CUKUROVA UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

PhD THESIS

Production of Haploid Plants in Cucumber (*Cucumis sativus* L.) *via* Gynogenesis

Fildaus NYIRAHABIMANA

Biotechnology Department

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Jury : Prof. Dr. İlknur SOLMAZ (Advisor) ……

: Prof. Dr. Yıldız Aka KAÇAR ……

: Assoc. Prof. Dr. Özhan ŞİMŞEK ……

: Prof. Dr. Hatıra TAŞKIN ……

: Assoc. Prof. Dr. Gökhan BAKTEMUR ……

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Thesis Number:

Prof. Dr. Sadık DİNÇER
Director
Institute of Natural and Applied Sciences

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Advisor: Prof. Dr. İlknur SOLMAZ

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ABSTRACT

Cucumber (*Cucumis sativus* L.) is a widely grown creeping vine plant belonging to *Cucurbitaceae* family. The haploidization technique is modern and improved breeding technique used to enhance economically essential crops including cucumber to meet consumers' preferences and needs worldwide. The aim was to analyse the effects of factors such as genotype, thermal pre-treatments and nutrient media combinations that are considered in haploid production via ovary culture process in four commercial cucumber varieties. A total of seventeen nutrient media and commercial cucumber varieties (three F1 and one open-pollinated cucumber varieties) haploid plant production performed through ovary culture process. Effects of temperatures pre-treatments as well as heat 35°C/3 days and cold 4°C/4 days pre-tretments with eight induction media and nine embryo maturation/regenerations nutrient media were evaluated in two distinct experiments of this study. In experiment one: Results of study demonstrated that embryo-like structures (ELSs) formation rate was high in induction medium 2 (IM2) contained of 2 mg L^{-1} 6-Benzylaminopurine (BAP), 0.5 mg L^{-1} and 1-Naphthaleneacetic acid (NAA) was successful in all varieties Ptk40 (20%), Botanik (20%), Beith Alpha (16%), and Sardes (13.3%) respectively compared to other induction medium combination used in this study. Due to findings of this study, nutrient medium containing KIN and 2,4-D seem to be successful during induction of haploid embryo formation in cucumbers. There was no plant formation occurred in this experiment. Experiment two's results demonstrated that embryo formation rate was high IM8 (2,4-D, PUT) induction media for all varieties [Botanik (88%) Sardes (60%), Ptk40 (32 %) and Beith alpha (20%). The 117-plantlets from Botanik variety, twelve from sardes and two of Ptk40 regenerated within IAA, BAP medium combination. 57 plants were germinated in 0.1 mg L^{-1} IBA. 19 diploids and 1 tetraploid of them were determined by flow cytometry. Based on findings of study, 2,4-D, PUT in nutrient medium was successful in this experiment. Further studies are required to optimise the embryo regeneration medium composition due to the cucumber growing seasons, genotypes, and mediums.

Keywords: *Cucumis sativus* L., Breeding, Haploid, Genotype, Gene expression

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

DOKTORA TEZİ

Hıyar (*Cucumis sativus* L.)'da Haploid Bitkiler Ginogenesis Yoluyla Üretimi

Fildaus NYIRAHABIMANA

Danışman: Prof. Dr. İlknur SOLMAZ

Biyoteknoloji Anabilim Dalı

ÖZ

Hıyar (*Cucumis sativus* L.), *Cucurbitaceae* familyasına familyasında bulunan, ve yaygın olarak yetişticiliği yapılan bir sebzedir. Bu çalışmada, Dört ticari hıyar çeşidinde ovaryum kültürü yoluyla haploid bitki üretilmesi amacıyla genotiplerde sıcaklık ön uygulamaları ve farklı besin ortamı kombinasyonlarının tespit edilmesi amaçlanmıştır. Gibi faktörlerin etkilerinin analiz edilmesi amaçlanmıştır. Toplam 17 besi ortamı ve ticari hıyar çeşidinin (3 F1 ve 1 açık tozlanan hıyar çeşidi) haploid bitki üretimi, ovaryum kültürü yolu ile gerçekleştirilmiştir. Sekiz giriş ortamı ve dokuz embriyo teşvik/rejenerasyon besin ortamı;sıcak (35°C/3 gün) şoku ön uydulama ve 4 gün soğuk (4°C) şokun etkileri bu çalışmanın iki farklı aşamasında değerlendirilmiştir. Çalışmanın birinci aşamasında 2 mg L^{-1} 6-Benzilaminopurin (BAP), 0,5 mg L^{-1} ve 1-Naftalinasetik içeren giriş ortamı 2'de (IM2) embriyo benzeri yapıların (ELS'ler) oluşum hızının yüksek olduğunu göstermiştir. Naftalin Asetik Asit (NAA), bu çalışmada kullanılan diğer indüksiyon ortamı kombinasyonuna kıyasla sırasıyla tüm çeşitlerde Ptk40 (%20), Botanik (%20), Beith Alpha (%16) ve Sardes (%13,3) çeşitlerinde başarılı bulunmuştur. Bu çalışmanın bulgularına göre, KIN ve 2,4-D içeren besi ortamının hıyarda haploid embriyo oluşumunun uyarıtımasında başarılı olduğu görülmektedir. İkinci deneyin sonuçları, embriyo oluşum oranının tüm çeşitler [Botanik (%88) Sardes (%60), Ptk40 (%32) ve Beith alpha (%20)] için yüksek IM8 (2,4-D, PUT) indüksiyon ortamı olduğunu göstermiştir. 117 bitkicik Botanik çeşidinden, 12 bitkicik sardes çeşidinden ve Ptk40 çeşidinden iki bitkicik IAA, BAP ortam kombinasyonunda rejenere edilmiştir. 57 bitki 0.1 mg L^{-1} IBA'da çimlenmiştir. Flow sitometri ile 19 diploid ve 1 tetraploid bitki ploidi seviyesini belirlenmiştir. Çalışmanın sonuçlarına göre 2,4-D ve besi ortamındaki PUT, bu deneyde başarılı bulnumuştur bulunmuştur. Hıyar yetiştirme mevsimleri, genotipler ve besin ortamı nedeniyle embriyo rejenerasyon ortamı optimize etmek amacı ile daha fazla çalışma yapılması gerekmektedir.

Anahtar Kelimeler: Hıyar, Ginogenesis, Haploidizasyon, Haploid, Gen ifadesi

GENİŞLETİLMİŞ ÖZET

Hıyar (*Cucumis sativus* L.), *Cucurbitaceae* familyasında bulunan ve tüm dünyada ekonomik değeri yüksek olan 2n = 2x =14 kromozomlu bir sebzedir. Hıyar, Hindistan kökenli olup, tropikal ve subtropikal bölgeler başta olmak üzere geniş alanlarda üretilmektedir. Dünyada en fazla hıyar yetiştiriciliği yapan ülkeler incelendiğinde, Çin'in en fazla üretimi yaptığı (70,3 milyon ton), Türkiye (1,62 milyon ton), Ukrayna (1,92 milyon ton) ve Rusya Federasyonu (1,03 milyon ton) Çin'den sonra geldiği belirlenmiştir. Türkiye'de hıyar çeşitlerinin çoğu açık alanda yetiştirilmektedir ve serada yetiştirilen çeşitlerin tamamına yakını F1 melezlerdir. *Cucurbitaceae* familyası, özellikle tropik ve subtropikal bölgelerde yayılış gösteren 118 cins ve 825 türü içermektedir. Kabakgiller arasında hıyar (*Cucumis sativus* L.), karpuz (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), kavun (*Cucumis melo* L.), kabak (*Cucurbita moschata* Duchesne ex Poir.), yaz kabağı (*Cucurbita pepo* L.), ve kış kabağı (*Cucurbita maxima* Duch. ex. Lam.) bulunmaktadır. Sebze ıslahında öncelikle saf hat geliştirme önemlidir. *Cucurbitaceae* familyasında klasik ıslah yoluyla yeni sebze çeşitlerinin geliştirilmesi zaman almaktadır. Bu süreci hızlandırmak, seleksiyonu geliştirmek ve yetiştirme verimliliğini artırmak için kabakgil türlerinde haploid bitki üretme tekniği uygulanmaktadır.

En önemli sebzelerden biri olan hıyarda biyoteknolojik yöntemler kullanılarak ıslah çalışması yapılması oldukça önemlidir. Hıyarda haploid bitkiler *in vitro* yöntemlerle gynogenensis yoluyla elde edilmektedir. Elde edilen bu haploidler, bazı kimyasallarla double haploid duruma getirilerek ıslah programlarında yer alacak mutlak homozigot hatların üretim süreci 2-3 yıl gibi kısa bir sürede sağlanabilmektedir. Yeterli haploid bitki elde edildiğinde, kromozom katlaması olduğu taktirde F1 hibriti üretim için faydalı materyal sağlanabilecektir. Haploid bitkiler gametofitik kromozom sayına sahipken, double haploidlerde kromozom sayısı iki katına çıkmaktadır. Haploid ve DH'lerin üretimi özellikle ilgi çekici bir biyoteknolojik yöntemdir. Haploidi teknolojisinin ve homozigot bitkilerin üretilmesine yönelik protokollerin ilerlemesinin tarım sistemleri üzerinde önemli etkisileri olmuştur. Klasik yetiştirme teknikleriyle karşılaştırıldığında, gametik embriyogenez, homozigot soyların oluşturulmasını sağlar ve bunların üretimi için gereken süreyi azaltmaktadır. Bu, tek bir adımda heterozigot ana bitkilerden %100 homozigot hatların hatların doğrudan yaratılmasına izin verir.

Haploid ve double haploid bitkiler elde etmek için, çeşitli teknikler vardır; in vitro ovüle-ovaryum ve anter-mikrospore kültürleri en etkili olanlardır. Ve son zamanlarda kabakgillerde ve bahçe bitkilerinde yaygın olarak uygulanmaktadır. Doğada kabakgiller familyasına dahil olan sebze türlerinde kendiliğinden haploid oluşumuna rastlanmamıştır. Bu nedenle kabakgillerde haploid embriyo ve *in vitro* bitki üretimi ile gynogenesis üzerine yapılan çalışmalar son yıllarda yoğunlaşmıştır. Haploidi tekniği, tüketicilerin ihtiyaç ve tercihlerini karşılamak için hıyar

genotipleri de dahil olmak üzere ekonomik açıdan önemli bitkileri geliştirmeye yönelik ıslah çalışmalarında yararlı bir yöntemdir.

Bu araştırma 2021 ve 2022 yetiştirme sezonlarında Çukurova Üniversitesi Ziraat Fakültesi Bahçe Bitkileri Bölümü araştırma serasında bulunan yay çatılı plastik serada yürütülmüştür. Çalışmada, dört ticari hıyar çeşidinde yumurtalık kültürü yoluyla haploid üretiminde önemli olan genotipler, sıcak ve soğuk şok uygulamaları ve besin ortamı kombinasyonları gibi çeşitli faktörlerin etkilerini incelemek amaçlanmıştır. Bu çalışma iki bölüme ayrılmaktadır. Birinci bölümde, dört adet giriş ortamı kombinasyonunu [(IM1: MS, 30 g L^{-1} sakaroz, 7 g L^{-1} agar (standart ortam = kontrol); IM2: 0,5 mg L^{-1} 2 4-Diklorofenoksiasetik asit (2,4-D) ve 1 mg L^{-1} Kinetin (KIN); IM3: 1 mg L^{-1} 2,4-D ve 5 mg L^{-1} Kinetin; IM4: 1,5 mg L^{-1} 2,4-D, 10 mg L^{-1} KIN] analiz etmek amaçlanmıştır ve dört adet rejenerasyon ortamı kombinasyonu (RM1: kontrol; RM2: MS, 30 g L^{-1} sakaroz, 7 g L^{-1} agar, 0,5) mg L^{-1} 1-Naftalenasetik asit (NAA), 2 mg L^{-1} 6-Benzilaminopurin (BAP); RM3: 1 mg L^{-1} NAA, 2 mg L^{-1} BAP; RM4: 1 mg L^{-1} NAA, 4 mg L^{-1} BAP)] piyasadan temin edilen üç adet F1 (Petektar40 : Ptk40; Botanik ve Sardes) ve açıkta tozlanan (Beith Alpha) hıyar çeşitleri kullanılmıştır.

Bu çalışmada ön işlem olarak sıcaklık şoku (3 gün boyunca 35°C) uygulanmıştır. Kullanılan dişi çiçek tomurcukları antezisten 1 gün önce toplanmıştır. Tüm eksplantlar (hıyarların ovaryumları) farklı ortamlarda kültüre alınmıstır. Daha sonra alınan eksplantlar 35°C sıcaklıktaki etüvde, karanlık ortamda 3 gün bekletilmiştir. Daha sonra, aydınlık koşulda 25±1°C'de bekletilmiştir. Bu işlem ve 9 gün boyunca 16/8 saatlik (aydınlık/karanlık) fotoperiyotta (3000-4000Lx ışık) transfer edilmiştir. Bu aşamada ilk gözlemler yapılmış ve 90 mm Petri kaplarındaki ovaryum dilimlerinin yüzeyinde ovaryumların genişlemesi ve embriyoların filizlendiği görülmüştür. Bu çalışmanın sonucunda, giriş ortamında (IM2) embriyo benzeri yapıların (ELS'ler) oluşum oranının [Ptk40 (%20), Botanik (%20), Beith alpha (%16) ve Sardes (%13,3)] yüksek olduğunu göstermiştir. IM2 ortamından elde edilen sonuçlar, diğer kombinasyonlara göre daha başarılı olduğu tespit edilmiştir. Bu ortamdan (IM2) elde edilen kallus oluşum oranlarının, Ptk40 %65, Sardes %60, Botanik %40 çeşidinde olduğu Beith Alpha'da kallus oluşumunun olmadığı tespit edilmiştir. Bu sonuçların ardından RM4, ELS oluşum oranlarını sırasıyla Ptk40 (%12) ve kallus oluşumunun %8 olduğu gözlenmiştir. Rejenerasyon ortamı kombinasyonlarında ELS ve kallus oluşumuna göre en yüksek değerler sırasıyla Beith Alpha (%90,47 embriyo) ve Ptk40 (%93,33 kallus) genotipleri ile RM4'te bulunmuştur. Çeşitlerimizde bitkicik rejenerasyonu düşük olsa bile, besin ortamına sıcaklık şoku ön uygulamasıyla eklenen 2,4-D ve KIN (IM2), hıyarlarda ovaryum kültürünün embriyonik uyartımında uygulanabilir olduğu belirlenmiştir. Elde edilen bitki benzeri yapılar, yaşamlarının erken evresinde ölmüştür.

Çalışmamızın ikinci denemede giriş ortamı kombinasyonları IM5: MS (Murashige ve Skoog), 0,06 mg L^{-1} TDZ; IM6: 0,15 mg L^{-1} 2,4-Diklorofenoksiasetik asit (2,4-D), 1,5 mg L^{-1} Kinetin (KIN); IM7: 0,1 mg L^{-1} 2,4-D ve 1 mg L^{-1} BAP; IM8: 5 mg L^{-1} 2,4 D ve 40 µM Putresin ve

beş adet rejenerasyon ortamı kombinasyonları, RM5 : 0,05 mg L^{-1} NAA ve 0,2 mg L^{-1} BAP; RM6: 0,5 mg L^{-1} NAA, 0,5 mg L^{-1} BAP; RM7: 0,01 mg L^{-1} IAA, 1 mg L^{-1} BAP; RM8: 0,1 mg L^{-1} IAA, 1 mg L^{-1} BAP ve RM9: 0,1 mg L^{-1} IAA, 2 mg L^{-1} BAP şeklindedir. Kullanılan dört ticari hıyar çeşidimizin dişi çiçek tomurcukları, antezisden bir gün önce toplanmıştır. Daha sonra 4 gün boyunca 4°C'de sıcaklık ön uygulamasına tabii tutulmuştur. Tüm hıyar ovaryumları (eksplantları), farklı şekillerde hazırlanmış giriş ortamı kombinasyonunda kültüre alınmıştır. Daha sonra tüm kültürler, ışık koşullarında 25±1°C'de ve 16/8 saat'lik aydınlık/karanlık bir fotoperiyotta (3000-4000Lx ışık şiddeti) iki hafta daha bekletilmiştir. Bu aşamada, gözlemlenen embriyo benzeri yapılar ve kallus oluşumları kaydedillmiştir. Tüm eksplantlar, rejenerasyon ortamı kombinasyonlarında alt kültürüre alınmıştır.

İkinci denemenin sonuçlarına göre IM8 indüksiyon ortamında embriyo benzeri yapılar ve kallus oluşum oranı yüksek olup, tüm çeşitlerde başarılı olmuştur [Botanik (88 embriyo/100 kültür dilimleri), Sardes (60 embriyo/100 kültürlenmiş dilimler) dilimi, geliştirilen dilim başına 150 embriyo), sırasıyla Ptk40 (32 embriyo/100 kültürlenmiş dilim) ve Beith Alpha (20 embriyo/100 kültürlenmiş dilim). Bu sonuçlara göre besin ortamına eklenen 2,4-D ve PUT, hıyar genotiplerinde ovaryum kültürünün embriyo uyartımında soğuk (4°C) şok uygulandığında başarılı olduğu görülmüştür. 124 bitki, IAA, BAP ile içeren besin ortamı içinde rejenere olmuştur. Bunlardan Botanik'te 117, Sardes çeşide on iki ve Ptk40'ta iki adet bitkiciğe benzer yapılar tespit edilmiştir. Botanik çeşidinden 57 gerçek bitki, IBA içeren besin ortamında çimlenmiştir. Sardes ve Ptk40 çeşitlerden elde edilen bitkiciğe benzer yapılarda albino oluşumu gözlenmiştir. Flow sitometri ile 19 diploid ve 1 tetraploid bitki ploidi seviyesini belirlenmiştir. Tüm çalışma boyunca yapılan gözlemler sonucunda, hıyar çeşitlerinin embriyo olgunlaşması, rejenerasyonu, çimlenme besin ortamı kombinasyonu, büyüme mevsimleri, genotipler, ön sıcaklık uygulamaları ve besin ortamı kompozisyonu göz önüne alınarak daha fazla çalışmanın gerekli olduğu belirlenmiştir.

EXTENDED SUMMARY

Cucumber (*Cucumis sativus* L.) is a cucurbit of 2n=14, a major and commercially attractive *Cucurbitaceae* vegetable crop with a highly valued position in the vegetable market. Economically, it is represented by pickling and fresh market cultivars all over the world. Cucumber is widely cultivated and consumed for its fresh and distinct flavour. It is a monoecious, open-pollinated plant that takes 6-8 years to breeders to develop varieties. Cucumber originates from India and is known to have existed for 5000 years. It has spread from India to the east of China, west of Asia, North Africa and Southern Europe. It was taken into culture in the Middle Ages in Europe. Cucumber is in amongst cultivated species in Türkiye and worldwide. It is very essential horticultural crop that is cultivated both in greenhouses and open fields in Türkiye. Türkiye is in the amongst important producers in terms of world cucumber production and was ranked the second world producer with 1.92 million tons after China of 70.3 million tons along with Russian Federation with 1.62 million tons and Ukraine of 1.03 million tons production in 2021. Vegetables are of great importance in human nutrition and health. Improvement studies are carried out on existing varieties to increase the quality and productivity of vegetables. *Cucurbitaceae* plant family contains 118 genera and 825 species spreading particularly in the tropical and sub-tropical regions. The cucumber (*Cucumis sativus* L.), watermelon (*Citrullus lanatus (Thunb.)* Matsum. & Nakai), melon (*Cucumis melo* L.), pumpkin (*Cucurbita moschata* Duchesne ex Poir.), summer squash (*Cucurbita pepo* L.), and winter squash (*Cucurbita maxima* Duch. ex. Lam.) are the most economically significant and crucial cultivated crops within this plant family. These crops have a global presence and have greatly influenced modern society. Vegetable breeding primarily relies on creating pure and stable lines through breeding. Developing new vegetable varieties through traditional breeding in the Cucurbitaceae family is time-consuming. To expedite this process, enhance selection, and improve breeding efficiency, the technique of producing haploid plants has been implemented in cucurbit species.

Biotechnological techniques are very useful in breeding studies of cucumber, which is one of the most important vegetables. In cucumber, haploid plants are obtained through gynogenesis by using in vitro methods and by turning these haploids into double haploid state with some chemicals, absolute homozygous lines that will take place in breeding programs can be provided in a short period of 2-3 years. If the sufficient haploid plants are obtained, after chromosome folding the F_1 hybrid can provide useful material for production. Haploid plants have a gametophytic chromosomal number, whereas double haploids have undergone chromosome duplication. Generation of haploids and (double haploids) DHs is an especially appealing biotechnological tool, and the advancement of haploidy technology and protocols for producing homozygous plants has had a considerable impact on agricultural systems. Compared to traditional breeding techniques, gametic embryogenesis enables the creation of homozygous lines and reduces time needed for their

generation. This permits the direct creation of fully homozygous lines from heterozygous parent plants in a single generation. There are various available techniques to obtain haploid and double haploid, of that *in vitro* ovule-ovary and anther cultures are most effective and widely applied recently in cucurbits as well as horticultural crops. Spontaneous haploid formation has not been found in nature in vegetable species included in cucurbits family. Therefore, studies on haploid embryo and *in vitro* plant production along with gynogenesis in cucurbits have intensified in recent years. Ovule and ovary culture is now the privileged approach owing to its low effectiveness, but it has been employed on species which do not respond to more effective processes.

The haploidy technique is useful method in breeding studies to improve economically pivotal crops including cucumber genotypes to meet the needs and preferences of consumers on market. This research study was carried out in the growing seasons of year 2021 and 2022 particularly in the plastic greenhouse of the Horticulture Department, Faculty of Agriculture, Çukurova University. The aim of this study was to analyse the effects of various factors that are very considered in haploid production via ovary culture in four commercial cucumber varieties such as genotypes, heat and cold shock pre-treatments, and nutrient media combinations. This study was divided into two main parts. Part one was about to analyse four induction media combinations [(IM1: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar (standard medium = control); IM2: medium with 0.5 mg L^{-1} 2,4- Dichlorophenoxyacetic acid (2,4-D) and 1 mg L^{-1} Kinetin (KIN); IM3: the 1 mg L^{-1} 2,4-D and 5 mg L^{-1} Kinetin added in the standard medium; IM 4: 1.5 mg L^{-1} 2,4-D, with 10 mg L^{-1} KIN and four regeneration media (RM 1: control; RM2: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 0.5 mg L^{-1} 1-Naphthaleneacetic acid (NAA), 2 mg L^{-1} 6-Benzylaminopurine (BAP); RM3: 1 mg L^{-1} NAA, 2 mg L^{-1} BAP; RM4 supplemented with 1 mg L^{-1} NAA, 4 mg L^{-1} BAP)] combinations and three F1 (Petektar 40 = Ptk40; Botanik and Sardes) and open-pollinated (Beith Alpha) cucumber varieties obtained from the market were used. In this experiment the heat shock pre-treatment (35°C for 3 days) was applied. The used female flower buds were taken 1 day prior to anthesis. All explants (cucumber ovaries) were cultured in different media. And then, cultures were kept in oven at 35°C/3 days in dark conditions. After, cultures were transferred to 25±1°C in light conditions with a photoperiod (a lux intensity light of 3000 to 4000Lx) of 16h/8h light/dark for a further 9 days. During this stage the first observation was performed and the ovules enlargement and embryos sprouting on the surface ovaries slices in Petri dishes was seen. This experiment resulted that induction media (IM2) was successful in embryo-like structures (ELSs) formation noticed in all varieties [Beith Alpha (22%), Sardes (13.3%), Ptk40 (12%), Botanik (8%)] compared to other induction media combination used in the first experiment. The callus formation rate obtained from this medium (IM2) were Ptk40 (50%), Sardes (46%), Botanik (16%) and Beith alpha has no callus formation. Following these results RM 4 gave the ELSs formation rates Ptk40 (6%), and callus formation (32%) respectively. There was no formation of ELS and callus obtained from IM2 in other varieties. According to the ELS and callus formation in regeneration medium

combinations, the highest number over developed ovaries' slices was found in RM4 with genotypes of Beith Alpha (90.47 %) ELS and Ptk40 (93.33%) (callus) respectively. Even if plantlet regeneration was low in our varieties, 2,4-D and KIN (IM2) added to the nutrient media with heat shock pre-treatment seem to be applicable in the embryonic induction of ovary culture in cucumbers. The obtained plantlet-like structures died at the early stage of their life.

In second experiment of our study, the induction media (IM5: MS (Murashige and Skoog), 0.06 mg L^{-1} TDZ; IM6: 0.15 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 1.5 mg L^{-1} Kinetin (KIN); IM7: 0.1 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP; IM8: 5 mg L^{-1} 2,4 D and 40 µM PUT and five regeneration media as well as RM5: 0.05 mg L^{-1} NAA and 0.2 mg L^{-1} BAP; RM6: 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} BAP; RM7: 0.01 mg L^{-1} IAA, 1 mg L^{-1} BAP; RM8: 0.1 mg L^{-1} IAA, 1 mg L^{-1} BAP, and RM9: 0.1 mg L^{-1} IAA, 2 mg L^{-1} BAP combinations. The used female flower buds of our used four commercial cucumber varieties were taken one day before anthesis. Subsequently, they were pre-treated at 4°C for 4 days. All cucumber ovaries (explants) were in distinct prepared induction media combination. Then, all cultures were kept at 25±1°C in light conditions with a photoperiod (a lux intensity light of 3000-4000Lx) of 16 hr/8 hr light/dark for a further two weeks. At that time the observed embryo like structure and callus formation were recorded. The subculture of all explants to the new and fresh prepared regeneration media combinations was performed.

