cells and embryo-like structures (ELS) in *Cucumis* species (Ikeuchi et al., 2013; Kurtar et al., 2016).

In twelve weeks, the observation on the progress of achieving embryogenic callus on medium 5a (0.05 mg/L NAA + 4.0 mg/L BAP) showed that the callus survived but in a slow growth pattern. The calli were selected randomly and divided into five different media sets. Each set was treated with 5a-5e media separately (Figure 5). Medium 5a, which maintained the hormonal combination of NAA (0.05 mg/L) + BAP (4.0 mg/L), was used as a positive control for the embryogenic callus initiation. After four weeks, the difference between the growth rate of callus in each medium was observed. Notably, the highest formation of embryogenic callus was observed in medium 5b (BAP 4.0 mg/L), suggesting that BAP alone, at a high concentration, was more effective than combinations with NAA.

Medium 5b showed the best response by the highest formation (87%) of embryogenic callus or embryo-like structures (ELS) with globular and pre-globular shaped callus, followed by media 5a, 5c, 5d, and 5e (Figure 5). Embryogenic callus initiation culture results were summarised in Table 4. The embryogenic callus formation percentage was calculated by the number of calli which gave rise to embryogenic calli over the total number of calli which were transferred to embryo maturation media and expressed as a percentage (%) (Naïtchédé et al., 2023). The features of embryogenic and non-embryogenic were listed in Table 5. The association between unique hormonal combinations and embryogenic callus percentage were determined by the Chi-Square Test of Independence. The extremely low p-value of .001 ($X^2(4, N = 260) = 18.056, p = .001$) suggests that media selection has a significant impact on discrimination between embryogenic and non-embryogenic callus.

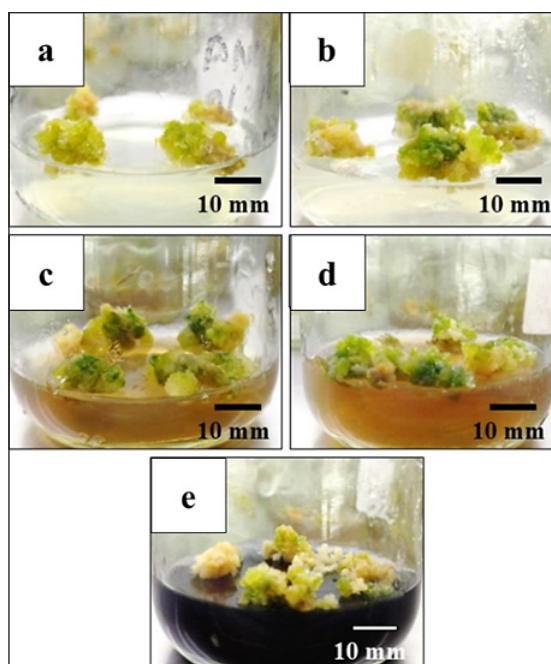




Figure 5. Embryogenic callus growth on initiation media, (a) NAA (0.05 mg/L) + BAP (4.0 mg/L), (b) BAP (4.0 mg/L), (c) NAA (0.05 mg/L) + BAP (4.0 mg/L) + AgNO₃ (10 mg/L) + ascorbic acid (0.5 mg/L), (d) BAP (4.0 mg/L) + AgNO₃ (10 mg/L) + ascorbic acid (0.5 mg/L), and (e) BAP (4.0 mg/L) + AgNO₃ (10 mg/L) + ascorbic acid (0.5 mg/L) + activated charcoal (2.5 g/l)

Table 4
Embryogenic callus initiation from *C. melo* anther culture in five different media

Experiment	Media (Label)	Hormonal Combination	Percentage of Embryogenic Callus Formation	Percentage of Non-Embryogenic Callus Formation
Embryogenic callus initiation (52 replicates)	Figure 5a	NAA (0.05 mg/L) + BAP (4.0 mg/L)	75%	25%
	Figure 5b	NAA (0 mg/L) + BAP (4.0 mg/L)	87%	14%
	Figure 5c	NAA (0.05 mg/L) + BAP (4.0 mg/L) + AgNO ₃ (10 mg/L) + Ascorbic Acid (0.5 mg/L)	73%	27%
	Figure 5d	NAA (0 mg/L) + BAP (4.0 mg/L) + AgNO ₃ (10 mg/L) + Ascorbic Acid (0.5 mg/L)	60%	40%
	Figure 5e	NAA (0 mg/L) + BAP (4.0 mg/L) + AgNO ₃ (10 mg/L) + Ascorbic Acid (0.5 mg/L) + 2.5 g Activated Charcoal	52%	48%
-	-	MS0	0%	100%