According to the results of this experiment 2, the embryo like structures and calli formation rate was high in induction media of IM8 which was successful in all varieties [Botanik (88 embryos/100 cultured slices), Sardes (60 embryos per 100 cultured slice, 150 embryos per developed slices), Ptk40 (32 embryos per 100 cultured slice), and Beith alpha (20 embryos per 100 cultured slices] respectively. Based on this results, 2,4-D, PUT added to the nutrient medium was successful with cold (4°C) shock in the embryo induction of ovary culture in cucumber genotypes. The 124 plants regenerated within nutrient medium supplemented with IAA, BAP. From them Botanik has 117 plantlets, Sardes twelve plantlets and two plantlets of Ptk40. The 57 true plants were regenerated and rooted in IBA containing nutrient medium. The 19 diploids and 1 tetraploid plantlets of them were determined by flow cytometry. Due to all observations made during the whole study, we concluded that further research studies are required to optimise the embryo maturation, regeneration, and germination nutrient media compositions of cucumber varieties focusing on growing seasons, genotypes, temperature pre-treatments, and nutrient medium composition.

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SYMBOLS AND ABBREVIATIONS

%	: Percent
°C	: Degree Celsius
µg	: Microgram
µM	: Micromole
2.4-D	: 2,4-Dichlorophenoxyacetic acid
$AgNO_3$	: Silver nitrate
BAP	: 6-Benzylaminopurine
$Ca(ClO)_2$	: Calcium hypochlorite
CBM	: Cucumber basal medium
CRD	: Completely randomized design
DH	: Double haploid
ELS	: Embryo-like structure
FAOSTAT	: Food and Agriculture Organization Corporate Statistical Database
g	: Gram
$g\ L^{-1}$	: Gram per litre
Gy	: Gamma ray
HCl	: Hydrochloric acid
hr	: Hour
IAA	: Indole-3-acetic acid
IBA	: Indole-3-butyric acid
IM	: Induction medium
KIN	: Kinetin
NAA	: Naphthalene acetic acid
m	: Meter
MAS	: Marker-assisted selection
mg/L	: Milligram per litre
Min	: Minute
mm	: Millimeter
mM	: Millimole
MS	: Murashige and Skoog
NAA	: 1-Naphthaleneacetic acid
NaOCl	: Sodium hypochlorite
NaOH	: Sodium hydroxide
PUT	: Putrescine
RM	: Regeneration medium

SPD : Spermidine

SPM : Spermine

TDZ : Thidiazuron

w/w : Weight for weight

1. INTRODUCTION

Cucumber (*Cucumis sativus* L. or *C. sativus* L.) belongs to the *Cucurbitaceae* family with 98 genera and 975 species, found around the world especially in tropical and sub-tropical regions. It is a diploid cucurbit with 2n = 14 (Deepa et al., 2018). It is known as one of the oldest vegetable plants domesticated in India and have existed for 5000 years originated from India. Cucumber is commonly most cultivated species in Turkey and the world. Besides cucumber the genus *Cucumis* contains around 30 diverse species spread across two geographically diverse locations. Northeast Africa, Arabia, and the eastern Mediterranean region serve as the principal gene centers for *Cucumis* genus, with South Africa serving as the secondary gene center (Tatlioglu, 1993). The word *Cucumis,* which was given to the cucumber as a genus name, was first used by Varro in 36 years BC (Günay, 1970). It has been cultivated in warm regions in all countries (Robinson, 1999). Cucumbers are monoecious, gynoecious (Mibus and Tatlioglu, 2004) and parthenocarpic (Guan et al., 2019). Most cucumbers varieties are easy to grow and reach maturity in 50 to 65 days (Ozsan et al., 2017). The fruits of cucumber are eaten as a vegetable and as pickles in the immature stage. Cucumber comprises 12 mg vitamin C, 45 IU vitamin A, 96 g water, 0.6 g protein, 2.2 g carbohydrate, 12 mg calcium, 0.3 mg iron, 0.03 mg vitamin Bl, 0.02 mg vitamin B2, 24 mg phosphorus, 0.1 g fat, 0.3 mg niacin, and 15 mg magnesium in its 100 grams edible portion (Tatlioglu, 1993).

Scientific classification of cucumber plant	
Kingdom: Plantae	**Clade:** Tracheophytes
Clade: Angiosperms	**Clade:** Eudicots
Clade: Rosids	**Order:** Cucurbitales
Family: *Cucurbitaceae*	**Genus:** *Cucumis*
Species: *C. sativus*	**Binomial name:** *Cucumis sativus* L.

The *Cucurbitaceae* family, or cucurbit plants, is one of the most promising plant groups, with roughly 975 species distributed across 98 genera, including several economically valuable and popular fruits and vegetables that are broadly spread in tropical and subtropical regions around the world (Xu and Chang, 2017; Salehi et al., 2021). The most economical important and essential garden crops in this family are cucumber (*Cucumis sativus var. sativus* L.), watermelon (*Citrullus lanatus (Thunb.)* Matsum. & Nakai), melon (*Cucumis melo* L.), pumpkin (*Cucurbita moschata* Duchesne ex Poir.), summer squash (*Cucurbita pepo* L.) and winter squash (*Cucurbita maxima* Duch. ex. *Lam*.) species. All are practiced over the world as well as throughout cultures and have left an obvious influence on modern domestication (Nyirahabimana and Solmaz, 2022; Dong et al., 2016). Most of the vegetables within the cucurbit have been employed not only for nourishment but

also for medicinal purposes (Ugwu and Suru, 2021). Cucurbit plant seeds are famous due to their purgative content, high antioxidant, terpenoids, carotenoids, phytochemical, anti-inflammatory, anti-diabetic, and saponin (Rolnik and Olas, 2020). It is very essential and economically horticultural crop that is cultivated both in greenhouses and open fields worldwide. According to the data of 2019-2021, the cucumbers and gherkins world production average is 93,528,796 million tons of cucumbers are produced in the world. Turkey is in the amongst important world cucumber producers and was ranked again the second with 1,897,681 million tons after China of 72,918,087 million tons along with Russian Federation with 1,654,008 million tons and Mexico of 1,130,180 million tons of production (FAOSTAT, 2021).

The basis of vegetable breeding is inbred pure lines production. Pure lines, which can also be used directly as cultivars or as the source of superior new varieties and high producing hybrids, were seen as the first step in vegetable breeding (Veilleux, 1994). According to classical breeding, it takes time to release pure line for the next breeding studies for several varieties vegetables especially cucurbits plants (Lower and Basset, 1986). For such reasons as shortening the period of time, facilitating selection, and increasing breeding efficiency, the haploid plant production technique has been applied in several cucurbit species. Biotechnological approaches are applied in cucumber breeding studies, which is one of the most consumable vegetables (Hooghvorst and Nogues, 2020). Cucumber as it is mentioned as one of the horticultural crops, it is very useful to monitor the development of new varieties releases (Gürsoy et al., 2012). Breeding studies with commercially grown cucumber plants in the world are very important. Due to the natural foreign pollination of vegetable species in the *Cucurbitaceae* family, breeding studies take a very long time (Lower and Edwards 1986).

The interest in haploids studies began by Blakeslee et al (1922) who have been started with the observations of haploid plants in *Datura stamonium* L. plant. With the understanding of the significance of haploids, studies in many crop species have become widespread. In early studies in cucurbits, Aalders (1958) isolated haploid embryos from seeds of cucumber, while Swaminathan and Singh (1958) examined haploid shoots on a diploid plant grown from watermelon seed treated by X-ray. According to the successfully previous research studies, it has been reported that haploid plant in cucumber varieties is obtained through the application *in vitro* androgenesis, gynogenesis and parthenogenesis, that are commonly used in most cucurbits haploid production (Kurtar and Balkaya 2010). In addition, cucumber studies on haploid embryo stimulation through pollination using irradiated pollen and ovary culture have been also yielded successful results (Diao et al., 2009; Moqbeli et al., 2013; Li et al., 2013). Furthermore, haploids are plants that consist of gametophytic chromosome number (Ren et al., 2017), and doubled haploid is a plant that has undergone chromosome duplication. The evolution of haploidy technology and procedures for producing homozygous plants has had a significant impact on agricultural systems, and the manufacture of haploids and double haploids (DHs) provides an especially appealing

biotechnological tool (Germana, 2011). In contrast to traditional breeding procedures, gametic embryogenesis allows for the formation of homozygous lines and reduces the time required to produce such lines, allowing for the development of entirely homozygous lines from heterozygous parents in a single phase (Kurtar et al., 2016). This special property has particular consequences that can be exploited to the advantage of the plant breeders (Snape, 1989; Ishii et al., 2016).

1.1. Haploid Plants

Plants that carry half of the normal chromosome number (2n) of a species, that is, the chromosome number of reduced gamete cells (n number), are called "haploid". Haploids are morphologically scaled down examples of diploids and develop slightly weaker and haploids that have experienced chromosomal duplication are known as doubled haploids (Emiroğlu and Gürel 1993). The generation of haploids and DHs serves as an especially appealing biotechnological tool, and the advancement of haploidy technology and protocols for producing homozygous plants has had a considerable impact on agricultural systems (Germana, 2011). Haploidization methods are also called useful approaches in plant breeding. If sufficient haploid plants are obtained, after chromosome doubling, F1 can provide useful material for hybrid cucumber production. To date, several haploid cucumber breeding methods have been described, but the number of dihaploid lines obtained and their application in breeding programs have remained rather weak (Gałązka and Niemirowicz-Szczytt, 2013).

There are various existing techniques to acquire haploids and DHs, of that *in vitro* anther and ovule-ovary cultures are most effective and widely applied recently (Chambonnet and De Vaulx, 1985; San Noeum and Ahmadi, 1982; Xue et al., 1983). Currently, studies on haploid embryo and in vitro plant production via gynogenesis in cucumber have intensified (Baktemur et al., 2022; Sorntip et al., 2017; Domblides et al., 2019; Deng et al., 2020). Ovule-ovary in haploid studies on cucumber genotypes the haploid forming potential of their cultures. It has been indicated to be affected by many conditions, low and high temperature treatments, female gametophyte developmental stages, containing genotype, plant growth regulators and other components in the environment and culture conditions (Gémesné et al., 2002; Diao et al., 2009; Gałązka and Niemirowicz-Szczytt, 2013).

1.2. Advantages of Haploid Plants

The haploidization method contributes much more to the selection efficiency (Deng et al., 2020). Previous studies have been mentioned that using the haploid method together with molecular marker technology, can be shorten classical breeding up to 3 to 4 times and reduced to 3-4 years to produce 100% homozygous lines (Sari and Solmaz, 2021). In addition, by increasing the breeding efficiency, significant contributions are made to the competitiveness of the seed production industry (Dunwell, 2010). It is seen as a great potential to obtain haploid plants

primarily in combination breeding studies to protect our native genotypes and to provide them with agronomical important superior traits and in domestic hybrid variety breeding. The obtention of a complete homozygous lines from heterozygous cultivars could be reached in a single generation instead of various selfing generations (Kurtar et al., 2016; Germana, 2011). Furthermore, studies on haploid embryos stimulation and plant production over *in vitro* gynogenesis in cucumber varieties have been intensified in recent years (Gemes-Juhasz et al., 2002; Diao et al., 2009; Wei et al., 2010; Li et al., 2012; Li et al., 2013; Moqbeli et al., 2013; Plapung et al., 2014; Tantasawat et al., 2015; Golabadi et al., 2017; Ozsan et al., 2017; Zou et al., 2018; Erol and Sarı, 2019; Deng et al., 2020; Deng et al., 2021; Pradeepkumara et al., 2023).

1.3. Disadvantages of Haploid Plants

There are still several problems encountered that lead to the haploid plants production disadvantages such as haploid plants production frequency is very low (Sari and Solmaz, 2021) and the selection is still difficult (Gu et al., 2003), the process of tissue culture to develop haploids requires the highest level of management and expertise to develop obtained haploid and double haploid plant for accuracy selection (Yi et al., 2015). Even if the efforts are made latest, the haploid breeding programs have not yielded the desired and expected results (Campbell et al., 2001). Besides haploids, various ploidy levels are also obtained means that the doubling of haploids (diploidization) may not always lead to the formation of the homozygous plant (Domblides et al., 2019; Hooghvorst et al., 2020). The haploid production is associated with a high incidence of albino (colourless) plants (Hooghvorst and Nogués, 2020).

1.4. Problem Statement

Turkey ranks fourth in the world with a vegetable production of 25 million tons after China, India and the USA. In particularly, fewer studies have been undertaken on the cucumber (*Cucumis sativus* L.). It is reported that cucumbers are the most produced and consumable vegetable in Türkiye where from 2015 to 2021 ranked the second cucumber producer in world following China. And it is followed by cabbages, tomatoes, fresh beans, and squashes. The most important disadvantage of cucumber production is that it is done by small-scale and scattered family businesses and specialization cannot be achieved. Turkey can export products with market gaps during off-season. In order to reduce foreign dependency in seed supply, breeding studies to increase domestic hybrid production are insufficient (Abak, 2004). Natural haploid formation is not observed in many species. Although the frequency of spontaneous emergence of haploid plants in nature in some ways varies depending on the species and genotypes within the species, it mostly remains at very low levels as 0.1-0.001% (Pochard and Dumas de Vaulx, 1971). For these reasons, haploids that occur spontaneously in nature could not be used in breeding programs.

Today, the selection of suitable cultivars and the application of quality seedlings are of great importance in successful vegetable as well as cucumber cultivation. In the horticultural sector, with high input in small areas or under cover, it is an agricultural branch that covers an intensive labor and cost. In Türkiye, significant progress has been made in the fields of variety, seed and seedling cultivation in the last twenty-five years in vegetable as well as cucumber agriculture. In addition, the use of excess seeds for spare seedlings in traditional seedling production also increases the expenditures for seeds. This situation emerges as a factor that increases the input cost in traditional seedling production, especially considering the rapid increase in hybrid seed prices (Balkaya et al., 2015). Currently, Turkey breeding programs, preferences were given only to economically important cucurbits crop such cucumber, melon, watermelon, and squashes where more pest and virus-resistant crops are being tested by the means of marker-assisted selection (MAS). The traditional breeding of species belonged to the *Cucurbitaceae* take a long time because most of them are recalcitrant plants (Zheng et al., 2019). In plant breeding efforts, haploid plants are being used because they decrease the time required to produce 100% pure homozygous seedlings, which promises to enhance cucumber production. Whereas, producing pure plants for breeders by classical methods consumes time and are not cost effective for labor.

1.5. Objectives of This Study

1.5.1. General Objectives

In vitro gynogenesis has primarily been utilized on *Cucurbitaceae*, and earlier research has shown that this technology is still imperfect with a low embryogenesis rate (Deng et al., 2021). Cucumber is cross-pollinated and exhibits significant heterosis. Because of their favorable characteristics, hybrid cucumber types have predominated. To improve cucumber breeding efficiency and speed up parent purification, haploid gametophyte cultures can be cultured to stimulate embryoid development, allowing to produce homozygous DH plants in one-two years. This double Haploid (DH) approach is a powerful strategy for speeding up plant breeding operations (Nyirahabimana and Solmaz, 2022). Diverse factors have been shown to influence cultivation of ovules and ovarian in haploid studies of cucumber lines. These influences include genetics, exposure to differing degrees of temperature shock, developmental phases of the female gametophyte, growth regulators, and numerous substances present in the surrounding environment and cultural settings (Gemes-Juhasz et al., 2002; Diao et al., 2009; Gałązka and Niemirowicz-Szczytt, 2013; Ozsan et al., 2017). Our study hypothesized the production of haploid plant with DHs and improving the protocol as well as using temperature pre-treatment was well as cold and heat shocks, several nutrient media combinations, and commercial varieties of cucumber. It has been reported that improving successful varieties frequently depends on knowledge of specific genotypes and traits of parent crops present in the genetic resources (Tantasawat et al., 2015). In due course, high-quality individuals identified from this study will be used as the generation

commercial cucumber breeding population and economically valuable crop which is essential to several small-scale farmers. Our findings will be useful to the improvement of yield, quality and quantity and they will greatly enable the enhancement of the commercial cucumber breeding program in Turkey.

1.5.2. Specific Objectives

The objectives of this research study are as follows: (i) to produce the haploid plants and obtain DHs suited in cucumber breeding by ovary culture, (ii) to determine the suitable ovary cultures induction media components for haploid plants production, (iii) to improve the protocol for saving haploid embryos and plant regenerated from ovary culture in cucumber varieties which are less viable because most of them are dying at early stage of their life.

2. LITERATURE REVIEW

2.1. Methods Used to Obtain Haploids in Cucumber and its Relatives

Gynogenesis, parthenogenesis, or androgenesis effectiviness in cucurbit plants is extremely reliant on the genotype, and prior inestigation have found considerable differences in responses between varieties. The donor plants' environments also influence the incidence of *in vitro* gynogenesis and androgenesis. This phenomenon displays that the induction rate may be related to the parental plants' photoperiod response because surrounding environment impacts their vigor, physiological status and indigenous hormonal level, conditioning of microspores and ovarians to induce tissue culture reactions (Metwally et al., 1998; Seguí-Simarro and Nuez, 2004; Solmaz et al., 2011; Gürsoy et al., 2012; Wang et al., 2014; Dong et al., 2016; Asadi et al., 2018).

Haploid plants production interest began in 1921 with Dorothy Bergner *Datura stamonium* L. haploid production (Blakeslee et al., 1922). In early studies in cucurbits, Aalders (1958) allocate haploid embryos from cucumber seeds, meanwhile Swaminathan and Singh (1958) obtained haploid shoots on a diploid plant grown from watermelon seeds treated by X rays. Haploid and double haploid plants are crucial segments of cucurbits plant breeding. Since the 1970s, anther and microspore culture and unfertilized ovule and ovary cultures have enabled haploids to be obtained in many plants as promising techniques (Yang and Zhou, 1990).

Particularly, *in situ* haploid parthenogenesis, *in vitro* gynogenesis and androgenesis cultures are 3 different common techniques applied in advanced crop breeding studies which have been generated an origin of production of haploids and homozygous DHs plants. Spontaneous haploid formation has not been observed in nature in vegetable species belonging to the *Cucurbitaceae* family. Obtaining *in vitro* haploid plants by androgenesis has not been at a level to be used in cucumber genetic and breeding studies (Kurtar, 1999; Chambonnet and De Vaulx, 1985).

In recent years, studies on haploid embryo and *in vitro* plant production by gynogenesis have been emphasized. In studies on *in situ* haploid embryo induction by pollination with irradiated pollen and *in vitro* plant production from these embryos, cucumber (Truong-Andre, 1988; Sauton, 1989; Niemirowicz-Szczytt and Dumas de Vaulx, 1989; Przyborowski and Nlemirowicz-Szgzytt, 1994; Gülat and Abak, 1999), melon (Sauton and Dumas de Vaulx, 1987; Savin et al., 1988), positive results were obtained in watermelon (Gürsöz, 1990; Sari, 1994) and pumpkin (Kurtar et al., 2002) species. Cucumber is a monoecious, open-pollinated crop. Because it is monoecious acquiring of hybrid cultivars throught traditional breeding techniques requires both suitable facilities and time (Ozsan et al., 2017).

2.1.1. Parthenogenesis

To provide haploid plant in cucumber, Aalders (1958) cultured the embryos of the light seeds remaining on the water by applying the method of floating the seeds of the immature fruits he harvested, and he obtained 13 haploid cucumber plants. Eight of these plants of the Cucurbitaceae family, which have the first monoploid feature, were grown and made diploid by applying colchicine. However, no new individuals were obtained from these plants.

Chambonnet and De Vaulx (1985) provided haploid plants from embryo sac cells by culturing *in vitro* unfertilized ovules in squash (*Cucurbita pepo* L.) study. When plant regeneration was observed in embryos consisting of ovules in suitable conditions and nutrients, the plants were transferred to a free hormones' nutrient medium. Best results were obtained in ovules taken 1 or 2 days before flowering without fertilization, and 4-7 plants out of 100 ovules were obtained. The researchers, who determined that most of the plants were diploid, but some of them were haploid-diploid chimeras, aneuploid or polyploid, compared with the genetic analysis results of diploid plants, 8 heterozygous F1 plants that did not resemble the parent plant were observed.

Previously, Niemirowicz-Szczytt and Dumas de Vaulx (1989) used 300-900 Gy doses of Co^{60} (Cobalt60) treated with gamma rays to pollinate female flowers of the Polan F1 cucumber variety. The rate of fruit binding in pollination with irradiation pollen was observed to be 50-60%, with an average of 250 seeds per fruit. Even if, it has been discovered that the majority of the seeds are empty on the inside. The researchers discovered that when pollinating with control pollens (non-irradiated pollens), fruit retention was 100%, 400 seeds/fruit was an average number, and nearly all seeds contained normal embryos.

Gürsöz et al (1991), on haploid embryo formation with the stimulation technique with irradiated pollen in watermelon, they obtained a total of 761 embryos. The researchers reported that 72% embryos were at globular stage and observed 17 plant transformations from a total of 761 embryos. All of the plants obtained were found to be haploid via flow cytometry analysis.

Przyborowski and NiemirowiczSzczytt (1994) used the irradiation pollen technique to study the impacts of genotype and growing season on four Polan F1 and one native cultivar. The radiation source was Co^{60} gamma rays at a dosage of 300 Gy. The Polan F1 variety utilized by the researchers yielded the best results, with a 1.34% embryo rate.

Moreover, Çağlar and Abak (1996) applied gamma rays originating from Co^{60} with 300, 450 and 600 Gy doses to the male flowers of the cucumber cultivars they used in their research on gamma rays' effects to the viability and germination of cucumber pollen. In their viability tests, they determined that all three doses didn't cause any significant loss in pollen viability. The viability rate was over 80% throughout the year.

Furthermore, Caglar and Abak (1997) obtained the highest haploid frequency in 27 distinct cucumber genotypes at low doses (200 and 300 Gy) in their study conducted between 1991 and 1996 utilizing the irradiated pollen approach. The haploid embryos were germinated easily in E20A

media and were subsequently micro-cut into plantlets. The chromosomal number of haploid plantlets was doubled in vitro and then transplanted to the greenhouse. According to reports, the entire study can be completed in one working year.

Later in 1997, the study based on the evaluation of the effect of haploid embryo induction in two greenhouse cultivars and one open pollinated cultivar using the irradiated pollen technique and four different dosages of gamma rays (100, 200, 300, and 400 Gy) has been performed. Authors have been concluded that the most beneficial dose was 100 Gy, however not all embryos retrieved at this dose were under development-friendly conditions. At the treatment of 100 Gy, they obtained two complete seeds. Authors reported that the recovered embryos germinated on E20A medium (Lotfi et al., 1997).

Yanmaz et al (1997), tried 250, 300 and 350Gy irradiation doses to obtain haploid embryos of the gherkin (*Cucumis melo var. Flexuosus*) type and reported that they obtained embryos and plants at 300 to 350 Gy doses.

Several research has been performed by the irradiated pollen technique as well as the investigation of effect of low doses (0.2 and 0.3 kGy) gamma rays (Co^{60} source) in cucumber lines of different varieties and different genetic levels. Although researchers found differences in the number of embryos among the cultivars studied in their first experiment, they determined the approximate 3.3% plant have been transformed. In their second trial, authors tried lower radiation doses and applied 0.05 kGy and 0.1 kGy radiation doses. They reported that only diploids were formed at a beam dose of 0.05 kGy, and 0.1 kGy promoted the improvement for more haploid embryos (Faris et al., 1999).

Taner et al (2003), analysed the effects of pollen technique on formation of haploid embryos using gamma rays in gherkin (250, 300 and 350 Gy) and melon (300 and 350 Gy) and the correlation between pollen vitality and beam level. Pollen vitality in gherkin was found to be 28.8% at 250 Gy and 28.0% at 300 Gy, and the lowest viability was reported to be 19.4% and 350 Gy. They indicated that pollen vitality of pollen might be reduced based on the age and dose. On the other hand, in melon, 72 hours after the spraying with radiated pollens, it was determined that 64.54% of pollen grass tubes reached the ovary and encouraged the formation of haploid embryos in anthers applied at a dose of 300 Gy.

Besides, Claveria et al (2005), performed the study base on the optimization of the double haploid production in cucumber by in vitro embryo rescue method of induced parthenogenetic embryos. The collected cucumber male flowers were irradiated with 250 and 500 Gy from Co60 radiation source. Number of parthenogenetic embryos obtained from 500 Gy irradiation demonstrated a significant increase.

The two commercial F1 hybrids (Soltan and Monarch) and four local cucumber cultivars as plant materials have been used to produce parthenocarpic cucumber haploid embryos. The male flowers were taken the one day prior to anthesis and irradiated with 250 Gy gamma rays and they

pollinated the female flowers with irradiated pollen the next day. The fruits were harvested 21-23 days after pollination and the seeds were removed in an aseptic environment and were sterilized. Then seeds were developed in an E20A liquid nutrient medium prepared in Petri dishes by shaking them for 10 days. At the end of 10 days, the Petri dishes were observed on a fluorescent light source and the embryo developed seeds were transferred to E20A solid medium. In total of 18774 seeds, 49 embryos were rescued (Lotfi et al., 2008).

A study of the potential use of RAPD markers to characterize haploid and double haploid cucumbers was performed. In their study female flowers of 3 cucumber genotypes were pollinated by the pollen irradiated at 0.3 kGy gamma rays, 0.8 Gy/min Co^{60} source were applied to obtain haploid lines. In 3 to 5 weeks after pollination, the embryos were sterile rescued from ovaries under the stereo microscope and the embryos were transferred to E20A nutrient medium. Embryos which developed into plants, were cultured in MS medium. Depending on the variety and experiment, the mean number of embryos produced in plants ranged from 7.8% to 41.9% (Smiech et al., 2008).

Galazka et al (2015) used eight cucumber genotypes, the 4 F1 cucumber varieties and their 4 F2 lines as plant materials in the study on haploid embryo induction in cucumber. The genotypes of donor plants were examined through a comparison of the number of haploid embryos and DH plants produced by each genotype. In order to ensure haploid embryo induction, the donor plants were irradiated with 300 Gy dose of cobalt source, and 4 fruits per plant were kept. After 5-6 weeks, fruit were harvested, and embryo rescue was performed. The embryos were isolated by embryo recovery method. The embryos transferred to E20A medium. As a result of the study, a sum of 293 embryos and 93 haploids were gained in F1 varieties The 0.19-0.4 embryos per fruit were obtained in F1 varieties and 0.46-0.59 embryos from F2 varieties.

Salehian et al (2023) analysed cucumber haploid and DHs production through the pollination of female flowers with pollen treated by gamma rays. The anthers were irradiated with distinct doses (200.0, 300.0, and 500.0 Gy). Regarding the findings, pollination is most effective haploids obtention when pollen has been treated at 300.0 Gy dose. The regenerated plantlets' ploidy level has been identified using flow cytometry. Almost all plants were 78.39% haploid and 21.6% mixoploid. The haploid plants' chromosomes number were doubled via *in vitro* treatment of nodal explants with various colchicine concentrations. The use of 500.0 mg/L colchicine for 24 hrs led to the most successful regeneration rate (85.4%) of doubled haploid.

2.1.2. Androgenesis

a. Androgenesis in Cucumber

Subsequently, Kumar and Murthy (2004) investigated the effects of polyamines, spermidine, and putrescine concentrations ranging from 5-10000 µM in their anther culture studies on two cucumber genotypes Calypso and Green Long. Authors used 2.0 µM 2,4-D, 1.00 µM BA, 25 M sucrose in Gamborg B5 medium as basal medium. They reported that the best results have

been obtained from the medium containing 200 μM spermidine, and that the highest doses of 500-1000 μM putrescine and spermidine were not beneficial for embryogenesis.

Mohamed and Refaei (2004), conducted the study on cucumber anthers, have used MS medium combined with 6 mg/L 2,4-D and 100 g/ L sucrose. They transferred the calli to MS medium with 0,05 mg/L kinetin and NAA added, and they have been concluded that there were 2.6 plants per anther.

While Song et al (2007) established a six-step protocol that includes temperature pre-treatment, embryogenic callus medium, culture conditions, embryo induction medium, embryo germination medium, and genotypic effects to develop an efficient anther culture protocol which may be useful in cucumber genotypes. Authors reported that varieties grown in cold regions gave good results to cold applications, and varieties grown in temperate regions gave good results to the heat shock pre-treatment. The best medium for embryogenic callus induction was MS supplemented with 4.44 μM BA, 2.26 μM 2,4-D, 4.64 μM KIN hormone combination. For embryo induction hormone combination contained 0.54 μM NAA, and 13.32 μM BA, 2.22 μM was efficiency to embryo germination. Researchers observed that the nutirent medium containing BA, 6% sucrose and 1.2% agar gave the best results. As a result of the study, three embryos per anther and 42 (93% success) double haploid plants from 45 anthers were obtained. Suprunova and Shmykova (2008), have been found that cucumber anthers with 8% sucrose MS+100 mg/L serine, 800 mg/L glutamine and various plant growth regulators (2.0 mg/L 2,4-D and 1.0 mg/L BA; 1.0 mg/L 2,4-D and 0.5 mg/L BA; 0.4 mg/L 2,4-D and 0.2 mg/L BA) for media modification and the result of callus was obtained.

Additionally, Zhan et al (2009) carried out a microspore culture in 10 different cucumber varieties. Cold pretreatment (4°C) for 2-4 days also improved embryoid induction, with the maximum embryoid output obtained from microspore pre-treated at 4°C for 2 days. The results also showed that low concentrations of exogenous hormones (0.5 mg/L 2,4-D, and 0.2 mg/L 6-BA) had a positive impact on embryogenesis, and that only those at the cotyledon stage of the embryo turned into plants, although B5 and NLN mediums had no significant difference in embryo induction.

Nguyen and Chen (2012) investigated the effects of cucumber anther culture pre-treatment, medium compositions and concentrations, and genotype utilizing anthers of 3 different cucumber cultivars (Kaluoer, HH1-8-57, and Jinlu Nongjiale). The results revealed that pre-treatment at 4°C for two days has significantly raised the embryonic callus formation ratio. And the best embryonic callus rate (81.3%) was obtained on (MS + 1.0 mg/L 2,4-D + 0.5 mg/L 6-BA + 3% sucrose + 0.8% agar); the highest rate (40.0%) embryoid induction was obtained on (MS + 0.1 mg/L NAA + 3.0 mg/L BA) nutrient medium. The examined cucumber cultivars varied greatly in their embryonic callus and embryoid induction ratios. The variety HH1-8-57 had the best embryo formation rate

(40.0%), and Jinlu Nongjiale variety had high embryonic callus formation rate (81.1%). Two of the three genotypes examined for ploidy level and produced haploid plants.

In the research of (Gałązka and Niemirowicz-Szczytt 2013) investigated the impact of cold pretreatment 4°C for two to four days for cucumber flower buds and applied the culture media composed by auxin (NAA, IAA, 2,4-D) and cytokinin (BAP, KIN, TDZ) based on used genotypes. After the inoculation of anthers in Petri dishes containing induction medium the incubation was at 25°C-35°C for 2-20 days. The study results have been shown that these conditions are favoured to increase the haploid induction rate from cucumber anther culture.

Moreover, the analysis of the effect of combinations of different genotypes and plant growth hormones as well as kinetin, BAP, and 2,4-D on callus and gametic embryo formation has been in cucumber anther culture performed. The highest callus formation in the combination of 1.5 μM kinetin and 2 μM 2,4-D but they achieved the highest embryo formation per anther in (2 μM 2,4-D and 1.0 μM BAP) combination (Hamidvand et al., 2013).

Abdollahi et al (2016) evaluated the nutrient medium and genotypes (landrace Esfahani and Beith Alpha) effects on cucumber anther culture. Interestingly, the observation of genotypes' distinction in used genotypes were noticed on MS culture medium with modified macronutrient (half, full, 1.5-fold, and double-strength). The callus induction also was performed by testing 25 distinct combinations of BAP and 2,4-D concentrations with 10 different combinations of BAP and NAA concentrations. While basal MS (vitamin and salt), 3% sucrose and agar of 0.8% kept fixed with pH 5.8. The callus formed from landrace Esfahani did not develop in structures, embryogenic or organogenic. Apparently, embryo formation and plant regeneration in Beta Alpha were affected during callus induction at initial conditions. After six weeks of culture, induced callus were recorded and the highest chromosome doubling rate (81%) was observed. They conclude that the genotype of donor plants is a key factor to the cucumber anther culture' s protocol alterations. The study has been proven that heat pre-treatment of anther at 32^{o}C for 2 days was to be optimal for embryo induction, and the maximum number of embryos per anther was 1.85 embryos.

Asadi et al (2018), assessed the haploid production induced by the anther culture using different culture conditions such as temperature and medium composition for two genotypes of cucumber (landrace Esfahani and Beta Alpha F1). The male flower pretreated at 4°C for 2 days and cultured anthers were exposed at high temperature 35°C for 1 hr, and then taken to 25°C/ 20 days under 12/12 light photoperiod in prepared medium until calli and/or embryos produced. By using 0.68-0.91 mg/L BAP and 1 mg/L 2,4-D the best production of 6.67% diploid/DH was obtained. Thus, in the induction stage maximum 2,4-D concentrations yielded more embryo from calli, many embryos were generated. After flow cytometry and SSR analysis were performed in produced 14 plants, ten plants (71.4%) were found DH and four (28.6%) heterozygous, i.e two of them diploid and two tetraploids.

Ghanbari and Golabadi (2020) investigated the effects of hormones' concentrations, compositions, temperature, and light pre-treatments towards callogenesis, embryogenesis and plant regeneration from cucumber anther explants. The open pollination cucumber landrace Isfahan and greenhouse inbred line were utilized. Cold (4°C), fresh flower, light treatment (brightness and darkness) and evaluation of five different levels of hormone concentrations of KIN (1-2.5 mg/L), BAP (0.1-3.5 mg/L) and 2,4-D (1-3.5 mg/L) was performed. The findings have demonstrated that callus formation of greenhouse inbred line was better during darkness treatment. While did not significantly affect the landrace genotype. The best combination was BAP: 2,4-D at 3.5: 1 mg/L (for both lines). In other hand cold pre-treatment indicated the significant effect on haploid embryos production

Amirian et al (2020), examined induction in male flower of three cucumber genotypes with *in vitro* anther culture. Two Beth alpha F1 (BT1 and BT2, gynoecious) and Dastgerdi (DTG, monoecious) in the application of single and combination $AgNO_3$ (3 mM), GA_3 (1.5 mM) and $CoCl_2$ (3 mM) spray after the gynoecious cucumber's first true leaf emergence. Androgenesis was performed in two gynoecious varieties, where donor plants were treated by $AgNO_3$ (3 mM) or GA_3 (1.5 mM) and the DTG genotype without any treatment. DTG variety demonstrated the best embryogenic callus formation rate (62.2%). The number of embryos per anther was 1.81. According to the findings, embryogenic callus formation rate (27.1%) obtained from gynoecious varieties and (0.23) embryos number per anther. Based on SSR marker and cytological and analysis 16 regenerated plants were spontaneous doubled haploid, 2 diploid, and 1 tetraploid plants.

b. Androgenesis in Other Cucurbits

Metwally et al (1998) conducted a study to analysis the effect of sucrose and 2,4-D (2,4-Dichlorophenoxyacetic acid) combinations on the formation of haploid plants of a summer squash variety through anther culture, solid MS (Murashige and Skoog, 1962) in anther culture medium; they tried sucrose at ratios of 30, 60, 90, 120 and 150 g/L and 2,4-D 0.1, 1.0, 2.5 and 5.0 mg/L. As a result of the research, they reported that the most regenerated plantlets obtained from the incubation medium supported with 150 g/L sucrose and 5 mg/L 2,4-D. At the same time, they subjected male flowers of summer squash species to cold pre-treatment for 4 days at 4°C and they obtained 1.9 plantlets per anther in MS medium containing 5 mg/L 2,4-D and 150 g/L sucrose.

Rakha et al (2012), conducted a study about anther culture in six interspecific Cucurbita hybrids and the study evidenced that the induction medium (1.0 mg/L 2,4-D) is good and suitable than mediums with other concentrations for callus regeneration. Auxin (NAA, IAA, 2,4-D and others) and cytokinin (BAP, KIN and others) have been known to be broadly useful in in vitro anther/microspore cultures as well as in vitro un-pollinated ovule-ovary cultures.

Abdollahi et al (2015) generated the embryo induction high-frequency (1.47 and 1.20 embryos) per anther were produced respectively in Crimson sweet and Charleston gray watermelon

cultivars by using MS medium modified by 0.44 mg/l 2,4-D and 0.372 mg/l KIN or 0.338 mg/l BAP. Plant growth regulators can be also combined in different ways such as combinations of auxins and cytokinins. The best ME/EC was recorded from medium combination of 2 µM 2,4-D and 1.5 µM KIN and also in combination with 1 and 1.5µM BAP, the embryos average was 1.90 and 1.85 respectively. The study has been supplied that the anthers of Charleston Gray watermelon did not show any response to anther culture on free plant growth hormones basal MS medium. Cold pre-treatment and/or heat shock has been applied to a variety of cucurbit species to attempt large scale of embryo and haploid plants in general.

Nguyen et al (2019) tested anther culture method on bitter melon (*Momordica charantia* L.) F1 hybrids. The study's findings demonstrated that the microspore developmental stage has a pivotal impact on callus formation frequencies. It was stated that substantially, callus formation ratio and callus morphological are depending on the composition and concentration of growth hormones in the nutrient medium and various pre-treatments. The male flower buds taken at 5-7 a.m kept into plastic bags in dark condition at 4°C for 1 day. Three variants of Murashige-Skoog (MS) nutrient media were used, differing in composition and concentration of growth regulators. Mediums combination were (1.0 mg/L 2,4-D and 1.5 mg/L BAP); (1.5 mg/L 2,4-D and 1.0 mg/L BAP); (1.5 mg/L NAA, 1.0 mg/L BAP and 0.5 mg/L KIN). The callus formation (93.75±2.55%) recorded as high frequency which was seen (1.0 mg/L 2,4-D and 1.5 mg/L of BAP) culture medium.

Androgenic responsiveness has been demonstrated to vary depending on genotype in various research on cucurbits and cucumber. This mechanism demonstrates that the embryo and callus induction frequency are associated to photoperiod response of the parental plants, and the surrounding environment, physiological status, and their indigenous hormonal level (Metwally et al., 1998, Seguí-Simarro and Nuez, 2004; Solmaz et al., 2011; Gürsoy et al., 2012; Wang et al., 2014, Dong et al., 2016; Asadi et al., 2018).

2.1.3. Gynogenesis

a. Gynogenesis in Cucumber

Haploid is an ideal material for breeding, unpollinated ovary culture is an efficient method to obtain cucumber haploid (Cheng et al., 2008). Several experts and scholars conducted preliminary studies on in vitro gynogenesis culture for various crops, even if was not a successful method, the in vitro culture of non-pollinated ovary plants had made some progress and achievements (Zhu et al., 2018). The ovary culture method has been gained the popularity where from the biggening still now is an applicable technology in cucurbits.

Gémesné et al (1996) used the starting medium containing 0.02 mg/L TDZ in the ovary culture studies on cucumber and zucchini plants. Then, media containing NAA and BA were used

for embryo regeneration and they reported that they obtained embryos in both cucumber and zucchini.

Gémesné et al (2002) cultured the ovary using 5 parthenocarpic cucumber breeding cultivars (7D4C, KS0C, D20F2A5, E10D14, Perez ML) (female parent) and Perez F1. The researchers collected the ovaries of the donor plants in 3 days, 6 hours before and at the anthesis day and cultured them in vitro. The 150 ovaries in each genotype were tested in the study. The 0.02 mg/L TDZ and 4% sucrose were added to cucumber basal medium (CBM) used as regeneration medium. The incubation of all cultures has been performed in darkness at 24°C, 28°C or 35°C for 2 to 10 days. After the ovaries were transferred to the differentiation medium (CBM) supplemented by 0.05 mg/L NAA, 0.2 mg/L BA and 3% sucrose, and were exposed to a 16 hrs/8 hrs (light/dark) light regime at 26°C. According to the findings of the study, the researchers reported that the maximum embryo was obtained from the application of 35°C temperature, the maximum embryo formation rate 18.4% per ovary was obtained, and the plant formation rate was 7.1%. While the maximum frequency of plant regeneration was 7.1%. The 87.7% regenerated plants were haploid according to flow cytometry analysis.

Kumar et al (2003) analyzed the impacts of temperature pre-treatments and growth regulators in their anther culture studies on two cucumber genotypes Calypso and Green Long. Optimal embryo and embryogenic callus formation was produced from 2.0 mM 2,4-D and 1.0 mM BAP combination. The flower buds were pretreated for 0 to 10 days at 4°C and at 32°C for one day. Better results reported to be incubated at 4°C for 2 days. Embryo regeneration was carried out in B5 medium with 0.09 M sucrose, 0.25 mM naphthalene acetic acid and 0.25 mM kinetin, while embryo development was performed in medium containing 5.0 mM ABA. Plantlets were formed from embryos in Gamborg B5 medium supplemented with 0.09 mM sucrose. Root tips of 24 plantlets from each cultivar were used for the analysis of ploidy levels, 21 haploid plants were obtained in Calypso cultivar and 17 haploid plants in Green Long cultivar.

Diao et al (2009) investigated the impact of TDZ concentration with thermal shock pretreatment, induction medium, the effect of silver nitrate to embryo induction. In the use of SSR markers, homozygous from heterozygous varieties were distinguished. The findings indicated that heat shock (35°C) application for 3 days resulted in a higher frequency (44.5% to 89.4%) of embryo formation than two or four days. The TDZ concentration has the positive impact on embryo development, and high occurrence rate (72.7%) from 0.04 mg/L TDZ. Authors explained that the supplementation of $AgNO_3$ in induction medium did not have a significant effect on the embryo formation frequency, but reduced the embryo sprouting time and increased the embryos' number on each ovary slice. The study reported that 33 plants were diploid chromosome number, while 17 of 33 diploid plants were spontaneously double haploid as a result of homozygosity analysis with SSR markers.

It has been reported that with the aim of increasing the haploid plant yield obtained from in vitro of unpollinated ovules, anther and microspore cultures in cucumber, the examination on the effects of genotype, flower bud length, plant growth regulator concentration and developmental stages of microspores was carried out. In terms of plant growth regulators, researchers determined that the optimal concentration for gynogenesis induction was 0.2 mg/L TDZ. They stated that of the ten genotypes evaluated, only Gordion cultivar was able to develop haploid plants by in vitro gynogenesis (Suprunova and Shmykova, 2008).

Therefore, Vasudevan et al (2008) analyzed the effectiveness of amino acids (leucine, isoleucine, methionine, threonine and tryptophan) and polyamines (spermidine, spermine and putrescine) together with (BA) on the enhancement of shoots in cucumber. The observation showed that the utmost number of shoot growth was in the combination of leucine (88 µM), BA (4.44 µM), and spermidine (68 µM) compared to the combination of only BA and BA with Leucine. Long shoots were transferred to MS medium supplemented with BA (4.44 µM), leucine (88 µM) and putrescine (62 µM) for root induction, while rooted plants were developed and successfully established to the soil with 90% viability.

Wei et al (2010) utilized 5 varieties with best gynogenesis response and 4 varieties with low response as donor plants to evaluate the correlation between cucumber gynogenesis, hormone and polyamine contents in unpollinated ovaries in cucumbers. Hormones of IAA, ZR, GA_3 and ABA in unpollinated ovaries explants immediately prior to culture and at 3, 6, 9, and 16 days of culture, free and soluble compounds like spermidine (SPD), spermine (SPM), putrescine (PUT), and cadecine (CAD) conjugated polyamines were also analyzed. The findings reported that there was no direct association between IAA, ZR, GA_3 and ABA components in the ovaries. However, the lowest IAA/ZR, IAA/GA_3 and ABA/GA_3 ratios were beneficially in the response of in vitro gynogenesis. Genotypes with better gynogenesis response, free and conjugated SPD + SPM. The best total polyamine components, and lower ratio of PUT/total polyamines compared to those with lower gynogenesis response explained. SPD was the largest polyamine strain in unpollinated ovaries before culture, SPD and SPM contents reduced dramatically after 6 days of culture, while PUT and CAD increased significantly, and PUT became the most important polyamine at 6 days culture.

Li et al (2012) performed a study to determine the most suitable starting environment for embryo promotion and suitable regeneration environment for plant transformation from embryos in the unfertilized cucumber ovaries from Henan cucumbers. The highest rate of embryo formation occurs when the explants, which have been heat treated for 3 days, are placed in media containing 0.06-0.08 mg/L TDZ, with 0.05 mg/L NAA and 0.4-0.6 mg/L BA content. As a result of the study, the researchers obtained embryos from 3 different types. It has been reported that genotype difference, temperature pre-application time, TDZ concentration in the nutrient medium and the

developmental stage of explants affect the embryo formation rate. It is stated that the 6-BA and NAA concentration in the nutrient media also affect the transformation of embryos into plants.

Li et al (2013) tested the effect of TDZ and silver nitrate in unfertilized ovaries of Chinese long cucumbers. The high growth frequencies (7.85-12.14%) were observed during anthesis and on 0.03-0.07 mg/dm³ TDZ. The analysis showed that embryo sacs were fully develop during anthesis, and best plant differentiation rate was obtained in CBM contained by 0.05 mg/dm³ NAA, 0.2 mg/dm³ BA and 5-10 mg/dm³ AgNO3. Analysis of flow cytometry showed that 80% of the grown plants were haploid.

However, in the study of Moqbeli et al (2013), the growth and production of in vitro haploid lines in some new cucumber cultivars was studied, six cucumbers as Summerstar, 502×605, Kashmir, Adergreen, Royal, 2010-3 varieties were used. For 0, 2, 3 or 4 days at 35 ± 1°C heat shock was applied after inoculating the unpollinated ovary pieces of each variety on induction medium containing different concentrations (0, 0.01, 0.02, 0.03 and 0.04 mg/L) TDZ. The first formed structures were observed 3 weeks after culture. The best embryogenesis rate was achieved in Summerstar cultivar in medium composed by 0.04 mg/L TDZ. The study reported that TDZ had a positive impact on embryo induction and best findings were observed with heat shock applications at 35±1°C/ three days.

Plapung et al (2014), carried out research on production of haploid plants in the ovule culture part of their study on the development of cucumber mosaic virus resistant cucumber lines. They kept the unfertilized cucumber ovaries from 68 cucumber lines in 10% Ca $(ClO)_2$ and 3% NaOCl for 20 minutes and then rinsed them 3 times with sterile distilled water. Cucumber ovaries were kept for 1 month in CBM medium modified with 5 ppm $AgNO_3$. For embryo promotion, ovules detached from the ovaries were transferred to the starting medium with kinetin, BA: IAA, 2ip (6- (gamma, gamma-Dimethylallylaminopurine): IAA ratios 2:1, 3:1 and 4:1 ppm and kept for two months to grow. The resulting green embryos were transferred to regeneration medium modified with BA: IAA, 2ip: IAA ratios of 2:1, 3:1, 4:1 and 5:1 ppm. They assigned that the plants were haploid by rooting the plants with plant transformation in MS environment and by chromosome count.

The impact of combining of different medium in some cucumber's cultivars by ovary culture to embryo induction and haploid plants were analyzed. In the study, three different media combinations were altered fixed rate by plant growth regulators for embryo induction and plant regeneration. The effects of media, genotype, and plant age examined weekly. Owing to the findings, the most effective embryo stimulating medium was MS + 1: 10; 2,4-D: Kinetin (MSB), the most effective regeneration medium is MS + 1: 4, NAA: BAP (MSR). In total, 273 embryos were obtained, and 137 plant regenerations were observed. According to the total number of applied ovaries, embryo transformation rate (84.26%) and plant regeneration rate (42.28%) were obtained (Çetinkaya, 2015).

Tantasawat et al (2015) evaluated the impacts of several factors containing varieties of donor plants, induction and regeneration mediums, and heat shock pre-treatment on ELS and callus induction over unpollinated cucumber ovary culture. Although the donor plants differed in ELS, and callus formation potential were observed in all applications. The use of 1 mg/L TDZ and 1 mg/L BA to the induction medium resulted in the highest percentage of ELS formation in the 42.3% to 91.4% range, and 60.4% average. However, the highest percentage of callus formation (70.8%) was obtained in the nutrient medium containing 2 mg/L BA, 0.5 mg/ L indole-3-acetic acid (IAA), 1 mg/L gibberellic acid (GA_3) and 32 mg/L putrescine. Researchers concluded that heat shock reduced the percentage of embryo-like structure induction, which had no significant effect on callus formation.

Liu et al (2015) studied on embryo formation and regenerated plant from unfertilized cucumber ovary culture. Un-pollinated ovary culture was applied in 7 different cucumber (Cucumis sativus L.) varieties. The findings demonstrated that heat shock at 35°C for 2 days could gain the highest embryo yield, and the differential medium with 0.20 mg/L NAA and 0.8 mg/L 6-BA was optimal for embryo differentiation. The highest embryo yield was harvested with ovary selected at 21-30 nodes of the plant stem. Thus, the plant regeneration rate was increased. The ratio of diploid plants in regenerated plants was 70%, and the DH plant ratio in diploid plants was 84%. The embryo yield was significantly different among different genotypes.

Golabadi et al (2017) carried out ovule-ovary culture study on local Iranian cucumber varieties. In their research, they examined the effects of temperature pretreatments, hormone combinations and silver nitrate ($AgNO_3$). The study has been reported that the highest embryogenesis rate was observed from 0.5 mg/L NAA + 0.7 mg/L 2,4-D + 1 mg/L KIN + 1.8 mg/L BAP hormonal combination together with the thermal shock pre-treatment application in their first experiment. While the highest rate of callus induction was noticed in lack of heat shock and hormonal combination of 1.5 mg/L NAA + 0.7 mg/L 2, 4-D + 1 mg/L KIN + 1.8 mg/L BAP. The research was carried out to display the effects of different media on cucumber gynogenesis.