Table 5
Characteristics of embryogenic and non-embryogenic callus from anther culture of *C. melo*

Embryogenic callus	Characteristics	Non-embryogenic callus
		
Pre-globular/ globular-shaped	Morphology	Irregular-shaped
Compact	Structure	Loose
Not friable	Friability	Easily crumbles, friable
Green or yellowish-green	Colour	Light green or yellowish green
Opaque	Transparency	Semi-transparent

DISCUSSION

Cold pretreatment is a commonly applied technique in plant tissue culture, often used to enhance callus or embryogenic responses. In this study, its role in *C. melo* anther-derived callus formation was explored and found to align with findings in other species, such as *Capsicum annuum* L. (Haridhi, 2023), where pretreatment promoted callus initiation. Concerning *C. melo*, even negative effects of cold pretreatment on ovary-derived calli induction and proliferation have been previously documented (Metwally et al., 1998; Rakha et al., 2012). This study has presented the advanced anther culture technique to show that cold application is beneficial for anther cultures, probably through its various effects on physiological processes, such as the delayed senescence of anther walls, promotion of symmetric divisions of microspores, and release of necessary substances (amino acids and heat-shock proteins), as reported before (Touraev et al.). Similarly, cold stress can induce the shift of microspore development from the gametophytic to sporophytic pathways, leading to the formation of callus or embryogenesis (Mishra & Rao, 2013). According to this study, a pre-cold pretreatment for 3 days was certainly recommendable for *C. melo* anthers, due to the fact that it can result in the maximum callus size in the proliferation stage. These results reveal and confirm that the better treatment for promoting callus growth is a 3-day cold pretreatment than the other cold treatments.

Sterilisation treatments and cold pretreatments are also important factors in the success of anther culture, in addition to cold pretreatment. Various factors, such as the physiological status of the donor plants, the size, age and type of the explant, and the concentration and

duration of a sterilising agent, affect the efficiency of sterilisation. Hence, it is impossible to design a single Objective protocol of disinfection, and often the optimal series of operations may have Species-specific optimal disinfection and get the highest number of viable and aseptic Acta explants (Ahmadpoor et al., 2022). Sodium hypochlorite or NaOCl is considered a standard sterilising agent, due to its strong antibacterial and proteolytic abilities. Its active ingredient is chlorine, which results in an irreversible oxidation of bacterial enzymes, leading to a disruption of the metabolic process. Nevertheless, its phytotoxicity is also well reported (Mavani et al., 2020). While higher concentrations of NaOCl can enhance sterilisation levels, it also elevates the possibility of tissue damage and cytotoxic effects (Fitri et al., 2020; Mahood et al., 2022). Therefore, the concentration and exposure duration must be optimised based on the tissue type, in which lower concentrations are used for delicate tissues, and stronger treatments are applied for more robust explants like seeds (Bridgen et al., 2018). Previous studies reported NaOCl treatment durations ranging from 4 to 20 minutes for Cucurbitaceae species (Nguyen et al., 2019; Rakha et al., 2012). Lower NaOCl concentrations were typically applied for extended durations to enhance the aseptic rate.

In this study, 30 minutes of 5.25% NaOCl treatment was considered relatively long, yet the enclosed anther within the flower bud tolerated the exposure and produced healthy calli. This may be due to the protective role of the outer floral parts (petals and sepals), which buffered the anther from direct chemical stress. In another study, certain tissues in Cucurbitaceae species are quite robust and not even affected by this treatment. For example, Kurtar et al. (2016) reported successful callus formation from anthers of *C. maxima* and *C. moschata*, although after the sepal removal during surface sterilisation.

The findings of this study confirmed the potential of 10, 20, and 30 min NaOCl + Tween-20 treatments to sterilise *C. melo* flower buds and aid the induction of anther-derived *C. melo* callus. Ten minutes was sufficient for sterilisation, and the treatment at 20 minutes (2F) led to the highest proliferating rate and the largest callus diameter (16.8 ± 0.374 mm), indicating the best conditions for callus growth. However, 30 min treatment (2G) induced a significantly smaller callus size (12.4 ± 0.812 mm). This observation suggests that the increased duration of NaOCl application has led to high cytotoxicity, thereby reducing the callus potential for further growth. Cytological and genotoxic effects of NaOCl in onion (*Allium cepa*) and garden pea (*Pisum sativum*) have been reported. It is able to interfere with the cell cycle primarily by inducing G2 arrest and inhibiting DNA synthesis, resulting in a blockage of cell development (Salazar-Mercado et al., 2019). According to this study, 20 minutes of surface sterilisation with 5.25% NaOCl + Tween-20 is desirable for *C. melo* anther cultures since it resulted in the highest callus size when they were in the proliferation stage. The treatment response rate: a moderate power but satisfactory for a trade-off between the sterilisation effect and the tissue viability.