The unpollinated cucumber ovaries of 3 genotypes (0701, 0702 and 0703) taken one day prior to anthesis. Following surface sterilization, the obtained ovaries were split into four halves and pretreated on 8 distinct altered cucumber basal media (CBM). The explants in various media were maintained in the dark at 35°C for three days before being transferred to 25°C in the dark for another two days. Following pretreatment, all cultured media were exposed to light for 9 days at 25°C with a photoperiod of 16/8. Several parameters, including as genotypes and culture media, were studied. Callus production, embryo creation, and plantlet regeneration were also seen. Regarding the embryo and plantlet developments, the study's findings showed that the combination of genotype 0703 and CBM contained 0.03 mg/L TDZ medium produced the greatest outcomes (Ozsan et al., 2017).

Sorntip et al (2017). The effects of donor plant genotypes and three culture media (induction, differentiation, and regeneration) were examined in unpollinated cucumber ovary culture to generate the efficacy protocol for doubled haploid (DH) production in recalcitrant genotypes. Differentiation and regeneration media have a major impact on the creation of embryo-like structures. During the ovaries were cultured on differentiation medium and moved to the fresh prepared medium enriched with multiple plant growth hormones and additives. Therefore, regeneration culture medium, gave 59.89% ELS which was reported to be high percentages of production in the study. However, no genotypes or induction (first step culture) medium had a significant effect on ELS or callus induction. When the ELSs were moved to the third stage culture media, they grew into shoots, and full plants were observed when all were rooted on the hormone free MS.

Recently, Erol and Sarı (2019) studied the impact of spermidine (SPD) and putrescine (PUT) on the formation of haploid embryos in cucumbers using ovule culture. Spermidine and putrescine were employed at concentrations of 40 µM/L, 80 µM/L, 120 µM/L, 160 µM/L, and 200 µM/L to study the effects of polyamines. The five cucumber varieties Sardes F1, Cemre F1, Toros F1, Altay F1, and Langa were utilized. Ovule culture, liquid culture, and irradiated pollen approaches were investigated as three distinct methods to produce haploid plants. For the liquid culture, an initial medium including MS medium and 0.18 µM/L TDZ was used, along with 160 µM/L SPD, 160 µM/L PUT and SPD + PUT 320 µM/L (1: 1, v: v). Notably improved outcomes were observed in mediums supplemented with Spd and Put. The induction medium led to the development of embryos from Sardes F1, Cemre F1, and Altay F1 varieties. With the exception of the Langa variety, all cucumber types successfully produced 14 haploid plants using the irradiated pollen technique, which employed a 300 Gy radiation.

A potential development in cucumber breeding is the creation of F1 hybrids that distinguish themselves from varieties by plant uniformity in fruit sizes, superior yield, ripening date, and quality. The objective of research was to improve the in vitro gynogenesis induction conditions in unpollinated ovule culture in order to increase the emergence of new breeding forms and speed up the creation of homozygous lines. The study used eight promising cucumber accessions. The induction medium culture of 30 g/L sucrose and 7 g/L agar with 200 mg/L ampicillin and 0.2 mg/L TDZ was employed to enhance gynogenic growth. It has been demonstrated that the half-open bud was the best explant for growing. All accessions produced the most embryo-like structures from ovaries that were 2.1-2.6 cm length (Domblides et al., 2019).

In order to grow regenerated plants, seven different unpollinated F1 cucumber ovary types were used. The utilization of three donor plants and MS + 0.06 mg/L TDZ medium, plantlets were successfully regenerated. Based on evaluations made with the use of flow cytometry, chromosome counting, and SSR analysis, the regenerated plants were classified as haploid, double haploid, and autotetraploid. Under MS + 0.04 mg/L TDZ + 1.0 mg/L 1 6-BA + 0.1 mg/L NAA + 12 mg/L

$AgNO_3$ combination medium, a high number of embryo like structures were developed in the ovary slices, there was no plant regeneration occurred after differentiation culture (Zhou et al., 2020).

Deng et al (2020) conducted an experiment to investigate interaction between cucumber genotypes, cold pre-treatment, and TDZ treatment in order to optimize haploid plant induction. Cold pre-treatment stimulated the ovary effectively. The findings demonstrated that 0.06 mg/L TDZ, cold pre-treatment for four days, and genotype interaction can be employed as an essential technique to develop gynogenesis effectiveness. The highest plant regeneration rate was 79.3%. Authors also observed the regenerated plants were diploid or haploid using flow cytometry and chromosome counting approaches.

Currently, ovary culture is an effective method for producing DH plants in cucumber. Cucumber ovary culture, on the other hand, has lower rate of embryo development and plant regeneration. The used ovaries in the study were collected at 6 hours before anthesis and were pre-treated at 4°C. The sterilization was performed; ovaries sliced, and placed on nutrient medium, then incubated at 33°C/2 days in darkness. After cultures transferred to 25°C growing chamber until plantlets regenerated (Deng et al. 2021).

Demirel and Onus (2021) analysed cucumber types and varieties' effects on haploid embryo induction and plants production in 2 distinct cucumber types (Cocktail and Beith Alpha) and 5 various varieties (Çengel F1, Ufuk F1, Sedir F1, Amisos F1, Ptk40 F1). The MS medium and vitamins, 1:10; 2,4-D: KIN hormones induction medium and 1: 4 NAA: BAP hormones for plant regeneration medium combinations were applied. Significant difference was obtained during embryo and plant formation Çengel F1with 494.44 % and 302.78%, while the lowest amount obtained in Ptk40 F1 and Ufuk F1 varities.

Instantly, the research study examined the effect of genotype and medium composition on cucumber haploidization through ovarian culture. And three nutrient mediums were examined on 31 cucumber accessions. The responses of all used genotypes varied. The best responses well found in some cultivars with nutrient media. According to nutrient medium, the best performance was obtained in medium contained 2,4-D. With flow cytometry haploid plant rate was 34.72% of the examined plants. While the proportion of diploid plants was 37.5% (Baktemur et al., 2022).

Although it was stated that cucumber is one of recalcitrant plant, more emphasis on haploid induction using gynogenesis is needed in the in vitro production of new cucumber varieties.

Presently, Pradeepkumara et al (2023) conducted a study on in vitro gynogenesis, where F1 donor mother plants were created utilizing the genotypes DC-48, which has a very long shelf life, and DC-83, which has a very short shelf life. Authors analysed the effects of sucrose concentration, plant hormones, cold and heat shock pre-treatment, and ovaries developmental stages on F1 variety and its parents. The most advantageous developmental stage for the effective production of ELSs and haploids was unfertilized ovules cultivated one day before anthesis. Basal MS based media comprised with 0.2 mg/L NAA and 5.0 mg/L Zeatin, most powerful induction numerous ELSs in

all cultivars. The best temperature treatments produced a high rate of direct regeneration were cold pre-treatment at 9°C/ 4 to 6 days followed by a heat shock treatment at 32°C/ 2 to 4 days. For ploidy level assessment SSR-based detection and flow cytometry were performed. In the 16 selected plants 3 of them were diploids, and the remaining 13 were haploids.

b. Gynogenesis in Other Cucurbits

Metwally et al (1998) produced haploid plants from C. pepo (cv. Eskandarani) from unfertilized ovules of squash plants' ovaries collected one day before anthesis in the application of cold treatment and solid MS medium supplemented by 2,4-D.

Ficcadenti et al (1999), have been conducted studies of gynogenensis on un-pollinated ovaries in their study on melon. It has been reported that the ovaries were cut and cultured for 4 days in MS medium modified with 0.09 μM TDZ, and then transferred to MS medium modified by 0.27 μM NAA and 0.88 μM BA. They have concluded that gynogenic embryos were observed at the end of 4-6 weeks, and a total of 82% gynogenic embryos were formed from all genotypes. According to the chromosome counts of regenerated plants, it is stated that 15.4% are haploid, 23.1% are diploid, and 61.5% are mixoploid.

Lotfi et al (2003), performed a study to compare the irradiated pollen technique and ovary culture in melon haploid plant yield. The researchers have been stated that after the ovaries were immersed in Tween 20 and removed, they rinsed with water and then kept in 100% Clorox solution for 15 minutes and rinsed with sterile distilled water three times. Divide the ovaries (8-15 mm long) into 6-10 parts, 0.4 mg/L thiamine, 100 mg/L myo-inositol, 40 g/L sucrose and 0.02 mg/L (0.09 μM) TDZ, and MS basic salts were transferred to the medium containing the basic salts. After 4-5 days, they transferred the ovary fragments to the same medium modified with 0.05 mg/L (0.27 μM) NAA and 0.2 mg/L (0.88 μM) BA instead of TDZ. They cultured a total of 122 ovaries from two different genotypes and mostly observed callus formation from the somatic tissues of these ovaries. It is reported that 3 plantlets were obtained from these ovaries at the end of 5 to 6 weeks and the ploidy level of them could not be determined.

Shalaby (2007), in his study on obtaining haploid plants from ovule culture in squash, in 12 genotypes; examined the appropriate ovary stage, the required temperature application and the effect of sugar concentration. She took the ovules of ovaries collected 1 day before the anthesis to MS media containing 3%, 6%, 9% sugar with 1 mg/L KIN and 1 mg/L 1 2,4-D. At 25 ± 1°C temperature and 16 hours light period, the environment has been renewed and preserved every 4 weeks. It has observed the highest regeneration rate in the environment with 3% sugar content. She also concluded that 32°C incubation conditions for 4 days were the most suitable temperature for embryonic stimulation. It has been found that 65% of the plants formed were haploid and 35% were dihaploid.

Unfertilized ovaries of Halep karasi watermelon genotype was picked on the day of anthesis, one day before anthesis and one day after anthesis. Ovary explants and ovules were cultured on two different (MS and CBM) mediums supplemented with 1 mg/L TDZ, 1 mg/L SPM (spermine) and 1 mg/L TDZ + 1 mg/L SPM. Ovary explants and ovules were incubated at 25-26°C, having 3000-4000 lux light density, possessing 16/8hr light/dark photoperiod for 8 weeks. After plant regeneration from ovules and ovaries they were transferred to growth regulator free MS and CBM medium. In ovule culture, the highest plant regeneration rate was 2.5% and obtained from MS + 1 mg/L TDZ and CBM + 1 mg/L SPM medium in ovary explants harvested one day after anthesis. The highest plant regeneration percentage in ovary explants was 10% and achieved from MS + 1 mg/L TDZ medium in one day after anthesis period. Totally 4 plants were obtained from ovary culture (Gürsoy et al., 2012).

Kaur et al (2017) analyzed the gynogenic response of muskmelon genotypes with temperature (heat treatment at 35°C for 2 days and cold treatment at 4°C for 4 and 8 days) and MS medium with (TDZ, and coconut water + proline + 2,4-D + kinetin). The highest callus formation rate was observed in medium containing (2,4-D + kinetin). While heat treatment showed best results on media supplemented with 0.04 mg/L.

Nitwatthanakul and Tiraumphon (2018), studied about various factors that have impacts on in vitro gynogenesis such as genotypes of donor plants and induction media in 3 melon varieties (Green Net, Honeydew and Pot Orange). The three induction mediums [M1, MS + 0.20 mg/L (BAP) and 0.40 mg/L (NAA); M2, MS + 0.30 mg/L BAP + 0.30 mg/L NAA; and M3, MS + 0.40 mg/L BAP + 0.60 mg/L NAA] were tested. Female flower buds were collected 1 day before anthesis. Callus formation was observed in 8 weeks of culture. There was no significant interaction between analyzed two factors. High callus formation frequency (75.54%) was observed in the medium containing 0.2 mg/L BAP and 0.40 mg/L NAA.

Unfertilized ovary culture constitutes an effective method for haploid breeding and can greatly shorten the breeding time. They evaluated the effects of several important factors on unfertilized ovary cultures of watermelon, including genotype, medium, the duration of induction and the development stage of the ovaries. The most effective induction medium and maturation medium were MS medium supplemented with 3 mg/L 2,4-D, 2 mg/L BAP, 0.5 mg/L NAA and MS supplemented with 0.8 mg/L BAP and 0.2 mg/L NAA, respectively. The duration of induction (13 days) significantly influenced ELS formation. The obtained 100 plantlets and used chromosome counting and flow cytometry to identify the ploidy levels of 50 of them, among which 48 were haploid and 2 were diploid (Zou et al., 2018).

Compared to the study carried out by (Zou et al. 2020) where they analysed heat shock treatment of donor plants in dark, genotype, sampling time, 2,4-D concentration and combinations of various hormone concentration factors on in vitro haploid production from unfertilized of winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) varieties

(Zaochunhongyu, Xinong No. 9 and Xiaolühuang). The 33°C (heat shock) pre-treatment in dark in 4 days was the greatest treatment for in vitro culture of un-pollinated ovary culture. The best response frequency 17.2% was observed from the ovaries collected one day prior to anthesis. The plant regeneration frequency 15.0% was obtained from (2,4-D 4.0 mg/L + 6-BA 2.0 mg/L + NAA 0.5 mg/L) growth regulator combination in Zaochunhongyu variety.

Zhu et al (2020) in their study, unpollinated watermelon ovaries were heat shocked at 37°C for 0h (A0) before anthesis and for 0 hr (A1), 4 hr (A3), 8 hr (A5), 12 hr (A7), and 24 hr (A8) respectively at the first day of anthesis. The ovule enlarged in the rate of 0% at 25°C to 96.8% at 37°C (24 hr). This demonstrated that the heat shock application has the effect on amino acids synthesis and transformation during ovule development to embryo like structure. The study's findings ensure new information on the complex link between in vitro gynogenesis and temperature treatments.

Isak et al. (2022), investigated the effect of plant growth regulators, heat treatment (25 °C or 35° C for 3 days followed by 25°C for 30 days) and floral stages (12 hr before anthesis, at anthesis, and 12 hr after anthesis) on the embryo and callus formation in watermelon cultivars (Ersin, Silva, Üstün) through unfertilized ovary culture. Distinct 2,4-D, BAP, and NAA concentrations and combinations were applied. The high frequency (97%) of callus formation and (6.94%) of ELS was obtained from induction medium combination (3 mg/L 2,4-D + 2 mg/L BAP + 0.5 mg/L NAA). The best floral stage was during anthesis which produced the highest (85%) callus formation and the 25°C was the good results (79%) heat pre-treatment for callus formation was noticed in the study.

3. MATERIAL AND METHOD

This study was carried out in Çukurova University, Institute of Natural and Applied Sciences, Department of Biotechnology and Faculty of Agriculture, Horticulture Department. Field phase of this study performed in the plastic greenhouse of Horticulture Department, Faculty of Agriculture, Çukurova University. Cucumber ovary tissue culture studies were performed in Cucumber ovary tissue culture studies were performed in Department of Horticulture, Plant Biotechnology and Prof. Dr. Saadet Büyükalaca' s Tissue Culture Laboratories, Faculty of Agriculture, Çukurova University (Adana, Türkiye) in 2021-2023. The area of the study is located at 37°01′47.5" Northern latitude and 35°21′58.2" Eastern longitude. Ovary culture was divided into 3 experiments such as first experiment (Summer growing season of 2021), second experiment (Autumn growing season of 2021) and third experiment (Summer growing season of 2022).

3.1. First Year (first and second experiments) Ovary Culture

3.1.1. Plant Material

In this study, a total of 4 commercial cucumber varieties such as Sardes F1 (long group), Ptk40 F1 (mini-group), Botanik F1 (pickling group) and Beith Alpha (open-pollinated group) were used as donor plants materials. Fruit characteristics of varieties are shown in Figure 3.1. The characteristics of the varieties are given below:

Sardes F1 (Bayer Tohum This variety is an appropriate plant for late spring and summer growing seasons, with a fruit height of 17-18 cm, a medium strong plant type with dark green fruit color without veins, and resistant to Powdery Mildew (PM), Cucumber Mosaic Virus (CMV), Cucumber Vine Yellow Virus (CVYV) and Zucchini Yellow Mosaic Virus (ZYMV). It is the most common variety grown by local farmers (Erol and Sari, 2019).

Ptk40 F1 (Petektar Tohum): Multi flowering, autumn, and early spring seasons, with fruit height of 10-12 cm, attractive smooth skins, high quality of green color fruit. And it is a parthenocarpic and hybrid cucumber for greenhouse production. Mostly, it is characterized by its short internodes, earliness, long shelf life, the firm and crisp fruits have an exceptional good flavour and taste (Anonymous 1, 2023).

Botanik F1 (Yüskel Tohum): Gherkin type prickly cucumber, very strong, medium internodes, armpit. Dark green, long handle, 12-14 cm, very good taste, and aroma, suitable for export, very high quality and is greenhouse. Fruit is parthenocarpic, spiny, cylindrical, medium, 11-13 x 1.3-1.4 cm, green color, and growing period in open field with resistance to CMV, CVYV Figure 3.1. (Anonymous 2, 2023).

Beith Alpha (Antalya Tarım): It has wide vegetation. The nodes are short and give very strong side shoots on each seat. It has green, long, cylindrical fruits and deliciously fragrant with very tasty and suitable for fresh consumption. It is also planted for spring, autumn and open

pollinated with abundant flowering structure. And it is resistant to high temperatures Figure 3.1. (Anonymous 1, 2023).

Figure 3.1. Fruit images of the varieties used in the experiment
(A) Sardes, (B) Ptk40, (C) Botanik, (D) Beith Alpha (Internet source: Anonymous 3, 2023)

3.2. Method

3.2.1. Cultivation of Plants

A total of four commercial cucumber varieties as plant materials taken from Antalya Tarım A.Ş. Three of them are F1 greenhouse types (Ptk40 F1, Sardes F1, and Botanik F1) and one open pollinated type (Beith Alpha) were used in this experiment. The seeds and seedlings preparation works performed by Antalya Tarım A.Ş. on 30/04/2021. When the seedlings were at the stage with 3-4 true leaves, on 24/05/2021, fifty seedlings from each variety and 250 healthy seedlings were planted in a plastic greenhouse of Horticulture Department, Faculty of Agriculture, Çukurova University, Adana within (100-50) x 50 cm spacing in double rows. Plant cultivation, irrigation, pruning, weeding, and disease and pest control processes were continued until the plants reach maturity for female flower buds sampling. The plants were grown as a single stem wrapped in rope Figure 3.2. Ovary culture for first experiment (Summer growing season) was established on

29/06/2021, second experiment on 14/10/2021 (Autumn growing season) and third experiment on 21/05/2022 (Summer growing season).

Figure 3.2. Experiment site at Çukurova University
(A) Greenhouse preparation, (B) Seedlings of four cucumber varieties, (C-D) Planting the seedlings, (E) Mature donor plants in a greenhouse

Cultivation and maintenance procedures were continued until the plants were mature enough to be sampled for ovule-ovary culture Figure 3.3.

Figure 3.3. (A-B) Donor plants maintenance in plastic greenhouse

Figure 3.4. Mature cucumber plant donor in a plastic greenhouse
(A) Healthy plants in the greenhouse (B-C) Female flower buds and ovaries on the plant one day before anthesis

3.2.2. Collection of Female Flowers Buds

In this study, female flower buds were harvested at 2-3 weeks after the second female flower emerges plants donor materials. The fresh unfertilized ovaries were taken one day before anthesis, where the corolla's color becomes greenish-yellow Figure 3.4. Most ovaries are featured nearly mature embryonic sacs, which made them ideal explants for initiating *in vitro* gynogenesis (Li et al. 2013). The collected female flowers were brought to the Tissue Culture Laboratory of Plant Biotechnology Laboratory, Horticulture Department, Faculty of Agriculture, Çukurova University and the sterilization phase was started.

Figure 3.5. Used female flower buds one day before anthesis
(A) Botanik F1, (B) Ptk40 F1, (C) Beith Alpha, (D) Sardes F1

3.2.3. Nutrient Media Preparation (First and Second experiments)

In the study, MS nutrient medium, which was used in ovule-ovary culture studies performed in cucurbit species, and which was reported to have positive results, was used in order to provide embryo induction as the basic nutrient medium. The basal nutrient medium was MS [Murashige and Skoog (Murashige and Skoog, 1962; PhytoTech Labs)] with supplementation of

different concentration of hormones as well as induction media combinations [(IM1: MS, 30 g L^{-1} sucrose, 7 g/L agar (control); IM2: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg L^{-1} kinetin (KIN); IM3: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 1 mg L^{-1} 2,4-D, 5 mg L^{-1} KIN; IM4: MS, 30 g L^{-1} sucrose, 7 mg L^{-1} agar, 1.5 mg L^{-1} 2,4-D, 10 mg L^{-1} KIN] and maturation media combination (RM1: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar (control); RM2: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 0.5 mg L^{-1} 1-naphthaleneacetic acid (NAA), 2 mg L^{-1} 6-benzylaminopurine (BAP); RM3: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 1 mg L^{-1} NAA, 2 mg L^{-1} BAP; RM4: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 1 mg L^{-1} NAA, 4 mg L^{-1} BAP) (Ozsan et al., 2017; Deng et al., 2020; Baktemur et al., 2022) (all used hormones were supplied by PhytoTech Labs)] Table 3.1. Before sterilization in the autoclave (HMC HIRAYAMA, Japan) at 121°C for 15 min, the pH of all nutrient medium was adjusted to 5.8 in the aid of 1 NaOH and 1 HCl.

Table 3.1. Nutrient media combinations used in first and second experiments

Medium code	**Induction medium**
IM1	MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar (control)
IM2	MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 0.5 mg L^{-1} 2,4-D, 1 mg L^{-1} Kinetin
IM3	MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 1 mg L^{-1} 2,4-D, 5 mg L^{-1} Kinetin
IM4	MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 1.5 mg L^{-1} 2,4-D, 10 mg L^{-1} Kinetin
Medium code	**Regeneration medium**
RM1	MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar (control)
RM2	MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 0.5 mg L^{-1} NAA, 2 mg L^{-1} BAP
RM3	MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 1 mg L^{-1} NAA, 2 mg L^{-1} BAP
RM4	MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 1 mg L^{-1} NAA, 4 mg L^{-1} BAP

IM1: Induction medium 1, IM2: Induction medium 2, IM3: Induction medium 3, IM4: Induction medium 4, RM1: Regeneration medium 1, RM2: Regeneration medium 2, RM3: Regeneration medium 3, RM4: Regeneration medium 4

The media prepared in Erlenmeyer flasks are wrapped with aluminum foil and stretch film for sterilization and placed in an autoclave for sterilization Figure 3.5. Autoclaving was completed according to the sterilization protocol for 15 minutes at 121°C Figure 3.6.

Figure 3.6. Media Preparation

Figure 3.7. Preparation of nutrient medium combinations
(A) Prepared media in autoclave container, (B) Autoclaving the media

The media removed from the autoclave were poured into sterile 90 mm Petri dishes in sterile cabinet (Laminar Flow Cabinet, ahm seed technologies) Figure 3.8. The media was allowed to cool, and the Petri dishes were wrapped with cling film and stored in room at 4°C to keep them sterile until use.

Figure 3.8. Pouring autoclaved media into sterile 90 mm Petri dishes in sterile cabinet

3.2.4. Ovary Culture Processes

❖ **Setting up and Optimization of Ovary Culture First Experiment**

A completely randomized design (CRD) in a four-factor factorial experiment (varieties [4 cucumber varieties], heat shock pre-treatment, induction [IM 1- IM 4] and maturation media [RM 1–RM 4]) was carried out with five slices of cucumber ovaries were placed in a 90 mm plastic Petri dish (Tantasawat et al., 2015). The heat shock pre-treatment used was 35℃ for 3 days in the dark in an oven (UN 110 Memmert, Germany). The growing chamber was under a lux intensity light of 3000 to 4000Lx for 16hr/8hr light/dark at 25℃ temperatures.

❖ **Treatments for Explant Material**

The unpollinated ovaries that were taken one day prior to anthesis were directly brought to the laboratory of plant biotechnology for tissue culture processes. Female flower buds were separated from their corolla for surface sterilization. The collected ovaries were washed with running water for 20 minutes and cleansed 3 times with autoclaved water. Subsequently, surface sterilization, cucumber ovaries were taken into the sterile cabinet. By using sterile pens, ovaries of each cucumber variety transferred into sterile containers or jars. Firstly, ovaries are kept in 70% of ethanol solution for one minute and were rinsed three times with distilled water. Later explants were kept in sodium hypochlorite (10%) with two to three drops of TWEEN20 for 10 min after that, ovaries were rinsed four-fold with sterile distilled water to free all chemicals. Wet ovaries were transferred on sterile filter paper to remove excess water Figure 3.9.

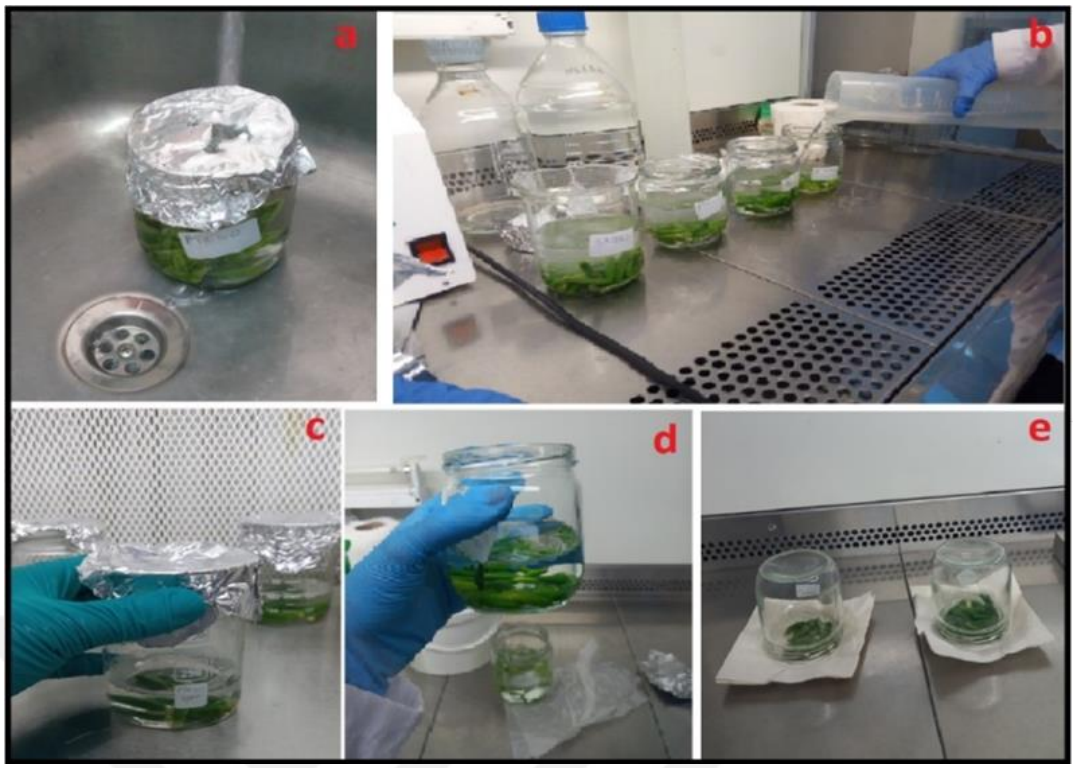

Figure 3.9. Sterilization processes
(a) flower buds surface sterilization under tap water, (b) to (d) flower buds surface sterilization under the ster cabinet, (e) removing excess water from ovaries on sterile filter paper

❖ **Ovary Culture in Induction Medium in Petri Dishes**

After sterilization, cucumber ovaries were peeled from their exterior and then split into cross- and horizontal-sections under sterile conditions and then ovary slices were placed in four prepared induction media into 90 mm plastic Petri dishes Figure 3.10.

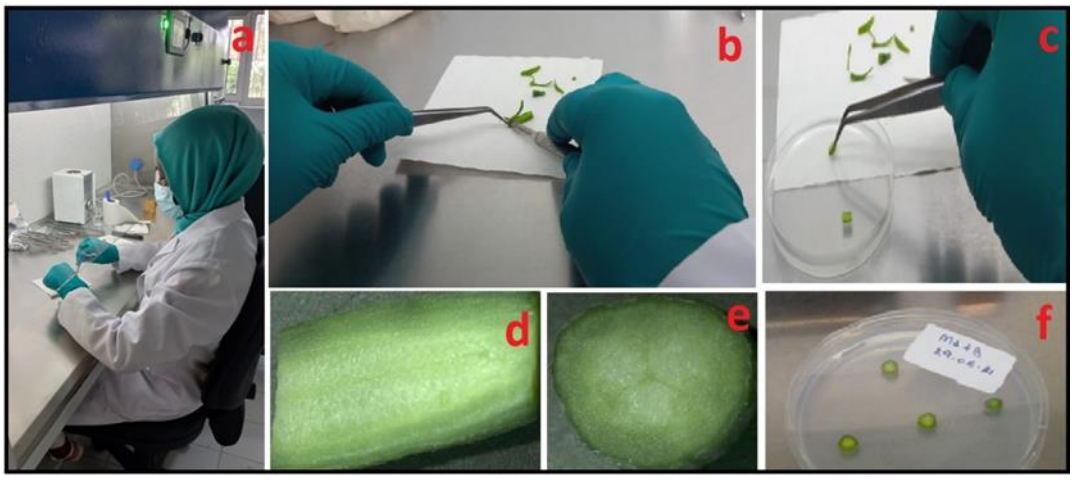

Figure 3.10. Cucumber ovary culture process
(a) Peeling ovary's exterior skin, (b) Split into horizontal- and cross-sections, (c) Placing ovary slice in 90 mm Petri dish, (d-e) Used ovary slices sections, (f) Inoculated ovary slices in plastic Petri dish

The cultures were incubated in the oven (UN 110 Memmert, Germany) for 3 days at 35℃ in dark conditions (Figure 3.11.), then transferred to the growing room (Figure 3.12.) of 25℃ under a lux intensity light of 3000-4000Lx for 16hr/8hr light/dark for 15 days to transfer the cultured ovaries in maturation media combinations Table 3.1. (Diao et al., 2009; Suprunova and Shmykova, 2008; Moqbeli et al., 2013; Erol and Sarı, 2019).

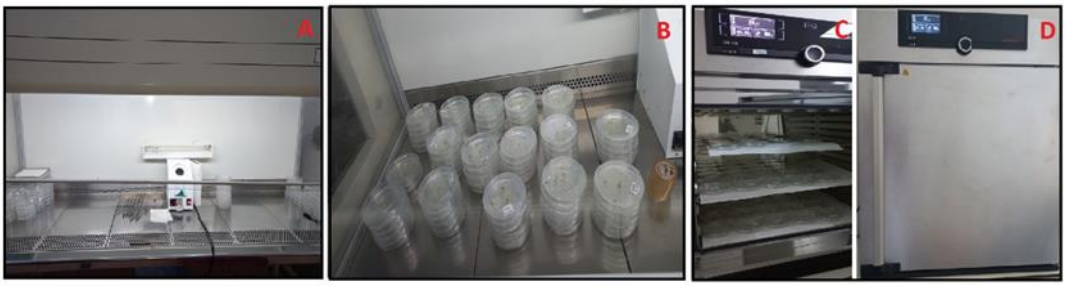

Figure 3.11. Heat shock pre-treatment process
(A-B) Petri dishes containing used cucumber ovaries' slices, (C-D) Heat shock (35°C/3days) pretreatment in oven

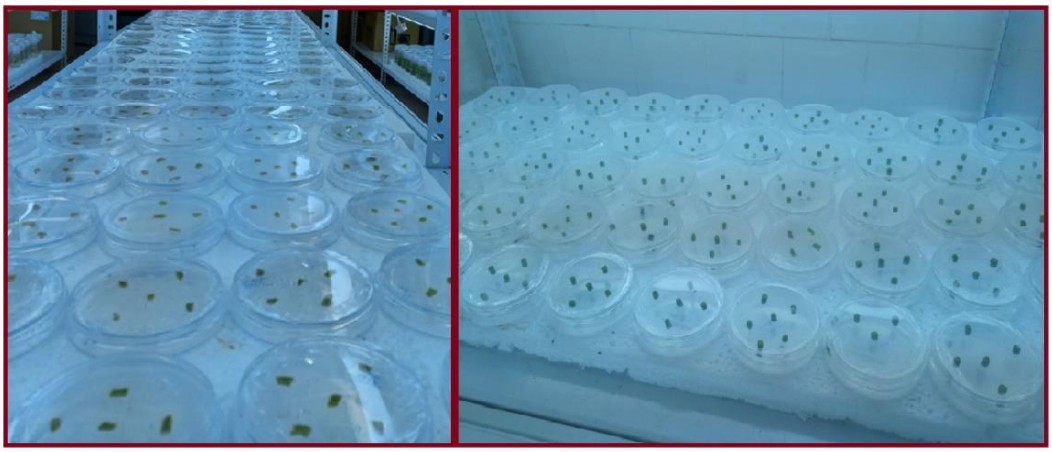

Figure 3.12. Growing chamber of 25℃ under a lux intensity light of 3000-4000Lx for 16hr/8hr light/dark conditions

3.2.5. Embryo Induction and Development

Throughout cucumber ovary culture, the effects of various factors as well as nutrient media and varieties were assessed. After 9 days from the initiation of culture, the first observation was made, and induced embryos and callus were observed and recorded. The observation of embryo was carried out under a binocular microscope Olympus SZ60 (Olympus SZ-PT, Japan) and a stereo microscope, Olympus SZ61 (Olympus Corporation, Shinjuku, Tokyo, Japan). This microscope with a high-quality optics, allowed the perfect following of ovules' transformation to the embryo like structure Figure 3.13. The developed explants were subcultured onto fresh regeneration media combinations after 15 days of induction (Ozsan et al., 2017; Baktemur et al., 2022). Subsequently our experiment was conducted, and cucumber ovary culture explants were subcultured after 30 days of the first subculture on fresh regeneration nutrient media.

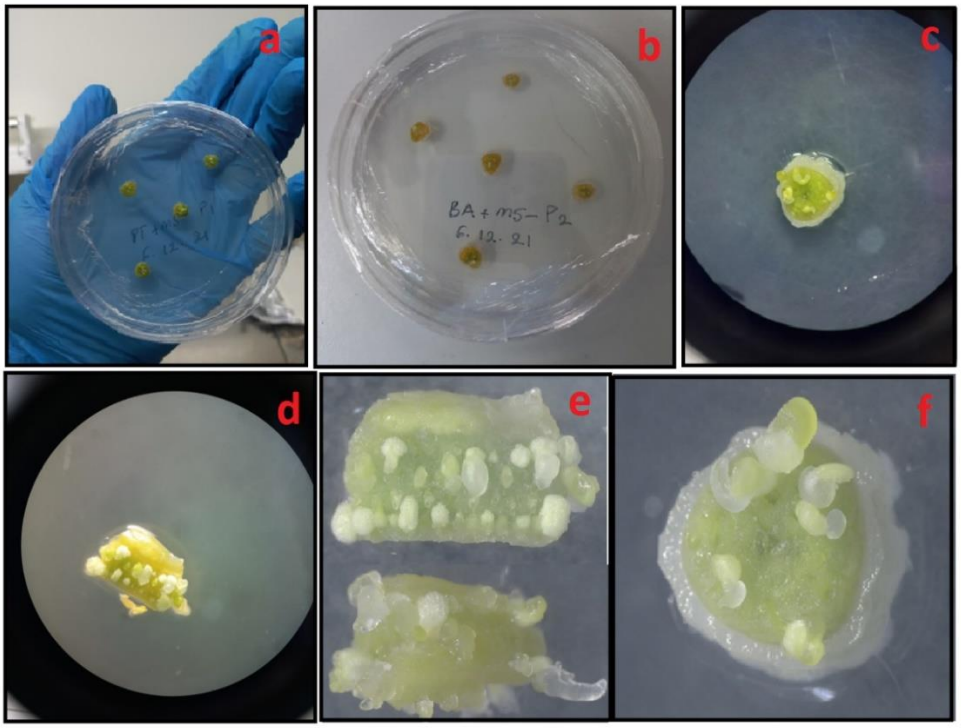

Figure 3.13. After 9 days from the initiation of culture embryo formation (a-b) Ovules of different cucumber varieties development in Petri dishes, (c-d) Observation under binocular microscope, (e-f) Observation under stereo microscope.

After 30 days from the first day of culture, embryo ratio was determined, callus formation Figure 3.14. was recorded consecutively, and plant regeneration rate was determined after 5 months of culture. The results of this study evaluated only as percentage (number of embryo per 100 cultured ovaries, number embryo developed per 100 ovaries, plant number per 100 embryos) (Baktemur et al., 2022). ELS or callus formation percentages were calculated from the number of ovary pieces making up the ELSs or callus/total number of ovary pieces × 100. After the ELSs sprouted, the healthy ones were rooted in standard medium containing 0.1 mg L^{-1} indole-3-butyric acid (IBA) plant growth hormone.

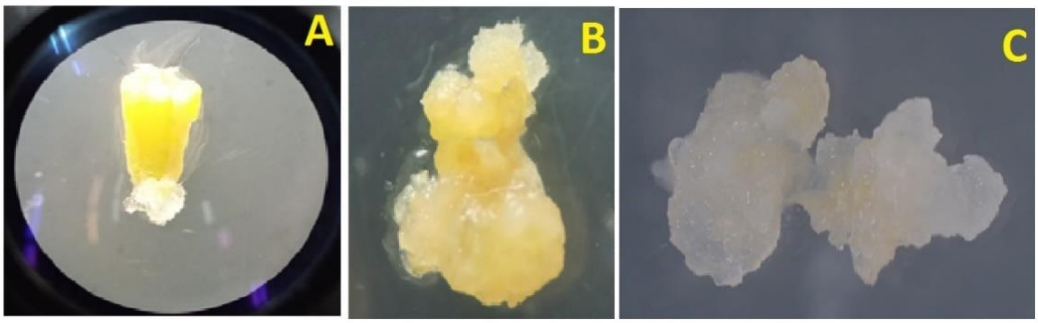

Figure 3.14. Callus formation after 9 days
(A-B) Callus structure under binocular microscope at 9 days, (C) Callus structure under stereo microscope after 9 days of culture

Callus and embryo were evaluated under binocular- and stereo-microscopes. Embryogenic callus, callus structure, shapes, and color also have been determined Figure 3.15. and Figure 3.16.

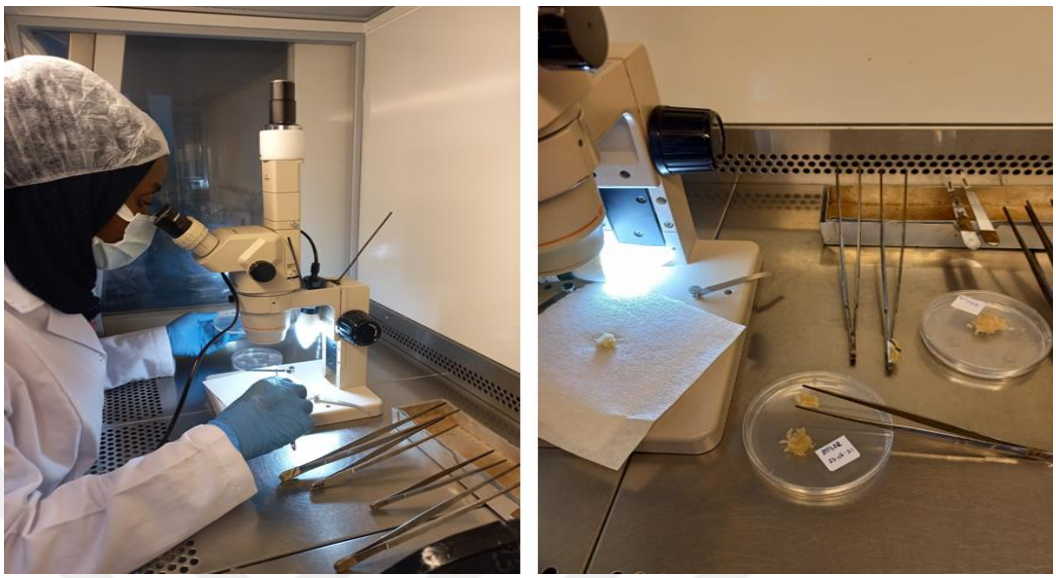

Figure 3.15. Analysis of produced callus under binocular microscope

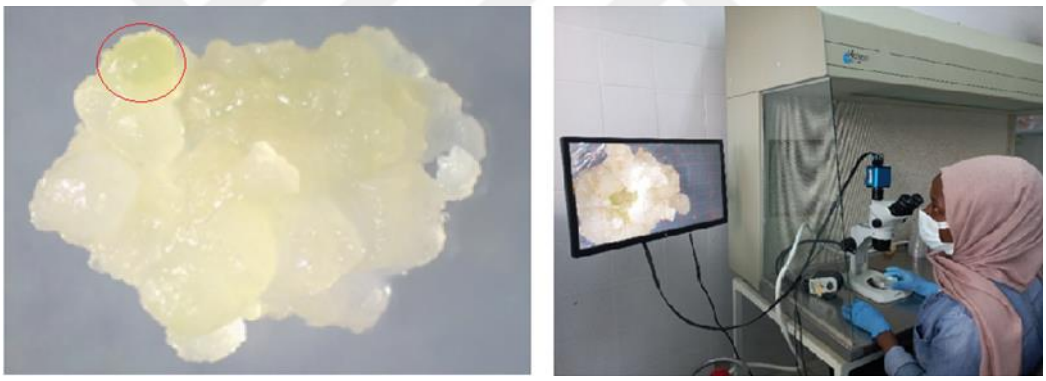

Figure 3.16. Analysis of produced callus (embryogenic callus) under stereo microscope

3.3. Second Year (third experiment) Ovary Culture

The third experiment of this study was performed in the summer growing season of 2022. The field work was carried out at the same plastic greenhouse region used in experiments number one and two on 19 April 2022. In this experiment cold shock (4°C) pre-treatment and various plant growth hormones' concentration were tested.

3.3.1. Plant Materials

In this experiment also plant materials were the same as used in experiment number one four commercial cucumber varieties' female flower buds. The fresh unpollinated ovaries were taken one day before anthesis around 8 a.m to 9 a.m, and at this time, the corolla's color is greenish-yellow Figure 3.17.

Figure 3.17. Used female flower buds one day before anthesis

3.3.2. Nutrient Media Preparation for Third Experiment

The nutrient induction media which named following the nomenclature of used nutrient media in experiment to differentiate with media used in experiment No.1 [(IM5: 4.4 g L^{-1} MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 0.06 mg L^{-1} Thidiazuron (TDZ); IM6: standard medium with 0.15 mg L^{-1} 2,4- Dichlorophenoxyacetic acid (2,4-D (PhytoTech Labs)) and 1.5 mg L^{-1} Kinetin (KIN (PhytoTech Labs)); IM7: 0.1 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP, IM8: 5 mg L^{-1} 2.4 D (PhytoTech Labs) and 40 µM Putrescine (PUT) and regeneration media as well as RM5: 0.05 mg L^{-1} NAA and 0.2 mg L^{-1} BAP, RM6: 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP, RM7: 0.01 mg L^{-1} IAA and 1 mg L^{-1} BAP, RM8: 0.1 mg L^{-1} IAA and 1 mg L^{-1} BAP, RM9: 0.1 mg L^{-1} IAA and 2 mg L^{-1} BAP (PhytoTech Labs) were tested in this experiment Table 3.2. and Table 3.3.

Table 3.2. Nutrient media component used in third experiment

Medium code	**Standard: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, and pH 5.8-5.9**				
	2,4-D (mg L^{-1})	**BAP (mg L^{-1})**	**TDZ (mg L^{-1})**	**Put (µM)**	**KIN (mg L^{-1})**
IM5	-	-	0.06	-	-
IM6	0.15	-	-	-	1.5
IM7	0.1	1	-	-	-
IM8	5	-	-	40	-

IM5: Induction medium 5, IM6: Induction medium 6, IM7: Induction medium 7, IM8: Induction medium 8

Table 3.3. Regeneration media combinations for 3rd Experiment

Medium code	**Standard: MS, 30 g L^{-1} sucrose, 7 g L^{-1} agar, and pH 5.8-5.9**		
	NAA (mg L^{-1})	**BAP** (mg L^{-1})	**IAA** (mg L^{-1})
RM5	0.05	0.2	-
RM6	0.5	0.5	-
RM7	-	1	0.01
RM8	-	1	0.1
RM9	-	2	0.1

RM5: Regeneration medium 5, RM6: Regeneration medium 6, RM7: Regeneration medium 7, RM8: Regeneration medium 8, RM9: Regeneration medium 9

3.3.3. Ovary Tissue Culture

After a collection of the unpollinated ovaries were collected were directly brought to the Tissue Culture laboratory of Prof. Dr. Saadet Büyükalaca, Horticulture Department, Faculty of Agriculture, Cukurova University for ovary culture processes. Cold shock pre-treatment applied to the female flower buds at 4°C/ 4 days during this experiment. The sterilization procedure performed as it was done in first and second experiments Figure 3.8. After the sterilization, the ovaries were peeled with the sterile pens and scalpels without damaging the ovules and sliced into pieces based on their size under sterile conditions like it was performed in other experiments of this study Figure 3.9. The explants were cultured in prepared nutrient media into 90 mm plastic Petri dishes Figure 3.18. After the ovaries inoculated on induction media (IM), the cultures stayed in growing chamber at 25±1°C in light conditions and a photoperiod of 16 hr/8 hr Figure 3.11. for 30 days. And then after the subculture was applied (induction media were changed to regeneration media combination), the fresh nutrient regeneration media combinations Table 3.3 were changed every 30 days.

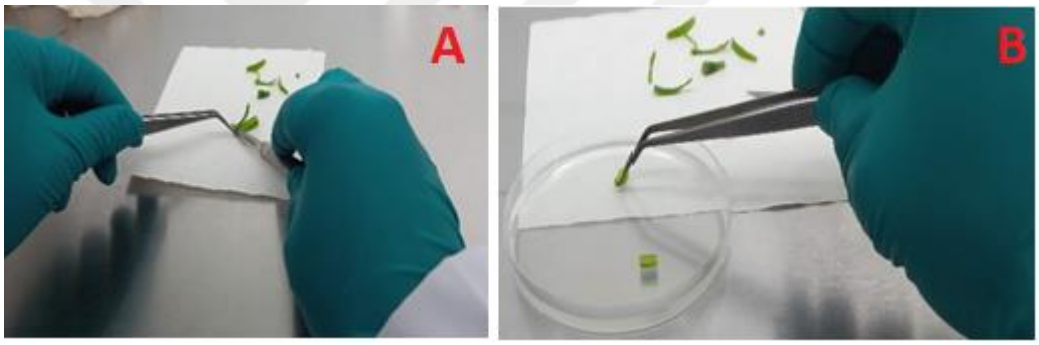

Figure 3.18. (A) Inoculating ovary slices on induction nutrient medium combination, (B) ELS on cultured ovary slice to be transferred to the regeneration medium combinations

Generally, plant growth regulators (PGRs), particularly auxins, have widely been used for gynogenesis embryo and callus induction. And considerably their optimum concentration varied from species to species or variety to variety (Kumar et al., 2003). In this study, a total of four commercial cucumber varieties such as Beith Alpha, Ptk40, Sardes and Botanik were used. Ovary culture was performed for four cucumber varieties for haploid induction experiments. The used ovaries were freshly collected one day before the anthesis. Fifty ovaries' slices were cultured into the prepared nutrient medium for each variety and medium combinations were IM5, IM6, IM7, IM8 Table 3.2. Therefore, all Petri dishes were cultured at 25℃ under a lux intensity light of 3000 to 4000Lx for 16 hr/8 hr light/dark. The embryo induction rate was identified after 30 days of culture. Plant like structures were regenerated in 0.1 mg L^{-1} indole-3-butyric acid (IBA). And subculture was performed every four weeks. The plant regeneration frequency was evaluated five months after ELS were observed Figure 3.19.

Figure 3.19. Regenerated and rooted plantlets

3.3.4. Micro-cutting of Regenerated Plant

A total of 57 regenerated and rooted plants were micro-cut for multiplying the obtained plants Figure 3.20. The Figure 3.21 shows the micro-cutting technique and Figure 3.22 represent the micro-propagated derived plantlets.