In addition to cold pretreatment and surface sterilisation, hormonal types and concentrations in the culture medium are critical for callus induction. The observation that media containing only high levels of 2, 4-D failed to induce callus implies that 2, 4-D alone at 2.0-5.0 mg/L was not suitable for anther-derived callus induction in *C. melo*. In contrast, Kurtar et al. (2016) found that comparable concentrations of 2, 4-D successfully induced callusing in winter squash (*Cucurbita maxima* L.) and pumpkin (*Cucurbita moschata* Duch.) Since both species are members of the Cucurbitaceae family, these contrasting results demonstrate species-specific hormonal needs. This inconsistency highlights that although 2, 4-D at 2.0-5.0 mg/L was effective for winter squash and pumpkin, it was ineffective for melon (*C. melo*) when applied without cytokinin.

2, 4-D is among the most widely used synthetic auxins for promoting plant growth. It has been shown that auxin, in combination with other plant hormones, is essential for cell division and directional cell differentiation, impacting overall plant development (Simon & Petrášek, 2011). However, an imbalance, such as an excess of auxins relative to cytokinins, can inhibit proper callus formation. Typically, an intermediate ratio between auxins and cytokinins is optimal for callus induction (Ikeuchi et al., 2013). This is supported by the high callus induction observed in treatments combining 2, 4-D with BAP (4d-4o), and aligns with Skoog and Miller's (1957) principle that an intermediate auxin-cytokinin ratio promotes callus development in plant tissues (Ikeuchi et al., 2013). Moreover, the 0% induction in 4a medium (without hormones) is also consistent with the Kurtar et al. (2016) results.

It has been noted with some past reviews that although low concentrations of 2, 4-D, and other synthetic auxins can promote growth of the plant, the hormone is shown to stimulate abnormal plant growth at high concentrations (Song, 2014). In addition, 2, 4-D can induce epigenetic and genetic changes, such as DNA methylation and mutation. In somatic embryogenesis, high levels of 2, 4-D have been shown to suppress typical embryo development by affecting genetic and physiological processes at the cell level (García et al., 2019). These findings reinforce the importance of species-specific optimisation of hormone concentrations, particularly 2, 4-D, to support successful callus induction and proliferation, as demonstrated in this study and the work of Kurtar et al. (2016).

Further investigation focused on identifying conditions that specifically promote embryogenic callus formation. In this context, the observation from this study slightly differs from Ikeuchi et al. (2013) and Kurtar et al. (2016), who reported that a higher ratio of cytokinin-to-auxin promotes embryo formation or shoot regeneration. In this study, the absence of NAA, an auxin, in media 5b may have reduced the inhibitory effects on embryogenesis typically associated with auxins, thus favouring the formation of embryogenic callus.

In media 5c-5e, the addition of AgNO₃, ascorbic acid, and activated charcoal aimed to reduce the phenolic exudation and to enhance the callus growth (Demirkaya

& Çömlekçioğlu, 2021). Dangi et al. (2014) stated in their study that the addition of antioxidants such as polyvinylpyrrolidone (PVP), ascorbic acid, and citric acid has demonstrated a beneficial effect on explant survival. Nevertheless, the overall response of these treatments in this study showed that these supplements (AgNO₃, ascorbic acid and activated charcoal) were not significantly effective in promoting embryogenic callus formation as compared to media 5b, which might imply that these additives are good for eliminating the phenolic stress but had less nutritional impact on the embryogenic calli, or perhaps their concentrations might not be well adjusted. Demirkaya and Çömlekçioğlu (2021) also found such tendencies in other species, suggesting that the use of these additives could be largely context dependent.

Chi-Square analysis reveals that there is a significant influence of culture medium, hormonal combinations and additives on embryogenic callus induction. BAP (4.0 mg/L) was the best elicitor for the induction, while silver nitrate, ascorbic acid and activated charcoal showed a negative effect on embryogenic callus induction, indicating the need for optimisation of these compounds in a tissue culture regime. Further studies are required in order to understand the exact nature of these effects, particularly the balance of auxin and cytokinin and the role of additives such as AgNO₃ and activated charcoal.

To characterise the obtained calli, in particular embryogenic ones, the ploidy of the calli was measured by flow cytometry. The ploidy status of the anther-derived calli samples analysed deviated from expectation. Even though calli were considered to be haploid, flow cytometry revealed that they included both mixoploid and polyploid cells (Figure 6). As the calli were the object of the present investigation, chromosomal variation was more apparent than that of the adventitious shoots as reported by Krishna et al. (2016).

The reasons for the remarkable levels of unexpected ploidy observed in this study are numerous. Mitotic abnormalities, in particular failed cytokinesis, are probably among the factors contributing to this phenomenon. These errors can lead to unbalanced chromosomal content (aneuploidy or polyploidy) (Muenchrath et al., 2016; Potapova and Gorbisky,

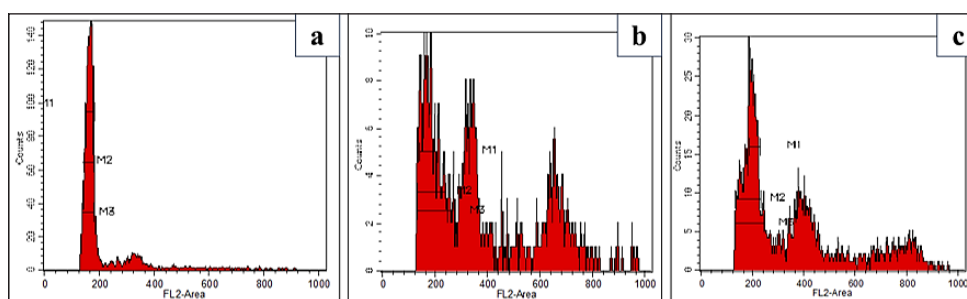


Figure 6. Histograms from Flow Cytometry analysis, (a) cotyledon as diploid external standard, (b) and (c) calli samples from anther culture

2017), which is the primary manifestation observed post analysis. Flow cytometry is also less sensitive for detecting aneuploidy, but aneuploidy can also occur. So, in order to obtain effective detection results, it is recommended to use flow cytometry combined with karyotyping (Eng et al., 2021). Chromosomes which fail to separate correctly due to errors in the mitotic machinery can cause arrest of the cell cycle at metaphase. At re-entry into mitosis, these cells may duplicate their chromosomes to generate polyploid cells (Eng et al., 2021; Zhou et al., 2017).

Polyploidization could also be associated with TCIV (Bairu et al., 2011; Krishna et al., 2016) or with anti-mitotic agents such as colchicine, trifluralin, oryzalin (Ebrahimzadeh et al., 2018), amiprofos-methyl (Kurtar et al., 2021), and carbamate (Zhou et al., 2017). Since there are no anti-mitotic agents in this experiment, TC IV could be one of the reasons for detecting polyploid, which could potentially alter the formation of chromosomal constitution and genetic materials (Debnath & Ghosh, 2022; Košmrlj et al., 2013).

A phragmoplast composed of microfilaments and microtubules forms between the daughter nuclei at the late anaphase stage, guiding the alignment and fusion of vesicles to a cell plate (Nishihama et al., 2002). Mistakes here may cause a disorganised cell plate, failure of cytokinesis and subsequently polyploidy or mixoploidy (Caetano-Pereira et al., 1998). Overexpression of specific effectors, such as the kinase-defective NPK1 and downstream effectors, disrupts phragmoplast expansion and leads to multinucleate and mixoploid cells (Eng et al., 2021; Nishihama et al., 2001). Endoreduplication, in which chromosomal DNA replication occurs without mitosis, can also alter the DNA synthesis, leading to increased ploidy levels by preceding the mitotic phase (De Almeida Engler & Gheysen, 2013; Sugimoto-Shirasu & Roberts, 2003). Besides, meiotic complications like nondisjunction can result in aneuploidy (Muenchrath et al., 2016) and, therefore, it changes the gene dosages within affected chromosomes (Potapova & Gorbsky, 2017). Normally, flow cytometry is incapable of detecting aneuploidy; thus, this condition requires both flow cytometry and karyotyping for a precise readout (Eng et al., 2021). Polyploidy or mixoploidy can also be obtained from cell fusion (syncytia) or nucleus migration (cytomixis), which generate multiple nuclei and mixed-ploidy cells (Caetano-Pereira et al., 1998). While mixoploids are generally genetically unstable, new methodologies permit ploidy sorting in chimaeras, opening the door for further research in polyploidisation (Regalado et al., 2015; Roy et al., 2001).

Ploidy can also be altered during initiation by tissue culture cellular competition. Diploid cells originating from wounded anther tissues may proliferate earlier and have advantages over haploid cells, leading to a reduction in haploid callus production. Asadi et al. (2018) recommend anther-side up position on the medium and gently scratching the sacs to promote microspore expulsion and to inhibit the diploid calli.