Figure 3.20. Regenerated plant that are going under micro-cutting technique

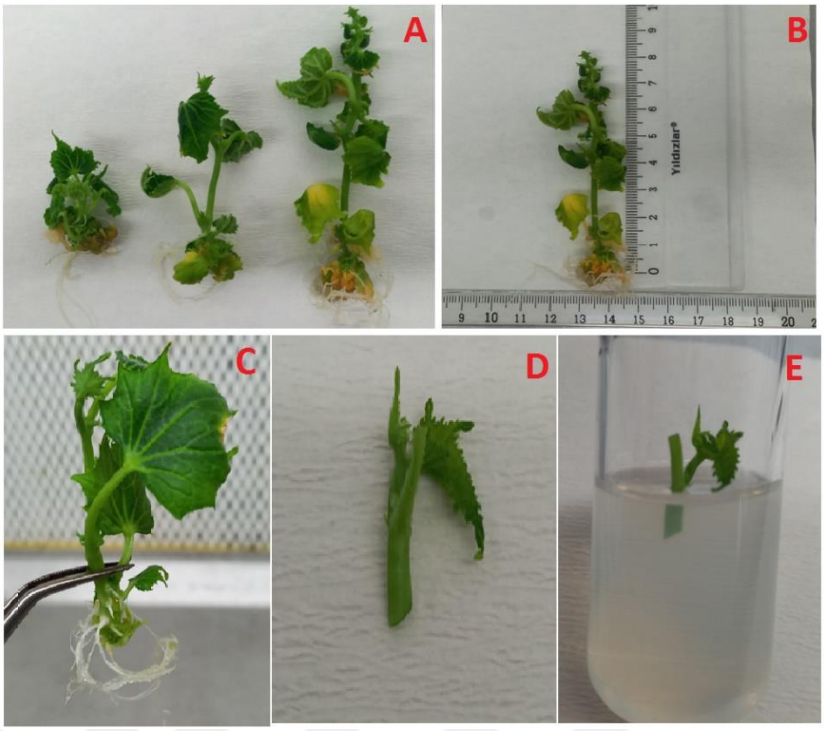

Figure 3.21. (A-B) Plant heigh, (C-D) Micro-cutting technique steps

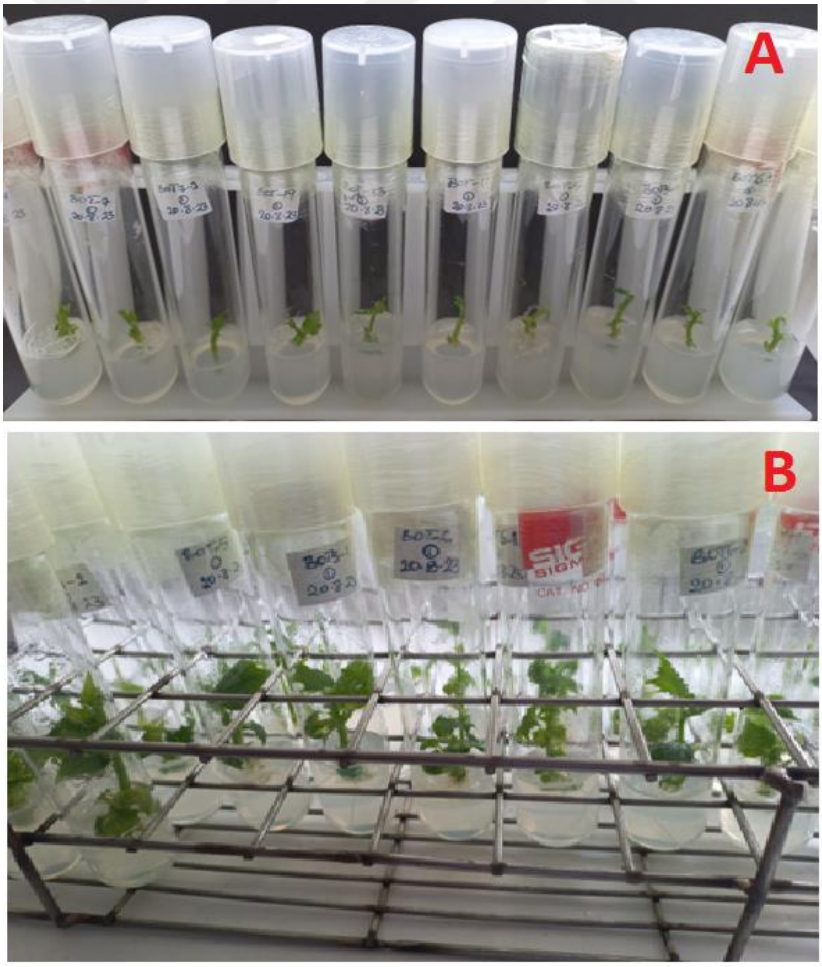

Figure 3.22. (A) to (B) Micro-propagated derived plants

3.3.5. Ploidy level analysis

Flow cytometry (Sysmex Cube 8) (Figure 3.24.) was used to determine the ploidy level. Fresh and young leaf samples of randomly selected twenty plantlets from in vitro embryogenic calli-derived plantlets (cucumber ovary culture regenerants) were collected from the micro-propagated plantlets (Figure 3.23.) and transported directly to the laboratory of IDA Yaşam Teknolojileri for ploidy identification. Concerning, leaf samples (0.5 cm^2 of leaf tissue per plantlet in individual glass tubes) were chopped with a razor blade and 400 µl of extraction buffer was added. Subsequently, all samples were incubated for 30 to 60 seconds. Afterwards, these samples were filtered using a special filter (Partec 50 m Cell Trics®). The addition of 1.6 ml of staining buffer to the sample tube for a brief incubation of 30 to 60 seconds before taking readings on a flow cytometer machine was performed.

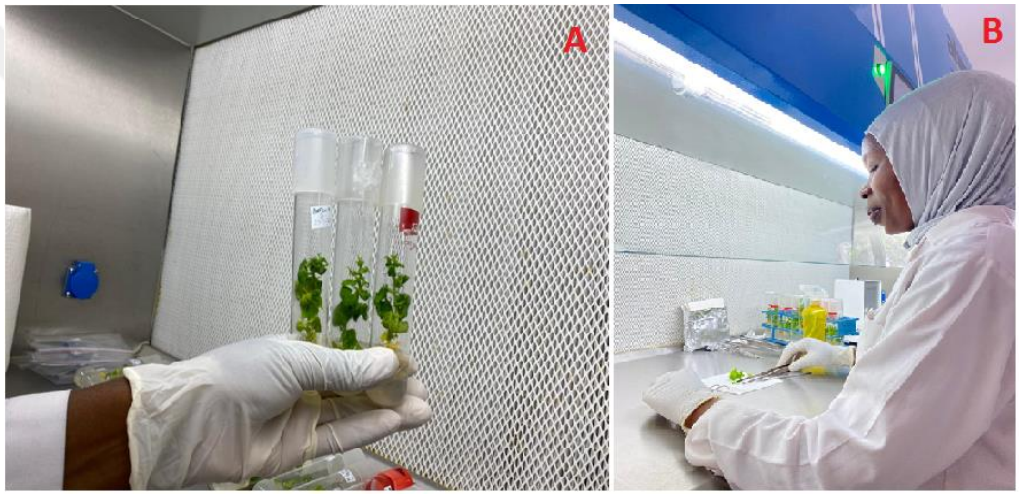

Figure 3.23. Fresh and young leaf samples from *in vitro* embryogenic calli-derived plantlets

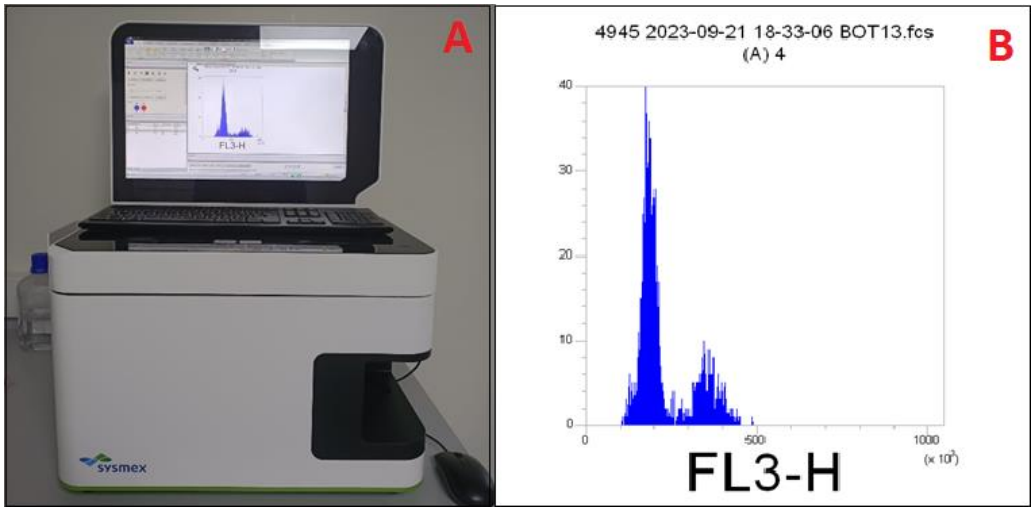

Figure 3.24. Flow cytometry analysis
(A) Flow cytometer machine, (B) Obtained peak from analysed samples

4. RESULTS AND DISCUSSIONS

The study was carried out to cover ovary culture technique with different nutrient media combinations (17 nutrient medium combinations) and temperature pre-treatments [heat (35°C/ 3 days) and cold (4°C/4 days) shocks pre-treatments]. Ovary culture experiment was repeated in three growing seasons (summer, autumn growing seasons of year 2021 and summer season of year 2022). The percentage analyses of the obtained research findings and observations are presented below.

4.1. Results and Discussion of Cucumber Ovary Culture First and Second Experiments

Ovary culture for experiment number one was established on 29/06/2021 from Summer growing season and 14/10/2021 Autumn growing season. The female flowers of the 4 commercial cucumber cultivars we used were collected 1 day before the anthesis and sterilization processes were applied, and the ovaries were peeled from the external skin and sliced into pieces and then cultured in the media combinations specified in Table 3.1. Fifty ovaries' slices were cultured into every prepared nutrient medium combination for each variety Figure 3.10. Later, cucumber ovaries were seeded on four distinct induction medium combinations (IM1, IM2, IM3, and IM4). The development of the cultured ovary slices was monitored after they had been pre-heated at 35°C for 3 days in dark condition in the oven . They were transferred in the growing chamber with a temperature of 25°C, a light intensity of 3000 to 4000 lux, a lighting duration of 16 hr, and darkness for 8 hr. In the 3rd week of observations, there was change in the number of undeveloped and developed ovaries. After 10 weeks of observation, the results were collectively evaluated. Ovules from ovary slices that swelled, enlarged, regenerated, and changed color from the cultured ovaries' slices were recorded as developed and remained as they were cultured, while ovules that did not show any regeneration were recorded as undeveloped. The ovule enlargement and callus formation were seen appearing into greenish and (or) yellowish cultures. And they continued to emerge and develop throughout the experiment Figure 3.12., 3.13.

All the ovaries' slices of used varieties (Beith Alpha, Ptk40 F1, Botanik F1, and Sardes F1) in the ovary culture experiments showed improvements. Due to the fact that the ovules in cucumber are even smaller than 0.5 mm (Figure 4.1.), the developed ovules showed regeneration ability only between 1-5 mm. Therefore, when the findings and visuals were examined, these structures were defined as callus- or embryo-like structures instead of clearly defining callus, embryo development or plantlet formation. The ratio of embryo induction was defined within 30 days of culture. And evaluation of plant regeneration rate was performed five months following the embryo enlargement considered as embryo-like structures, and embryogenic callus were observed.

Figure 4.1. Developed ovules' size into a cucumber slice (0.5 mm)

4.1.1. Results and discussion of Beith Alpha variety for first ovary culture experiment

The observation, evaluation were performed consectively and results of this experiment were recorded and calculated in pecentage (%). Ovules enlargement was considered as embryo-like structures (ELS) formation observed after 9 days from the first day of culture and callus formation displayed on the surface of cultured ovary slices there about two to three weeks after culture initiation. Our findings revealed that during embryo induction, the highest ovule or ELS development rate (16%) was obtained from induction medium 2 (IM2: 0.5 mg L^{-1} 2,4-D + 1 mg L^{-1} KIN). This result was followed by the growth rate (12%) of ELS that observed in the control medium, 4% ELS from induction medium 3 (IM3: 1 mg L^{-1} 2,4-D + 5 mg L^{-1} KIN) respectively. During this trial there was no ovule development observed in the induction medium 4 (IM4: 1.5 mg L^{-1} 2,4-D + 10 mg L^{-1} KIN). Based on regeneration medium combinations, the highest ELS formation rate (90.47%) was observed in RM4 (1 mg L^{-1} NAA + 4 mg L^{-1} BAP). According to the results of the study in case of callus formation IM3 found to be the most successful induction medium in Beith Alpha variety when it is compared to others. The callus formation rate (16%) was obtained after 9 days from the initiation day of culture Table 4.1, Figure 4.1.

Table 4.1. Observations of Beith Alpha variety in induction media after 9 days of culture

Variety	Medium	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 DSN (%)
Beith	IM1	50	3	3	12	100	0	0	0
Alpha	IM2	50	4	4	16	100	0	0	0
	IM3	50	5	1	4	20	4	16	80
	IM4	50	0	0	0	0	0	0	0

IM1: Induction Medium 1, IM2: Induction Medium 2, IM3: Induction Medium 3, IM4: Induction Medium 4, CSN: Cultured Slice Number, DSN: Developed Slice Number, EN: Embryo Numbe, CN: Callus Number

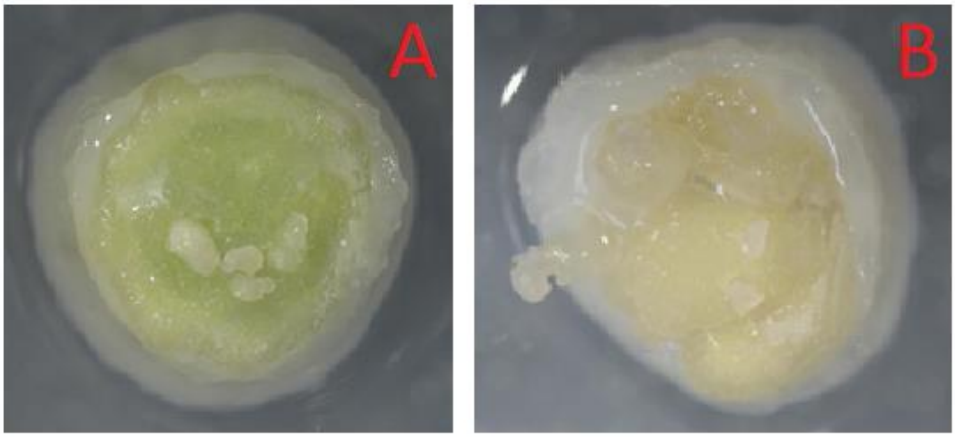

Figure 4.2. Induced ovules development on ovary slices
Beith alpha in: (A) IM4 after 9 days of culture, (B) Bieth Alpha during subculture process (after 1 month of culture)

According to the observation made during this experiment, and the results were recorded and calculated in percentage (%). The ELS development and callus formation were continued when the cultures were transferred to the regeneration media combination (RM1-RM4). The results showed that the highest ELS formation rate (90.48%) was obtained in regeneration medium 4 (RM4: 1 mg L^{-1} NAA + 4 mg L^{-1} BAP), followed by 28% from RM2 0.5 mg L^{-1} + 4 mg L^{-1} BAP medium combination, 12.5% of RM1 (control) and 2% from RM3 (1 mg L^{-1} NAA + 2 mg L^{-1} BAP) respectively. The callus formation rates of 20% were obtained RM3, 12% from RM4, and 2% of RM2 while there was no callus formation from the Beith Alpha variety obtained in RM1 Table 4.2.

Table 4.2. Observations of Beith Alpha variety in regeneration medium combination after one month of culture

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Beith Alpha	RM1	50	48	6	6	12	12.5	100	0	0	0	0
	RM2	50	50	8	14	28	28	175	1	2	2	12.5
	RM3	50	50	6	1	2	2	16.7	10	20	20	166.7
	RM4	50	42	21	19	76	90.48	90.48	3	12	14.28	14.28

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4, CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

Generally, the results showed that Beith Alpha responded in all tested induction and regeneration media combinations. Whereas the results of this experiment were recorded and calculated in percentage (%). The highest ELS formation average was 45.24% of RM4, 22% RM2, 6.25% RM1 and 2% RM3. Callus development ratio was 20% from RM3 and 7.142% of RM4 respectively. While there was no callus developed in media combination of RM2 and RM1 Table 4.3, Figure 4.3. The callus formation from Beith Alpha variety in different regeneration medium combination within 3 and five months of culture is shown in the Figure 4.4. and 4.5.

Figure 4.3. Callus development from Beith Alpha into regeneration medium 4 after 1 month

Table 4.3. Evaluation of Beith Alpha variety embryo and callus formation frequencies for first ovary culture experiment

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN(%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Beith Alpha	RM1	50	48	3	3	6	6.25	100	0	0	0	0
	RM2	50	50	11	11	22	22	100	0	0	0	0
	RM3	50	50	11	1	2	2	100	10	20	20	90.90
	RM4	50	42	21	19	38	45.24	90.48	3	6	7.14	14.28

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4, CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

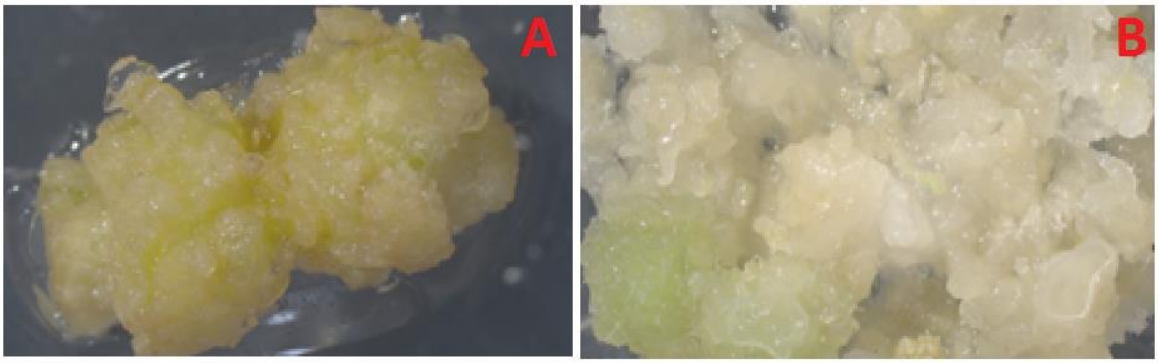

Figure 4.4. Callus formation from Beith Alpha variety in regeneration media after 3 months (A) Regeneration medium 2 and (B) Regeneration medium 4

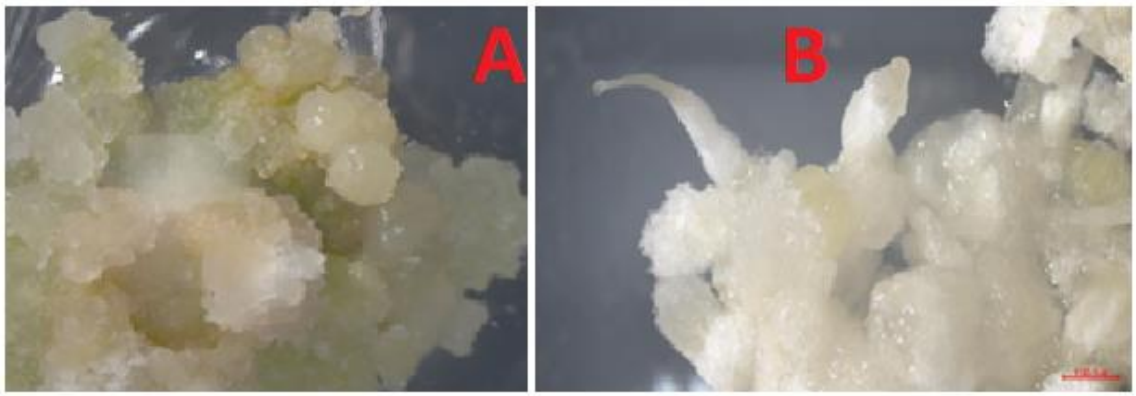

Figure 4.5. (A-B) Callus formation from Beith Alpha variety in regeneration media 4 at five months

4.1.2. Results of Beith Alpha variety for second ovary culture experiment

After 4 months from the first ovary culture experiment, the second ovary culture experiment was performed at 14/10/2021 for autumn growing season. The results of embryo induction from ovary slices in the second trial of Beith Alpha variety are explained below.

Compared to the result of Beith Alpha in the first experiment' findings, the results of second ovary culture development demonstrated that the highest embryo formation rate 16% with 50% callus regenerated from 0.5 mg L^{-1} NAA, 2 mg L^{-1} BAP medium combination 2 (RM2). The lowest values of embryo and callus formation frequencies were 2% with 44% in RM4 (1 mg L^{-1} NAA, 4 mg L^{-1} BAP) and RM3 (1 mg L^{-1} NAA, 2 mg L^{-1} BAP) nutrient media combinations respectively. There was no embryo and callus formation occurred in RM1 (control) Table 4.4.

Table 4.4. Evaluation of Beith Alpha variety embryo and callus formation in second ovary culture experiment

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Beith Alpha	RM1	50	0	0	0	0	0	0	0	0	0	0
	RM2	50	25	25	8	16	32	32	25	50	100	100
	RM3	50	25	25	1	2	4	4	24	48	96	96
	RM4	50	25	25	1	2	4	4	22	44	88	88

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4, CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

4.1.3. Results and discussion of Ptk40 variety for first ovary culture experiment

The results of this experiment showed that the embryo-like structures formation rate (20%) was high in induction medium 2 (IM2: 0.5 mg L^{-1} 2,4-D + 1 mg L^{-1} KIN) which was successful in Ptk40 variety, compared to 16% from IM 4 (1.5 mg L^{-1} 2,4-D + 10 mg L^{-1} KIN) and 12% of control (IM 1). There was on embryo like structures fromation observed in IM3 (1 mg L^{-1} 2,4-D + 5 mg L^{-1} KIN) medium combination with Ptk40 variety during 9 days from the initiation day of culture. In other hand, the best callus formation rate (52%) was in IM2 medium combination, followed by 8% of each IM3 and IM4 medium. No callus formation occured in I induction medium 1 (M1) during first 9 days of Ptk40 ovary culture in the experiment (I) of this research study Table 4.5.

Table 4.5. Observations of Ptk40 variety in induction media combinations after 9 days of culture

Variety	Medium	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN(%)	CN	CN/100 CSN (%)	CN/100 DSN (%)
Ptk40	IM1	50	10	6	12	60	0	0	0
	IM2	50	30	10	20	33.33	26	52	86.667
	IM3	50	4	0	0	0	4	8	100
	IM4	50	34	8	16	23.529	4	8	11.764

IM1: Induction Medium 1, IM2: Induction Medium 2, IM3: Induction Medium 3, IM4: Induction Medium 4, CSN: Cultured Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

Therefore, the ovaries' slices of Ptk40 variety were transferred after 30 days to the new fresh regeneration media combinations for callus- and embryo- like structures regeneration stages Figure 4.6. The embryo like structure frequency 8.33% was formed from RM2 (0.5 mg L^{-1} NAA + 2 mg L^{-1} BAP) and 8% from RM1 (control). The formation of embryo like structure did not occurred in medium combinations of RM3 (1 mg L^{-1} + 2 mg L^{-1} BAP) and RM4 (1 mg L^{-1} NAA + 4 mg L^{-1} BAP). Based of callus formation ratios, RM4 found to be good with (87.67%), RM3 (69.56%) and RM2 (50%), while there is no formation of callus obtained in RM1 (control) Table 4.6.

Table 4.6. Observations of Ptk40 variety in regeneration medium combinations after 1 month of culture

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Ptk40	RM1	50	50	4	4	8	8	100	0	0	0	0
	RM2	50	48	28	4	8	8.333	14.285	24	48	50	85.714
	RM3	50	46	32	0	0	0	0	32	64	69.565	100
	RM4	50	30	26	0	0	0	0	26	52	86.667	100

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4, CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number; CN: Callus Number

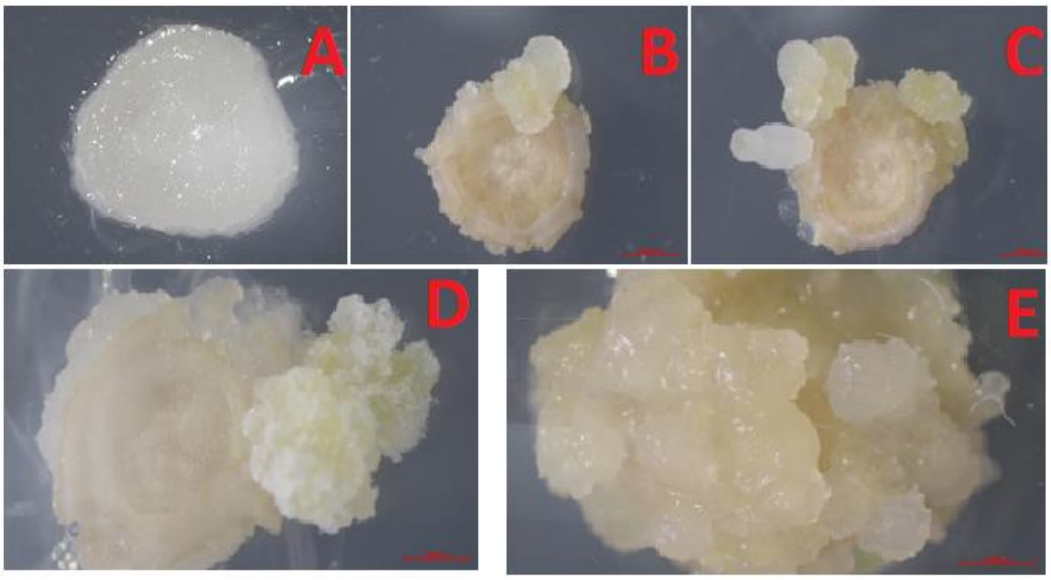

Figure 4.6. Callus formation in Ptk40 variety with various nutrient media after 1 month (A) Ptk40 in RM1 (control), (B-C) Ptk40 within RM2, (D) Ptk40 in RM3, (E) Ptk40 with RM4

Overall, the evaluation of Ptk40 variety used in all induction and regeneration media combination was performed. And the results of this experiment showed that best results was found in Ptk40 variety within IM2 and RM2 where we obtained the embryo development frequency (12.5%) and callus formation rate (52.08%), while 10% ELS and 53.33% callus formation were observed from IM4 and RM4 compared to RM3 with callus formation rate (39.13%) and the no ELS was developed in this trial. And then RM1 control produced ELS of 14% with on callus was formed from this medium combination Figure 4.7., Table 4.7.