CONCLUSION

The present study has developed an efficient protocol for callus induction and embryo formation in *C. melo* cv. Glamour through anther culture. Of the parameters tested, 3-day cold pretreatment, 20 min surface sterilisation with 5.25% NaOCl + Tween-20, and the hormone combination of 5.0 mg/L 2, 4-D 0.5 mg/L BAP provided the highest callus, proliferation, and regeneration rates. The formation of embryogenic callus was most efficiently triggered under 4.0 mg/L BAP without the addition of auxins, and it is indicated that a high cytokinin-to-auxin ratio is important to cause the development of embryo-like structures in this species. Flow cytometric analysis demonstrated that the majority of anther-derived calli showed mixoploidy or polyploidy and that no clear evidence was found for the regeneration of haploids under the conditions tested. This aberrant ploidy variation is probably derived from TCIV and mitotic anomalies, such as abnormal cytokinesis, endoreduplication, and chromosome doubling, rather than from anti-mitotic agents. The presence of these higher ploidy levels demonstrates chromosome instability within in vitro systems and the necessity of regular cytogenetic check-ups in haploidisation programs. In general, this protocol significantly improves callus and embryo induction in *C. melo*. Although the current study has some limitations, one of them is that it stops at the embryogenic callus and embryo-like structure (ELS) stage without converting them into whole, acclimatised plantlets, which temporarily limits its practical utility in breeding programs. The frequent occurrence of mixoploidy and polyploidy during the culture cycle also challenges routine haploid or doubled haploid production. These results are useful for genetic improvement and suggest potential for the development of *C. melo* breeding programs using androgenesis-based methods.

RECOMMENDATIONS

Future research in melon breeding should follow the following directions based on the findings and limitations of this study:

Complete Plantlet Regeneration: Germination and maturation media should be optimised to facilitate the successful conversion of embryo-like structures (ELS) into fully functional shoots and roots, followed by greenhouse acclimatisation.

Refinement of Microspore Reprogramming: Investigations are needed to fine-tune the durations and intensities of physical pretreatments (such as precise cold or heat shocks) in order to increase the initial efficiency of haploid microspore embryogenesis.

Mitigation of Ploidy Instability: In order to minimise tissue culture-induced variations and somaclonal mixoploidy, research should explore the use of anti-mitotic agents at critical stages or alter culture conditions (such as altering subculture intervals and reducing 2, 4-D exposure).

Advanced Cytogenetic Screening: Future protocols should incorporate routine karyotyping alongside flow cytometry to detect aneuploidy and chromosomal rearrangements, thus verifying the genetic integrity of regenerated lines.

ACKNOWLEDGMENT

The authors gratefully acknowledge the Ministry of Higher Education Malaysia for financial support under the Fundamental Research Grant Scheme (FRGS/1/2019/STG05/UMK/02/4). In this regard, we would like to sincerely thank the Centre for Biotechnology and Environmental Technology, University of Fallujah, Iraq, and College of Veterinary Medicine, Qassim University, Saudi Arabia, for their excellent assistance and assistance in providing the necessary facilities for the completion of this research successfully.

REFERENCES

- Ahmadpoor, F., Zare, N., Asghari, R., & Sheikhzadeh, P. (2022). Sterilisation protocols and the effect of plant growth regulators on callus induction and secondary metabolites production in *in vitro* cultures *Melia azedarach* L. *AMB Express*, *12*(1), Article 3. <https://doi.org/10.1186/s13568-022-01343-8>
- Ahmed, M. I., Fatimah, B. K., Dwi, S., Mohammed, A., & Pedram, K. (2015). Callus induction from ovules of kenaf (*Hibiscus cannabinus* L.). *Biotechnology*, *14*(2), 72-78. <https://doi.org/10.3923/biotech.2015.72.78>
- Ahmed, M. I., Fatimah, B. K., Dwi, S., Pedram, K., & Mohammed, A. (2016). Haploid induction in spring onion (*Allium fistulosum* L.) via gynogenesis. *Biotechnology*, *15*(1), 10-16. <https://doi.org/10.3923/biotech.2016.10.16>
- Asadi, A., Zebajadi, A., Abdollahi, M. R., & Seguí-Simarro, J. M. (2018). Assessment of different anther culture approaches to produce doubled haploids in cucumber (*Cucumis sativus* L.). *Euphytica*, *214*(11), Article 214. <https://doi.org/10.1007/s10681-018-2297-x>
- Bairu, M. W., Aremu, A. O., & van Staden, J. (2011). Somaclonal variation in plants: Causes and detection methods. *Plant Growth Regulation*, *63*(2), 147-173. <https://doi.org/10.1007/s10725-010-9554-x>
- Brauen, S., Erpf, P., & Wasem, M. (2021). On absolute and relative change. *SSRN Electronic Journal*. Advance online publication, 1-10. <https://doi.org/10.2139/ssrn.3739890>
- Bridgen, M. P., Van Houtven, W., & Eeckhaut, T. (2018). Plant tissue culture techniques for breeding. In J. Van Huylenbroeck (Ed.), *Ornamental crops* (pp. 127-144). Springer. https://doi.org/10.1007/978-3-319-90698-0_6
- Caetano-Pereira, C. M., Taschetto, O. M., Pagliarini, M. S., & Brasil, E. M. (1998). Spontaneous mixoploidy in maize anthers. *Cytologia*, *63*(3), 305-309. <https://doi.org/10.1508/cytologia.63.305>
- Dangi, B., Khurana-Kaul, V., & Kothari, S. L. (2014). Micropropagation of *Terminalia bellerica* from nodal explants of mature tree and assessment of genetic fidelity using ISSR and RAPD markers. *Physiology and Molecular Biology of Plants*, *20*, 509-516. <https://doi.org/10.1007/s12298-014-0247-1>

- De Almeida Engler, J., & Gheysen, G. (2013). Nematode-induced endoreduplication in plant host cells: Why and how? *Molecular Plant-Microbe Interactions*, 26(1), 17-24. <https://doi.org/10.1094/MPMI-05-12-0128-CR>
- Debnath, S. C., & Ghosh, A. (2022). Phenotypic variation and epigenetic insight into tissue culture berry crops. *Frontiers in Plant Science*, 13, Article 1042726. <https://doi.org/10.3389/fpls.2022.1042726>
- Demirkaya, B., & Çömlekçioğlu, N. (2021). Effects of biotin and ascorbic acid applications on haploid embryo induction in semisolid and double layer nutrient media in pepper (*Capsicum annuum* L.) anther culture. *International Journal of Agriculture, Environment and Food Sciences*, 5(2), 191-196. <https://doi.org/10.31015/jaefs.2021.2.8>
- Deng, Y., Tang, B., Zhou, X., Fu, W., Tao, L., Zhang, L., & 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*, 142(2), 253-268. <https://doi.org/10.1007/s11240-020-01839-w>
- Doležel, J., & Bartoš, J. (2005). Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany*, 95(1), 99-110. <https://doi.org/10.1093/aob/mci005>
- Doležel, J., Greilhuber, J., & Suda, J. (2007). Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols*, 2(9), 2233-2244. <https://doi.org/10.1038/nprot.2007.310>
- Ebrahimzadeh, H., Soltanloo, H., Shariatpanahi, M. E., Eskandari, A., & Ramezanpour, S. S. (2018). Improved chromosome doubling of parthenogenetic haploid plants of cucumber (*Cucumis sativus* L.) using colchicine, trifluralin, and oryzalin. *Plant Cell, Tissue and Organ Culture*, 135(3), 407-417. <https://doi.org/10.1007/s11240-018-1473-y>
- Eng, W. H., Ho, W. S., & Ling, K. H. (2021). *In vitro* induction and identification of polyploid *Neolamarckia cadamba* plants by colchicine treatment. *PeerJ*, 9, Article e12399. <https://doi.org/10.7717/peerj.12399>
- Fitri, M., Nazar, K., Meidyawati, R., & Azmi, R. (2020). Antibacterial effect of xanthorrhizol (*Curcuma xanthorrhiza* Roxb.) against the biofilm of *Fusobacterium nucleatum*. *International Journal of Applied Pharmaceutics*, 12(2), 57-61. <https://doi.org/10.22159/ijap.2020.v12s2.PP-06>
- Golabadi, M., Ghanbari, Y., Keighobadi, K., & 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.
- Habiba, R. (2016). Effect of genotypes, sucrose concentrations and their interaction on anther culture response on summer squash. *Journal of Agricultural Chemistry and Biotechnology*, 7(4), 113-120. <https://doi.org/10.21608/jacb.2016.40787>
- Haridhi, I. K. (2023). The effect of cold pre-treatment period on callus formation of pepper (*Capsicum annuum* L.) anther culture of local Aceh perintis genotype. *IOP Conference Series: Earth and Environmental Science*, 1183(1), Article 012040. <https://doi.org/10.1088/1755-1315/1183/1/012040>
- Hatfield, J. L., & Prueger, J. H. (2015). Temperature extremes: Effect on plant growth and development. *Weather and Climate Extremes*, 10(Part A), 4-10. <https://doi.org/10.1016/j.wace.2015.08.001>
- Ibrahim, A. M., Abbas, R. A., Aldabbagh, J. E., & Mohammed, A. (2023a). Factors affecting callus induction from anther and ovary of okra (*Abelmoschus esculentus* L.). *Indian Journal of Agricultural Research*, 57(5), 658-664. <https://doi.org/10.37077/25200860.2023.36.2.21>