Table 4.7. Evaluation of Ptk40 variety embryo and callus formation frequencies in first ovary culture experiments

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Ptk40	RM1	50	50	7	7	14	14	100	0	0	0	0
	RM2	50	48	31	6	12	12.5	19.35	25	50	52.08	80.64
	RM3	50	46	18	0	0	0	0	18	36	39.13	100
	RM4	50	30	19	3	6	10	15.79	16	32	53.33	84.21

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4, CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

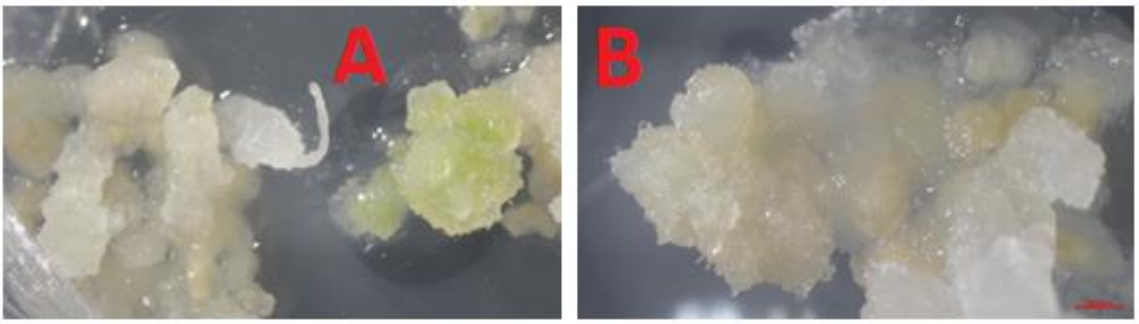

Figure 4.7. Formation of various structure including, (A) root like structurein and (B) emcryogenic callus of Ptk40 into RM4 after 3 months

4.1.4. Evaluation of embryo and callus formation for Ptk40 variety in regeneration medium combinations of second experiment

The results of autumn growing season (2nd experiment) showed the lowest embryo development in tested nutrient medium combinations. The highest callus formation rate (52%) from medium combination 3 (RM3: 1 mg L^{-1} NAA + 2 mg L^{-1} BAP) Table 4.8.

Table 4.8. Evaluation of embryo and callus formation frequencies for Ptk40 variety using different media combinations in second experiment

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Ptk40	RM1	50	25	0	0	0	0	0	0	0	0	0
	RM2	50	20	20	0	0	0	0	12	24	60	60
	RM3	50	28	28	0	0	0	0	26	52	92.86	92.86
	RM4	50	15	15	0	0	0	0	17	34	113.33	113.33

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4, CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

Figure 4.8. Callus formation in Ptk40 variety for 2nd experiment (Autumn season)

4.1.5. Results and discussion of Botanik variety for first ovary culture experiment

Furthermore, the experiment one of this research study, the Botanik variety used in the study was evaluatated in all used induction and regeneration media combinations. And the results of experiment were recorded and calculated in percentage (%). From the induction medium used in this experiment number one, IM2 (0.5 mg L^{-1} 2,4-D + 1 mg L^{-1} KIN) produced highest embryo- and callus- like structure rates (8%) and 16% respectively. In addition, induction medium combination 3 (IM3: 1 mg L^{-1} 2,4-D + 5 mg L^{-1} KIN) produced 4% of callus during 9 days of culture from the initiation of Botanik ovary culture day. The results of this experiment demonstrated that there was no response obtained from Botanik variety with medium combination IM4 (1.5 mg L^{-1} 2,4-D + 10 mg L^{-1} KIN), IM3 (1 mg L^{-1} 2,4-D + 5 mg L^{-1} KIN), IM1 (0 mg L^{-1} 2,4-D + 0 mg L^{-1} KIN: control) in the embryo and callus formation and IM3 for embryo formation Table 4.9.

Table 4.9. Observations of Botanik variety in induction media combination after 9 days of culture

Variety	Medium	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN(%)	CN	CN/100 CSN (%)	CN/100 DSN (%)
Botanik	IM1	50	0	0	0	0	0	0	0
	IM2	50	12	4	8	33.33	8	16	66.67
	IM3	50	2	0	0	0	2	4	100
	IM4	50	0	0	0	0	0	0	0

IM1: Induction Medium 1, IM2: Induction Medium 2, IM3: Induction Medium 3, IM4: Induction Medium 4, CSN: Cultured Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

Therefore, owing to the results obtained in this experiment after one month of subculture in a fresh regeneration media cominations, there was no response observed from Botanik with used nutrient media. The remained Botanik ovaries'slices in RM3 medium combination were subcultured again and again but they did not develop into either embryo nor callus structures Table 4.10.

Table 4.10. Observations of Botanik variety in regeneration medium combination after one month of culture

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Botanik	RM1	50	0	0	0	0	0	0	0	0	0	0
	RM2	50	0	0	0	0	0	0	0	0	0	0
	RM3	50	10	0	0	0	0	0	0	0	0	0
	RM4	50	0	0	0	0	0	0	0	0	0	0

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4, CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

Moreover, the performed evaluation in Botanik variety's ovary culture noticed that Botanik variety responded well only during embryo and callus induction culture and development in RM2 and RM3 medium combinations Figure 4.9. While, there was no development observed in embryo- and callus-like structure in regeneration medium combinations Table 4.11.

Table 4.11. Evaluation of embryo and callus formation frequencies for Botanik variety in different media combinations

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Botanik	RM1	50	0	0	0	0	0	0	0	0	0	0
	RM2	50	12	6	4	8	66.67	33.33	8	16	66.67	66.67
	RM3	50	10	2	0	0	0	0	2	4		20
	RM4	50	0	0	0	0	0	0	0	0	0	0

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM 3: Regeneration Medium 3, RM4: Regeneration Medium 4, CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

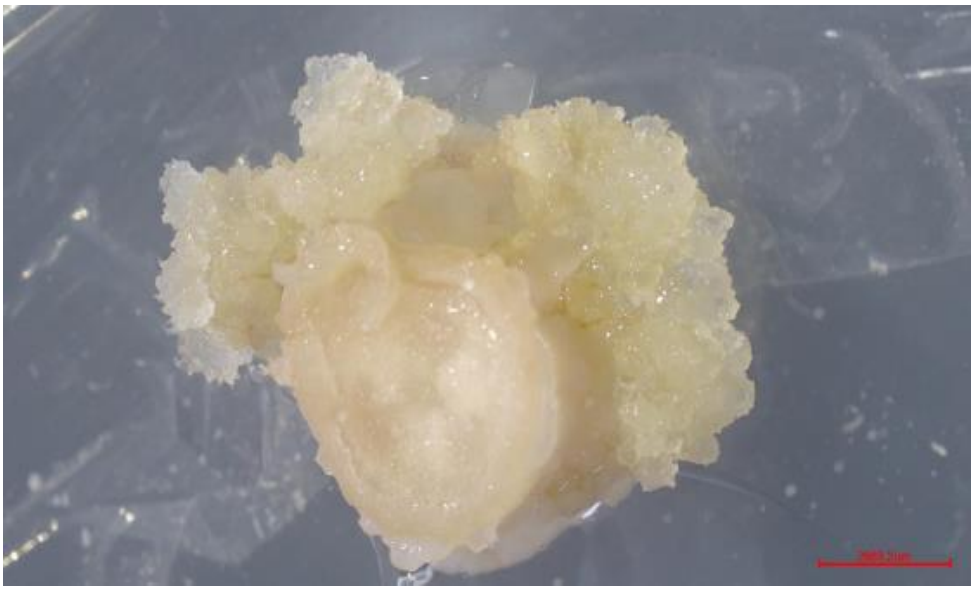

Figure 4.9. Callus formation from Botanik variety in RM3 medium combination after 1 month

4.1.6. Results of Botanik variety for second ovary culture experiment

The results of Botanik variety in second experiment indicated that there was no embryo development in all examined medium combinations. The good results was obtained in callus formation on the ratio (40%) of RM4 (1 mg L^{-1} NAA, 4 mg L^{-1} BAP) medium combination Table 4.12.

Table 4.12. Evaluation of embryo and callus formation frequencies for Botanik variety in different media combinations

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Botanik	RM1	50	10	10	0	0	0	0	1	2	10	10
	RM2	50	0	0	0	0	0	0	0	0	0	0
	RM3	50	15	15	0	0	0	0	5	10	33.33	33.33
	RM4	50	35	35	0	0	0	0	20	40	57.13	57.14

RM 1: Regeneration Medium 1, RM 2: Regeneration Medium 2, RM 3: Regeneration Medium 3, RM 4: Regeneration Medium 4,
CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

4.1.7. Results and discussion of Sardes variety for first ovary culture experiment

According to the experiment number one of this study, the results were recorded and calculated in percentage (%). Sardes variety was responded in all tested induction media combinations except from induction medium 4 (IM4: 1.5 mg L^{-1} 2,4-D + 10 mg L^{-1} KIN), IM1 which is control. Sardes resulted better in induction medium 3 (IM3: 1 mg L^{-1} 2,4-D + 5 mg L^{-1} KIN) with a frequency of 64% of embryo formation followed by 8% from IM2 (0.5 mg L^{-1} 2,4-D + 1 mg L^{-1} KIN) media combinations. The callus formation rates (60% IM3 and 36% IM2) were observed respectively after 9 days of culture from the initiation day of culture Table 4.13.

Table 4.13. Observations of Sardes variety in induction media combinations after 9 days of culture

Variety	Medium	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 DSN (%)
Sardes	IM1	50	0	0	0	0	0	0	0
	IM2	50	11	2	8	18.18	9	36	81.82
	IM3	50	31	16	64	51.61	15	60	48.39
	IM4	50	10	0	0	0	10	40	100

IM1: Induction Medium 1, IM2: Induction Medium 2, IM3: Induction Medium 3, IM4: Induction Medium 4, CSN: Cultured Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

Figure 4.10. Callus structure of Sardes variety in induction medium 3 after 9 days of culture

Due to the experiment, Sardes variety was developed in regeneration media combination Figure 4.9., and the results were recorded and calculated in percentage (%) as well as embryo formation rate (5.882%) and callus induction rate (70.59%) were obtained from RM3 (1 mg L^{-1} 2,4-D + 2 mg L^{-1} BAP). Whereas, the best callus formation rate (93.33%) was observed in RM2 (0.5 mg L^{-1} NAA + 2 mg L^{-1} BAP) and 10% from RM4 (1 mg L^{-1} NAA + 4 mg L^{-1} BAP). There was no embryo formation obtained from RM4, RM2, and RM1(conrol). While there was no development observed from the control medium with Sardes variety Table 4.14.

Table 4.14. Observations of Sardes variety in regeneration medium combination after one month of culture

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Sardes	RM1	50	50	0	0	0	0	0	0	0	0	0
	RM2	50	30	14	0	0	0	0	14	56	93.33	100
	RM3	50	34	26	2	4	5.88	7.69	24	48	70.59	92.31
	RM4	50	40	4	0	0	0	0	4	8	10	100

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4,
CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number; CN: Callus Number

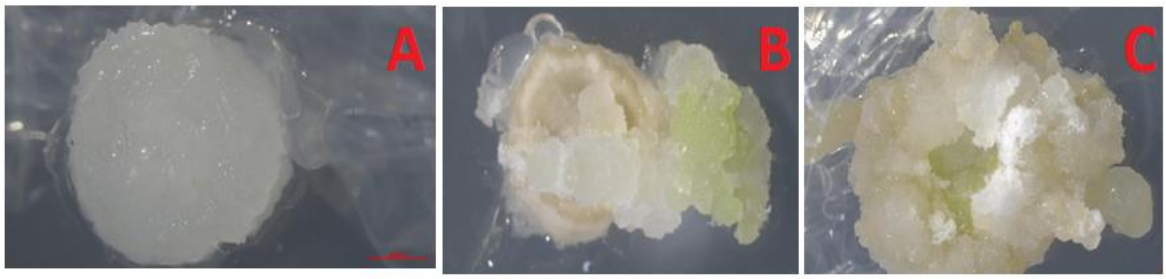

Figure 4.11. Negative and positive effect of nutirent media in Sardes variety after 1 month (A) Sardes in regeneration medum 1 (control), (B) Sardes in regeneration medium 2 (C) Sardes in regeneration medium 3

Furthermore, the ovary culture of Sardes variety was successfull in three regeneration media combinations except from the control nutrient medium Figure 4.12. The results of this experiment were recorded and calculated in percentage (%). The best embryo and callus develompent rate (50%) and (79.41%) were obtained from RM3 and (13.33%) ELS, callus (76.67%) were from RM2 respectively. There is no formation of embryo observed in the RM4 and RM1 medium combinations. The observation demonstrated that there was no development obtained in the control nutrient medium with Sardes variety Table 4.15.

Table 4.15. Frequency of embryo and callus formation for Sardes variety in different media combinations for the first experiment

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Sardes	RM1	50	50	0	0	0	0	0	0	0	0	0
	RM2	50	30	25	4	8	13.33	16	23	46	76.67	92
	RM3	50	34	42	17	34	50	40.48	27	54	79.41	64.29
	RM4	50	40	12	0	0	0	0	12	24	30	100

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4,
CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

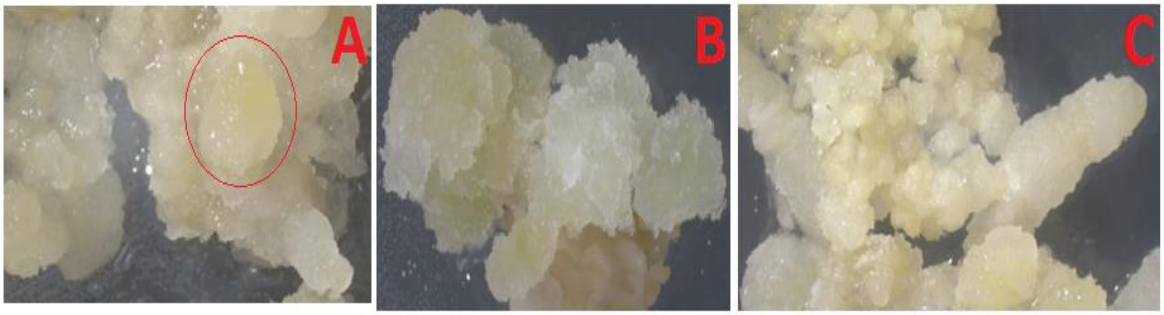

Figure 4.12. Effect of nutirent media in Sardes variety after 3 months
(A) Embryogenic callus formation in regeneration medium 3, (B) normal callus development, (C) development of embryogenic callus in regeneration medium 4

4.1.8. Results of Sardes Variety for second ovary culture experiment (2nd experiment)

However, there was no embryo induction observed, and the highest results of callus formation rate 90% obtained in this trial was from Sardes variety within RM3 (1 mg L^{-1} NAA, 2 mg L^{-1} BAP) medium combination Table 4.16.

Table 4.16. Frequency of embryo and callus for Sardes variety in different media combinations for the second experiment

Variety	Medium	CSN	RSN	DSN	EN	EN/100 CSN (%)	EN/100 RSN (%)	EN/100 DSN (%)	CN	CN/100 CSN (%)	CN/100 RSN (%)	CN/100 DSN (%)
Sardes	RM1	50	0	0	0	0	0	0	0	0	0	0
	RM2	50	30	30	2	4	6.67	6.67	10	20	33.33	33.33
	RM3	50	50	50	0	0	0	0	45	90	95	95
	RM4	50	40	4	0	0	0	0	34	68	85	85

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4, CSN: Cultured Slice Number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

In another hand, in the experience performed without heat shock pre-treatment application (control group) which was performed 06/07/2021, most ovary slices were found in callus formation from two weeks of culture. The embryo formation and callus development to the plant or other structure did not occur in this experiment. The evaluation made during this experiment compared to the heat shock pre-treatments showed that the formation of callus occurred but there were no further developments observed Figure 4.13. Highest callus formation rate (46%) was from Beith Alpha in induction medium combination (IM4), while embryo formation rate (4%) was obtained in Beith Alpha variety within induction medium combination (IM2) Table 4.17.

Table 4.1717. Evaluation of embryo and callus formation frequencies of ovary culture without heat shock pre-treatment in second experiment

Induction Medium code-	Regeneration Medium code	Variety	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN (%)	CN	C/100 CSN (%)	C/100 DSN (%)
IM1-	RM1	Beith Alpha	50	0	0	0	0	0	0	0
		Ptk 40	50	0	0	0	0	0	0	0
		Botanik	50	0	0	0	0	0	0	0
		Sardes	50	0	0	0	0	0	0	0
IM2-	RM2	Beith Alpha	50	7	2	4	28.57	5	10	71.43
		Ptk 40	50	11	0	0	0	0	0	0
		Botanik	50	20	0	0	0	20	40	100
		Sardes	50	1	0	0	0	1	2	100
IM3-	RM3	Beith Alpha	50	5	0	0	0	5	10	100
		Pkt 40	50	6	0	0	0	6	12	100
		Botanik	50	10	0	0	0	10	20	100
		Sardes	50	9	0	0	0	9	18	100
IM4-	RM4	Beith Alpha	50	23	0	0	0	23	46	100
		Ptk 40	50	0	0	0	0	0	0	0
		Botanik	50	5	0	0	0	5	10	100
		Sardes	50	5	0	0	0	5	10	100

IM1: Induction Medium 1, IM2: Induction Medium 2, IM3: Induction Medium 3, IM4: Induction Medium 4, CSN: Cultured slice number, RSN: Remained Slice Number, DSN: Developed Slice Number, EN: Embryo Number, CN: Callus Number

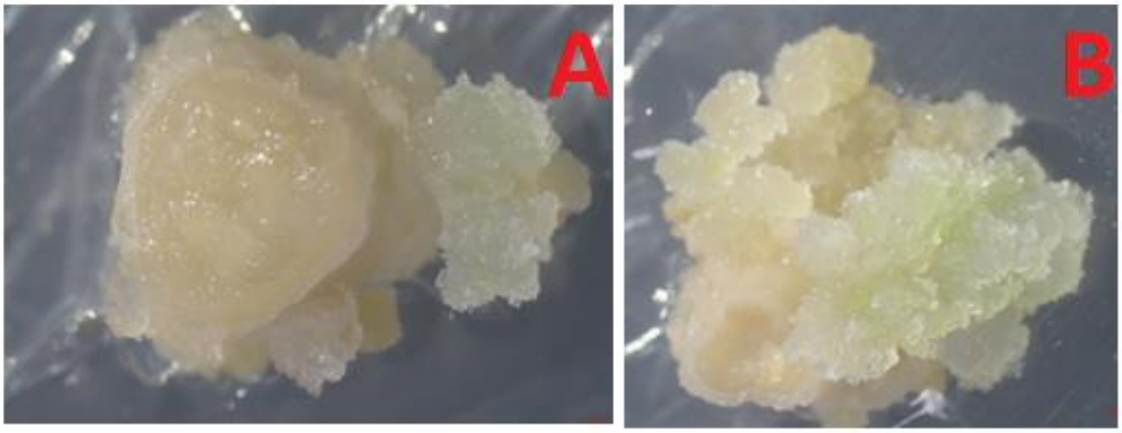

Figure 4.13. Some example of callus formation from Beith Alpha variety and regeneration medium without heat shock pre-treatment

4.1.9. Discussion for *in vitro* cucumber ovary culture of first and second experiments

Owing to the results of this study's part one, the findings demonstrated that the used four commercial cucumber varieties responded differently to the nutrient media combination and heat shock pre-treatments. Hence, the three-way interaction of variety × heat shock pre-treatment × nutrient media [induction (2,4-D, KIN) and regeneration (NAA, BAP)] treatments notably affected the haploid embryo and plant formation frequencies. Additionally, the results showed that the ELSs formation average was 42.24% embryo, and 7.14% callus from Beith Alpha variety with regenerating medium 4 (RM4) while induction media 4 within this variety produced no embryo and there was no callus development occurred. This followed by Beith Alpha within RM3 that produced 2% of embryo and 20% callus, whereas this variety in IM3 had the embryo formation rate (4%) and no callus obtained during this experiment. The combination of Beith Alpha and regeneration medium 2 (RM2) gave 22% embryo without callus formation, and embryo formation frequency (16%) was obtained from induction medium 2. On the other hand, there was no callus formation from this combination. This result was followed by Beith Alpha in control regeneration medium (RM1), where a 6.25% embryo without callus formation was obtained, whereas Beith Alpha in control induction medium (IM1) produced an embryo formation rate of 12%.

Due to the evaluation of embryo and callus formation in the Ptk40 variety, the results revealed that better results (20% embryo and 52% callus) were observed from induction medium 2 (IM2) and embryo and callus formation averages (12.5% embryo and 52.08% callus) from regeneration medium 2 (RM2) respectively, compared to induction medium 4 (IM4) which produced 16% embryo and 8% callus. During the regeneration medium combination trial, the observations showed that Ptk40 in RM4 yielded 10% embryo and 53.33% callus. Subsequently, during induction 12% embryo and 14% embryo for regeneration media combinations (control) were obtained. Yet there was no callus formation occurred. Furthermore, the average of callus formation in Ptk40 variety over developed slices number in the regeneration medium was 339.13%. The best callus formation from regeneration media combinations was observed as 86.67% RM4,

69.56% RM3, 50% RM2 respectively and there was no callus formation occurred in RM1 (control).

Based on embryo formation observed in the Botanik variety, the results showed that the highest embryo formation rate (8%) was produced by the IM2 medium combination; during regeneration medium the embryo average (66.67%) was produced over remained slices in the RM2 medium combination. There was no embryo formation observed in other nutrient medium combinations. According to callus formation, the best results were obtained from induction and regeneration medium combinations of IM2 (16%), IM3 (4%), RM2 (66.67%), and RM3 (40%), respectively. Yet, the embryo and callus formation ratio for other nutrient medium combinations was too low. There was no development observed. The highest formation of embryo from the Sardes variety with the used nutrient media combinations was 64% IM 3, 8% IM2 of induction, and only 5.88% regeneration (RM3). In addition, no embryo formation was obtained from the induction mediums of IM4 and IM1 (control). Regarding the callus formation from induction and regeneration media combinations, the best results were observed during culture induction as follows: IM3 (60%), IM4 (40%), IM2 (36%), and IM1 (control). The regeneration stage produced (93.33%) RM2, (70.59%) RM3, (10%) RM4, and (0%) RM1 (control), respectively. The average of this trial shows that Sardes is 50% embryo formation, 79.41% in RM3, 13.33% embryo with 76.67% callus in RM2, followed by 30% callus formation in RM4 without embryo formation in media combinations. Furthermore, due to the variety's performance in used induction and regeneration media combinations, the findings of these experiments showed that over a total of fifty Sardes ovary slices, 64% embryo, 60% callus was yielded from IM3, (induction) with 4% embryo, 48% callus (regeneration), and 8% embryo, 36% callus from IM2, with 0% embryo, and 56% callus for (regeneration), respectively. Subsequently, in fifty Ptk40 variety's ovary slices in induction media, the observed results were 20% embryo and 52% callus formation in IM2. And a good performance in regeneration medium 3 (RM3) gave 64% callus and 8% embryo with 48% callus regeneration in RM2 combinations. Beith Alpha was seen as the third variety because, on fifty ovaries slices, it responded as well as 16% embryo IM2, 12% embryo IM1 (control), and 4% embryo IM3, while no callus was obtained during the induction process. Based on the regeneration stages and subcultures, good results were produced from RM4 (76% embryo, 12% callus) and RM2 (28% embryo, 2% callus). Lastly, the lowest results were obtained in Botanik, where during induction no embryo or callus development was observed except for IM2 (8% embryo and 16% callus formation, respectively). Even in the regeneration stage, the same results were seen in RM2.

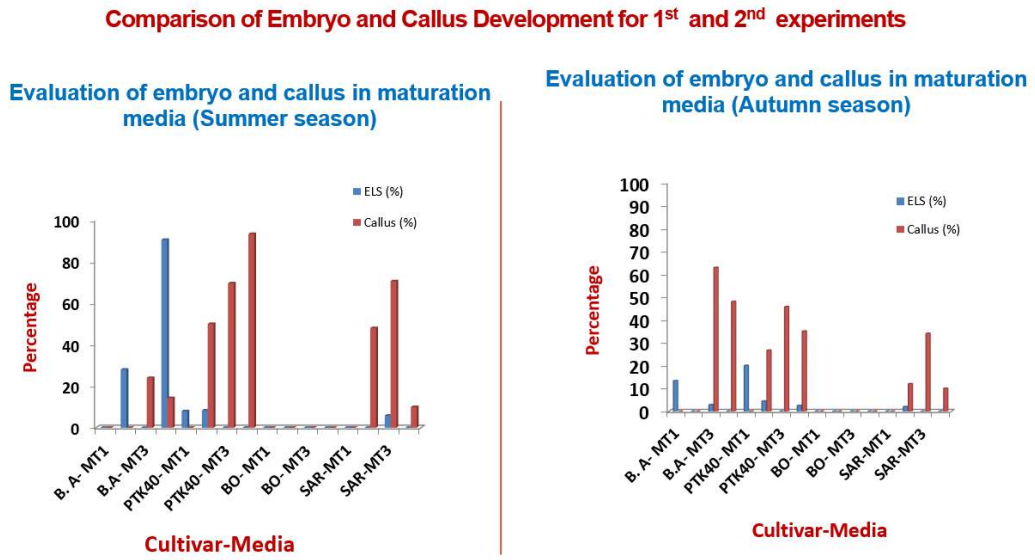

Figure 4.14. Comparison of embryo and callus development for Summer and Autumn seasons

The evaluation was to identify the factors affecting anther culture response traits in cucumber, particularly plant growth hormone concentrations via anther culture in six cucumber genotypes. The culture of anthers from each genotype on MS medium with three diverse levels of prepared hormone. All data were recorded on each cultivar for responding anthers, embryos, calli, and green point developments in percentage (%). Therefore, the planned comparisons between these genotypes as well as the further partition of phenotypic variance into its components are valid. For this reason, the hormone balance levels, such as genotype × levels interaction, of the cultivar 'Speed Way' were the best for all studied in vitro traits with respect to the three hormone balances on MS medium (Abd El-Maskoud et al., 2009).