- Ibrahim, A. M., Hameed, M. K., & Mohammed, A. (2023b). *In vitro* propagation of date palm (*Phoenix dactylifera* L.) cultivar Jawzi using shoot tip. *Basrah Journal of Agricultural Sciences*, 36(2), 267-284. <https://doi.org/10.37077/25200860.2023.36.2.21>
- Ikeuchi, M., Sugimoto, K., & Iwase, A. (2013). Plant callus: Mechanisms of induction and repression. *The Plant Cell*, 25(9), 3159-3173. <https://doi.org/10.1105/tpc.113.116053>
- Košmrlj, K., Murovec, J., & Bohanec, B. (2013). Haploid induction in hull-less seed pumpkin through parthenogenesis induced by X-ray-irradiated pollen. *Journal of the American Society for Horticultural Science*, 138(4), 310-316. <https://doi.org/10.21273/JASHS.138.4.310>
- Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., & Sadh, R. K. (2016). Somaclonal variations and their applications in horticultural crops improvement. *3 Biotech*, 6, Article 54. <https://doi.org/10.1007/s13205-016-0389-7>
- Kurtar, E. S., Balkaya, A., & Kandemir, D. (2016). Evaluation of haploidisation efficiency in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) through anther culture. *Plant Cell, Tissue and Organ Culture*, 127(2), 497-511. <https://doi.org/10.1007/s11240-016-1074-6>
- Kurtar, E. S., Seymen, M., Çetin, A. N., & Türkmen, Ö. (2021). Dihaploidisation in promising summer squash genotypes (*Cucurbita pepo* L.) via irradiated pollen technique. *Yuzuncu Yil University Journal of Agricultural Sciences*, 31(1), 42-51. <https://doi.org/10.29133/yyutbd.800475>
- Mahood, H. E., Abbas, M. K., & Zahid, N. A. (2022). Micropropagation of feverfew (*Tanacetum parthenium*) and quantification of parthenolide content in its micropropagated and conventionally grown plants. *Horticulturae*, 8(1), Article 50. <https://doi.org/10.3390/horticulturae8010050>
- Mavani, H. A. K., Tew, I. M., Wong, L., Yew, H. Z., Mahyuddin, A., Ghazali, R. A., & Pow, E. H. N. (2020). Antimicrobial efficacy of fruit peels eco-enzyme against *Enterococcus faecalis*: An *in vitro* study. *International Journal of Environmental Research and Public Health*, 17(14), Article 5107. <https://doi.org/10.3390/ijerph17145107>
- Metwally, E. I., Moustafa, S. A., El-Sawy, B. I., Haroun, S. A., & Shalaby, T. A. (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. <https://doi.org/10.1023/A:1005948809825>
- Midin, M. R., Nordin, M. S., Madon, M., Saleh, M. N., Goh, H., & Noor, N. M. (2017). Determination of the chromosome number and genome size of *Garcinia mangostana* L. via cytogenetics, flow cytometry and k-mer analyses. *Caryologia*, 71(1), 35-44. <https://doi.org/10.1080/00087114.2017.1403762>
- Mishra, R., & Rao, G. J. N. (2013). Effect of cold pretreatment and phytohormones on anther culture efficiency of two *indica* rice (*Oryza sativa* L.) hybrids—Ajay and Rajalaxmi. *Journal of Experimental Biology and Agricultural Sciences*, 1(2), 69-76.
- Muenchrath, D., Campbell, A., Merrick, L., & Lübberstedt, T. (2016). Ploidy—Polyploidy, aneuploidy, haploidy. In *Crop Genetics*, interactive e-learning courseware. Plant Breeding E-Learning in Africa. Retrieved from <https://pbea.agron.iastate.edu>
- Naïtchédé, L. H., Nyende, A. B., Runo, S., & Borlay, A. J. (2023). Plant regeneration from embryogenic callus-derived from immature leaves of *Momordica charantia* L. *Heliyon*, 9(11), Article e22122. <https://doi.org/10.1016/j.heliyon.2023.e22122>

- Nguyen, M. L., Ta, T. H. T., Huyen, T. N. B. T., & Voronina, A. V. (2019). Anther-derived callus formation in bitter melon (*Momordica charantia* L.) as influenced by microspore development stage and medium composition. *Sel'skokhozyaistvennaya Biologiya [Agricultural Biology]*, *54*(1), 140-148. <https://doi.org/10.15389/agrobiology.2019.1.140eng>
- Nishihama, R., Ishikawa, M., Araki, S., Soyano, T., Asada, T., & Machida, Y. (2001). The NPK1 mitogen-activated protein kinase kinase kinase is a regulator of cell-plate formation in plant cytokinesis. *Genes & Development*, *15*(3), 352-363. <https://doi.org/10.1101/gad.863701>
- Nishihama, R., Soyano, T., Ishikawa, M., Araki, S., Tanaka, H., Asada, T., Irie, K., Ito, M., Terada, M., Banno, H., Yamazaki, Y., & Machida, Y. (2002). Expansion of the cell plate in plant cytokinesis requires a kinesin-like protein/MAPKKK complex. *Cell*, *109*(1), 87-99. [https://doi.org/10.1016/S0092-8674\(02\)00691-8](https://doi.org/10.1016/S0092-8674(02)00691-8)
- Ozsan, T., Gozen, V., & Onus, A. N. (2017). Cucumber gynogenesis: Effects of 8 different media on embryo and plant formation. *International Journal of Agriculture Innovations and Research*, *6*(2), 419-422.
- Phillips, G. C., & Garda, M. (2019). Plant tissue culture media and practices: An overview. *In Vitro Cellular & Developmental Biology - Plant*, *55*(3), 242-257. <https://doi.org/10.1007/s11627-019-09983-5>
- Potapova, T., & Gorbosky, G. J. (2017). The consequences of chromosome segregation errors in mitosis and meiosis. *Biology*, *6*(1), Article 12. <https://doi.org/10.3390/biology6010012>
- Rakha, M. T., Metwally, E. I., Moustafa, S. A., Etman, A. A., & Dewir, Y. H. (2012). Evaluation of regenerated strains from six *Cucurbita* interspecific hybrids obtained through anther and ovule *in vitro* cultures. *Australian Journal of Crop Science*, *6*(1), 23-30.
- Regalado, J. J., Carmona Martín, E., Castro, P., Moreno, R., Gil, J., & Encina, C. L. (2015). Study of the somaclonal variation produced by different methods of polyploidization in *Asparagus officinalis* L. *Plant Cell, Tissue and Organ Culture*, *122*(1), 31-44. <https://doi.org/10.1007/s11240-015-0747-x>
- Roy, A. T., Leggett, G., & Koutoulis, A. (2001). *In vitro* tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). *Plant Cell Reports*, *20*(6), 489-495. <https://doi.org/10.1007/s002990100364>
- Salazar-Mercado, S. A., Torres-León, C. A., & Rojas-Suárez, J. P. (2019). Cytotoxic evaluation of sodium hypochlorite, using *Pisum sativum* L. as effective bioindicator. *Ecotoxicology and Environmental Safety*, *173*, 71-76. <https://doi.org/10.1016/j.ecoenv.2019.02.027>
- Simon, S., & Petrášek, J. (2011). Why plants need more than one type of auxin. *Plant Science*, *180*(3), 454-460. <https://doi.org/10.1016/j.plantsci.2010.12.007>
- Skoog, F., & Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symposia of the Society for Experimental Biology*, *11*, 118-131.
- Song, H., Lou, Q. F., Luo, X. D., Wolukau, J. N., Diao, W. P., Qian, C. T., & Chen, J. F. (2007). Regeneration of doubled haploid plants by androgenesis of cucumber (*Cucumis sativus* L.). *Plant Cell, Tissue and Organ Culture*, *90*(3), 245-254. <https://doi.org/10.1007/s11240-007-9263-y>
- Song, Y. (2014). Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. *Journal of Integrative Plant Biology*, *56*(2), 106-113. <https://doi.org/10.1111/jipb.12131>

- Sugimoto-Shirasu, K., & Roberts, K. (2003). "Big it up": Endoreduplication and cell-size control in plants. *Current Opinion in Plant Biology*, 6(6), 544-553. <https://doi.org/10.1016/j.pbi.2003.09.009>
- Thorpe, T. A. (2007). History of plant tissue culture. *Molecular Biotechnology*, 37(2), 169-180. <https://doi.org/10.1007/s12033-007-0031-3>
- Tomaszewska, P., Pellny, T. K., Hernández, L. M., Mitchell, R. A. C., Castiblanco, V., Vega, J. J. D., Schwarzacher, T., & Heslop-Harrison, J. S. (2021). Flow cytometry-based determination of ploidy from dried leaf specimens in genomically complex collections of the tropical forage grass *Urochloa* sp. L. *Genes*, 12(7), Article 957. <https://doi.org/10.3390/genes12070957>
- Zhou, K., Fleet, P., Nevo, E., Zhang, X., & Sun, G. (2017). Transcriptome analysis reveals plant response to colchicine treatment during chromosome doubling. *Scientific Reports*, 7, Article 8503. <https://doi.org/10.1038/s41598-017-08391-2>