In general, gynogenesis occurs in two or more stages, each with its own set of nutritional needs. Ovaries require low levels of growth regulators and to be maintained in the dark or light during induction; yet for regeneration they are transferred to medium with a higher concentration of growth regulators and incubated in light. The culture medium is a major factor in influencing the induction and growth of complete plants. However, it is still challenging to determine the best mixture for diverse plant species (Chen et al., 2011). In addition, based on the nutrient medium combination performance used in this study, IM2 (0.5 mg L^{-1} 2,4-D, 1 mg L^{-1} KIN) induction medium was successful in all used cucumber varieties in this experiment. Followed by IM3 (1 mg L^{-1} 2,4-D, 5 mg L^{-1} KIN). For regeneration medium RM2 (0.5 mg L^{-1} NAA, 2 mg L^{-1} BAP) was responded well in all used varieties and RM 3 (1 mg L^{-1} NAA with 2 mg L^{-1} BAP). The result of our study's fist experiment showed similar effects of the interaction between induction nutrient medium and heat shock pre-treatment that has a considerable impact on ELSs development and growth of callus (Tantasawat et al., 2015). The heat shock pre-treatment at 35°C in the oven for three days of darkness was performed after the inoculation of used cucumber ovaries' slices in the

prepared induction nutrient media combination. According to the observation during the experiment, the embryo and callus formation, with heat shock pre-treatment, during 9 days from day one of culture showed the sprouting of embryo and callus on the surface of ovaries' slices except from Botanik and Sardes varieties, which did not respond to the culture conditions; only Botanik and Sardes responded well in the use of IM2, and Sardes responded well in IM3 induction media combinations. The results demonstrate that in used four varieties, the ovary culture response of Sardes F1 generated high ELS rate (64%). All used cucumber varieties were observed to be recalcitrant for embryo and plant regeneration. Successfully, the ELS evaluation was performed where Beith Alpha was good in IM2, while Ptk40 was successful from IM 2 and RM 4 media combinations. Botanik and Sardes were developed well in IM3 induction medium. Compared to the control media, heat shock pre-treatment demonstrated the effects on all used cucumber varieties. The effect of heat shock application and used medium combinations was observed during the ELS, and callus formation and development (Golabadi et al., 2017).

According to the observation, the calli formed during this study were changing in color as well as light and dark brown, yellow and greenish yellow. During the callus development, the callus structure was also evaluated, whereas most of the calli were fragile, Figure 4.12., Figure 4.13., and Figure 4.14. Calli on medium were yellow, strongly dense, and calli on MS medium with the addition of 1.5 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP were green, strongly dense. Green-yellow, dense calli were obtained on medium supplemented with 1.5 mg L^{-1} NAA, 1 mg L^{-1} BAP, and 0.5 mg L^{-1} Kinetin. However, the effect of the developmental stage of microspores on the morphology of calli was not revealed. Despite receiving a large number of calli, the formation of embryoids was not observed (Nguyen et al., 2019). Owing to the ELSs and calli formation in the regeneration media combination (RM4), the highest number of ELS was found in varieties of Beith Alpha with 45.24% (ELS) and callus rate 7.14%, while the Ptk40 variety produced 10% ELS and 53.33% callus. Owing to the media used for embryo maturation and plant regeneration, the results demonstrated that medium RM2 produced 12.5% ELS and 52.1% callus in Ptk40, whereas medium RM3 formed 2% ELS and 20% callus from Beith Alpha. However, Botanik had 8% ELSs and 66.67% of callus formation found in the RM2 medium combination (Table 3.3).

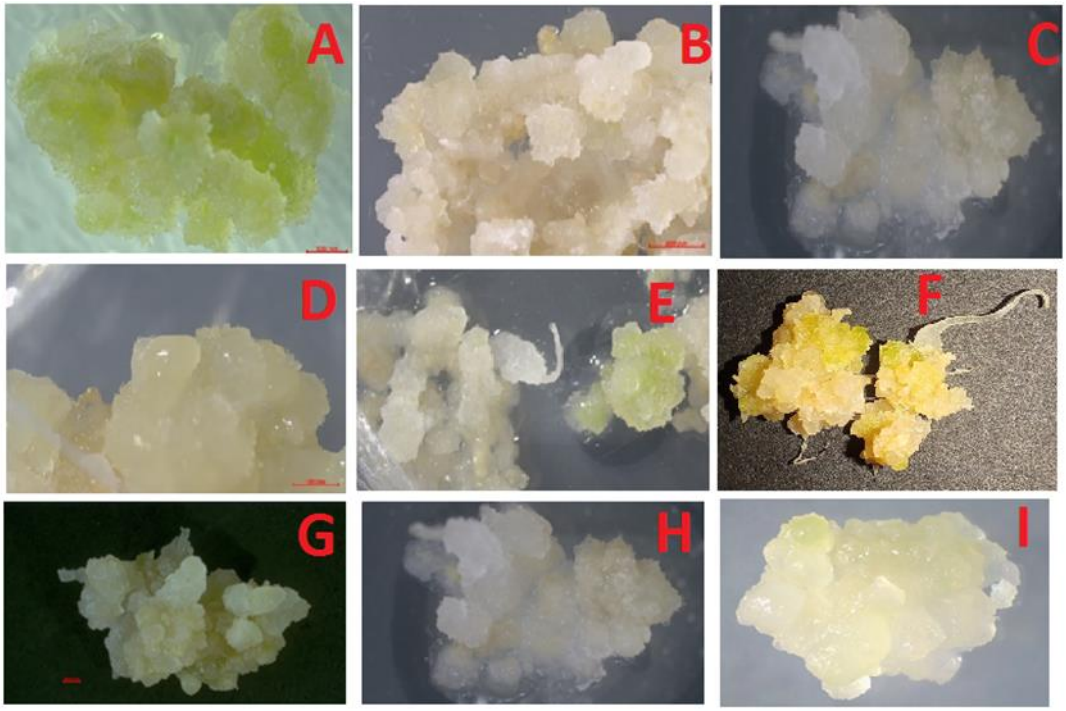

Figure 4.15. (A) to (I) The development of callus in distinct colors, shapes, structures

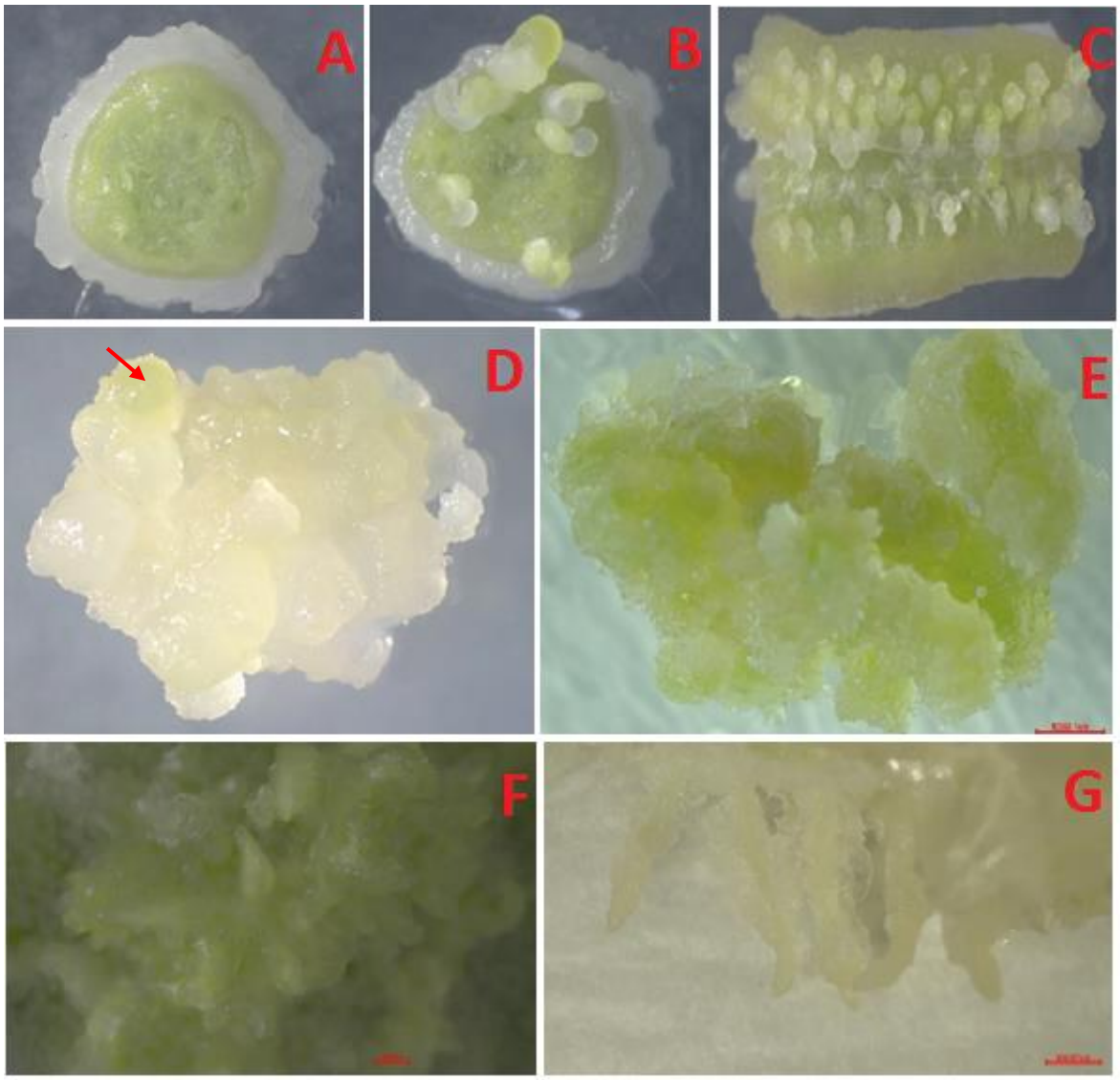

Figure 4.16. Embryo and callus formation developments
(A) Negative (no development) and positive developments: (B-C) Embryo like structures, (D) Red allow shows embryogenic callus, (E) Callus, (F) Developed callus, (G) Root like structure formation

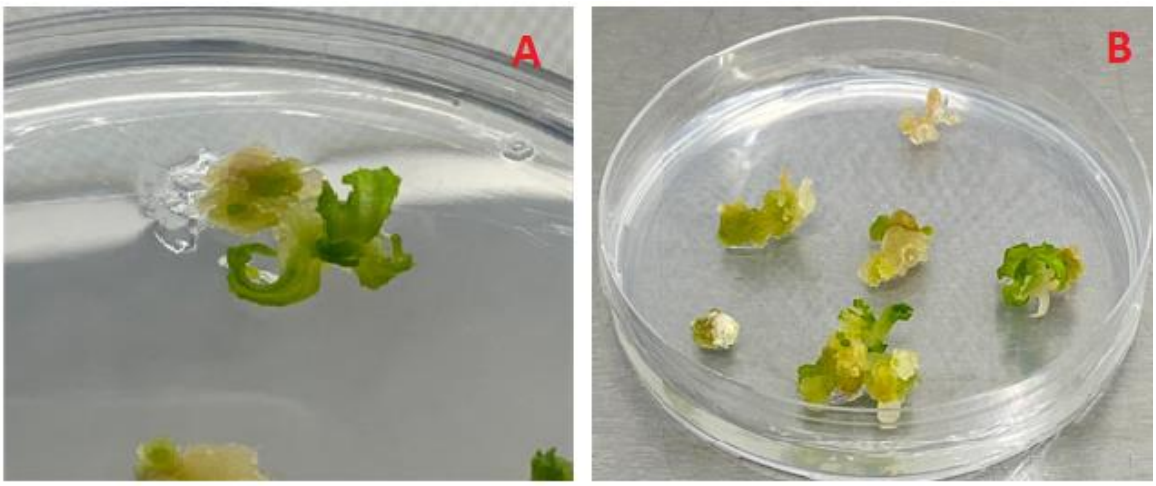

Figure 4.17. Plantlet-like structures (PLS) at early stage, (A-B) PLS development in Petri dish

Haploidy approaches are very substantial to accelerate modern plant breeding process. The pure lines of 100% homozygous needed in plant breeding could be acquired in a short time using in vitro gynogenesis. However, the desired level has not been attained. Yet, some promising results were obtained from various cucurbits, such as watermelon (Taşkın et al., 2013; Akbaş and Solmaz, 2019), melon (Ficcadenti et al., 1999; Koli and Murthy, 2013; Malik et al., 2011) and cucumber (Gemes-Juhasz et al., 2002; Diao et al., 2009; Wei et al., 2010; Li et al., 2012; Li et al., 2013; Moqbeli et al., 2013; Plapung et al., 2014; Tantasawat et al., 2015; Golabadi et al., 2017; Ozsan, Onus 2017; Zou et al., 2018; Erol and Sarı 2019; Deng et al., 2020; Baktemur et al., 2022). In addition, in haploid production, the used genotypes or varieties play a major role even though plant species can have favourable response for this technique. Current study, ovaries of four cucumber varieties of were planted on eight various hormones concentrations as nutrient media including four of induction and four for maturation to obtain haploid plants. Application of ovary culture method in cucumber utilizing diverse genotypes, culture medium, temperature pre-treatments have been performed with several researchers (Deng et al., 2021; Baktemur et al., 2022; Pradeepkumara et al., 2023).

Furthermore, the effects of heat pre-treatments and nutrient media over two breeding accessions and one local cultivar in cucumber ovary culture have been evaluated. The culture has been incubated four days at 35°C and distinct concentrations (0.1, 3 mg L^{-1}) for Indole-3-acetic acid (IAA), 2,4-D, Indole-3-butyric acid (IBA), Kinetin (KIN), NAA and BAP. On other hand, the various concentrations (0.1-0.8 mg L^{-1}) of TDZ, 2,4-D, IBA, KIN, BAP, NAA and $AgNO_3$. The results have been shown that heat shock was deemed to be successful by embryogenesis rate (38.98%) compared to without heat pre-treatment (28.60%). Based on first experiment, the hormone combination has been demonstrated a high embryogenesis rate (70.53%) reached from a combination of 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} KIN and 1.5 mg L^{-1} BAP (Golabadi et al., 2017).

Apart from cucumber genotypes of *Cucurbitaceae* family, Kurtar et al (2018), analysed the ovules culture of *Cucurbita maxima* and *Cucurbita moschata* at different flowering times. Ovules were cultured on a specific solid medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BAP), thidiazuron (TDZ), and naphthaleneacetic acid (NAA) to enhance callus formation and plant regeneration. The medium supplemented with 4.0 mg L^{-1} BAP, 0.05 mg L^{-1} NAA, 0.1 mg L^{-1} TDZ provided the highest response at anthesis time. Plantlets were rooted and elongated on a solid MS medium consisted of 0.01 mg L^{-1} IAA, 1 mg L^{-1} BAP. After the ploidy level analysis of 122 plants, it was found that 70 plants were haploid, 46 were diploid, and the rest were mixed ploidy.

Besides the cucumber genotypes, some studies were performed to examine various factors affecting haploid induction in other cucurbit plants. For instance, Shalaby (2007), used 12 squash genotypes to determine the appropriate ovary stage, the required temperature application and the

effect of sugar concentration to enhance haploids via ovary culture method. In the study, MS medium supplemented with 3%, 6%, 9% sugar with 1 mg L^{-1} kinetin and 1 mg L^{-1} 2,4-D were applied. The used ovaries were collected 1 day before the anthesis. Every four weeks the nutrient media were renewed at 25 ± 1°C temperature and 16 hours photoperiod of the growing chamber. It has observed the highest regeneration ratio was from 3% sugar content. The research stated that 32°C culture incubation for 4 days was the utmost suitable temperature for embryonic induction. The 65% of regenerated plants were haploid and 35% diploid. Moreover, Nguyen and Huyen (2023) evaluated heat treatment, microspore developmental stage with other factors that influence callus induction from melon microspores. The results indicated that from the used heat shocks that 40ºC for 48 hours was most suitable temperature for callus formation from melon microspore in all flower bud sizes used in the study.

In the study performed by (Tantasawat et al., 2015) examined 5 induction media 3 differentiation media and 5 genotypes for a cucumber ovary culture experiment. Before transferring to the differentiation media, cultures were incubated at 35°C/3 days and then at 25°C/11 days, the 25°C/ 2 weeks in the dark. The percentage of embryo like structures (ELS) ranged between 5.27% - 68.60%. According to the accessions, Big C and Chai Lai were found to be more successful than others with 44.60% and 44.74% respectively. Moreover, (Ozsan et al., 2017) analysed 8 different nutrient media using 3 cucumber cultivars and cucumber basal media (CBM) added with several doses of TDZ, KIN, and 2,4-D were applied. Heat shock pre-treatment (35°C/3 days) was applied under dark condition. Subsequently, explants stayed 2 days at 25°C in darkness. A medium combination of 0.03 mg L^{-1} TDZ with CBM, produced the highest embryo number with 17 embryos.

Also based on heat shock pre-treatment, in the study of Sun et al (2009), combined 0.25 mg L^{-1} 2,4-D with 0.5 mg L^{-1} NAA, and 0.5 1.0 mg L^{-1} BAP, respectively, and supplemented with 30 g L^{-1} sucrose with the heat shock pre-treatment of 35°C for 6 days were applied in the ovary culture of pumpkin (*Cucurbita moschata* cv. Miben). It was found that the ovaries at the stage of 1 day prior to anthesis were optimal for embryo induction. Heat treatment at 35°C for 6 d before embryo induction remarkably enhanced the potential of embryo formation in unpollinated ovary. High concentration of 2,4-D promoted embryo formation in unpollinated ovary and inhibited callus formation. The ovary explants cultured in MS medium containing 4.0 mg L^{-1} 2,4-D, 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} 6-BAP produced the highest embryo formation rate (20±3.8%) with the mean embryo number of 11.3±3.2 per ovary segment. Presently, ovule and ovary culture are the least prior method because of its quit efficiency. Our findings showed that the interplay of genotype and heat pre-treatment by diverse nutrient compositions had a beneficial influence on embryogenesis outcome. According to the observation at the end of the study, those that swell and enlarge, change color and regenerate callus-like or embryo-like structures were evaluated within the scope of ovary development Figure 4.15, Table 4.18.

Table 4.18. Evalution of embryo and callus formation frequencies from the experment one of the study

Cultivar	Medium	Plant Growth Regulator (mg/L)		No. cultured Petri dish	No. cultured slices/medium	No. infected slices/medium	No. remained slices/medium	ELS development (total)	ELS development (%)	Callus development (total)	Callus development (%)
		NAA	BAP								
Beith	RM1	0	0	10	25	35	15	2	13.33	0	0
Alpha	RM2	0.5	2	10	50	40	10	0	0	0	0
	RM3	1	2	10	50	15	35	1	2.85	22	62.85
	RM4	1	4	10	50	25	25	0	0	12	48
Ptk40	RM1	0	0	10	50	45	5	1	20	0	0
	RM2	0.5	2	10	50	5	45	2	4.44	12	26.66
	RM3	1	2	10	50	15	35	0	0	16	45.71
	RM4	1	4	10	50	10	40	1	2.5	14	35
Botanik	RM1	0	0	10	50	45	5	0	0	0	0
	RM2	0.5	2	10	50	50	0	0	0	0	0
	RM3	1	2	10	50	25	25	0	0	0	0
	RM4	1	4	10	50	35	15	0	0	0	0
Sardes	RM1	0	0	10	50	45	5	0	0	0	0
	RM2	0.5	2	10	50	0	50	1	2	6	12
	RM3	1	2	10	50	0	50	0	0	17	34
	RM4	1	4	10	50	0	50	0	0	5	10

RM1: Regeneration Medium 1, RM2: Regeneration Medium 2, RM3: Regeneration Medium 3, RM4: Regeneration Medium 4, ELS: Embryo like structure

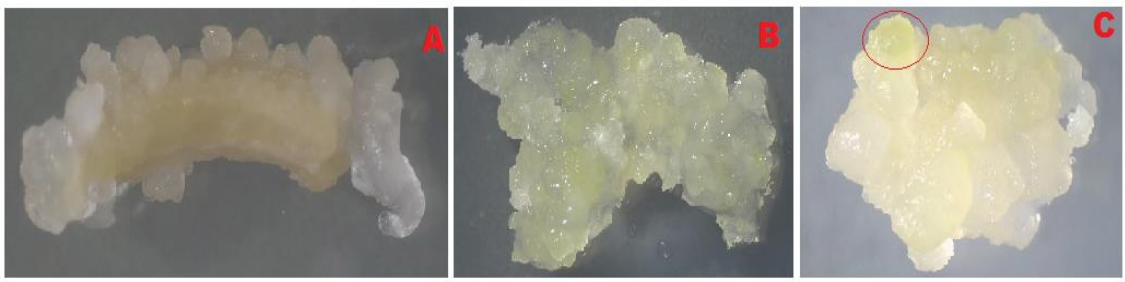

Figure 4.18. Ovary development
(A) Ovules enlargement, (B-C) Callus formation on the surface of cultured ovary slices

4.1.10. Histological Analysis of Structures Obtained from Ovary Culture in Cucumber

It has been observed that in some used nutrient media the ovules could not be enlarged. This may indicate that samples of female flower buds or ovaries were not collected at the right time to be used in ovary culture. Degeneration has been observed in some ovules. It shows that the samples taken from these cultured slices were not suitable on the medium used (like RM 4) for the development of the ovules of some varieties. This means that there is a great effect of genotype and culture medium. It was determined that the ovules development obtained from this medium were embryos developing in the embryo sacs (Figure 4.19.). For instance, this supports the possibility of haploid embryos to be obtained from the medium used (RM 2) in Sardes variety.

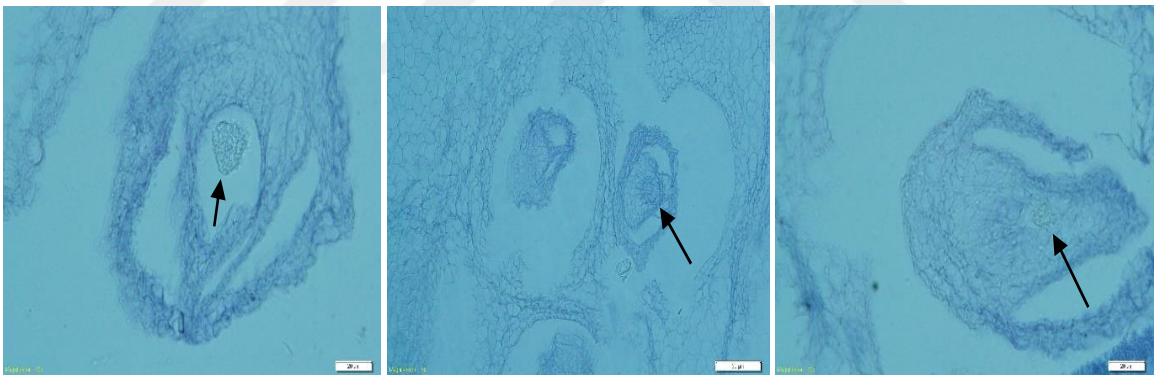

Figure 4.19. Embryos in ovules. Black arrows indicate embryos.

For control most embryos were developed from the skin tissue of ovary slices. For instance, Sardes F1variety responded in RM 3 (control= without heat shock pre-treatment): The green color calli structures were produced and a large number of embryo development in the outer tissues has been observed in the advanced globular stage Figure 4.17.

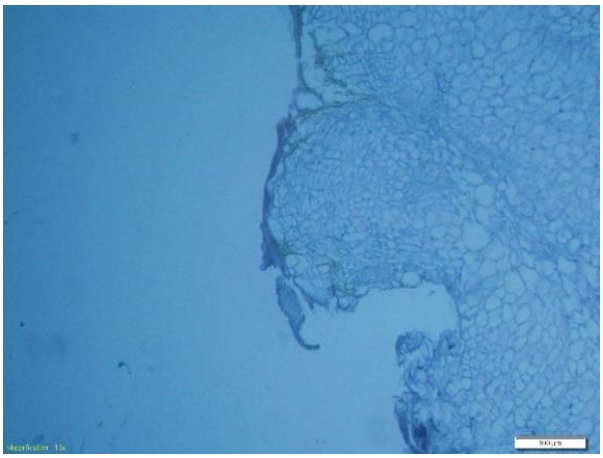

Figure 4.20. Embryo development in the outer tissues of ovary slice

Callus development was observed in the seed ovules. No embryo formation was found (Figure 4.18.).

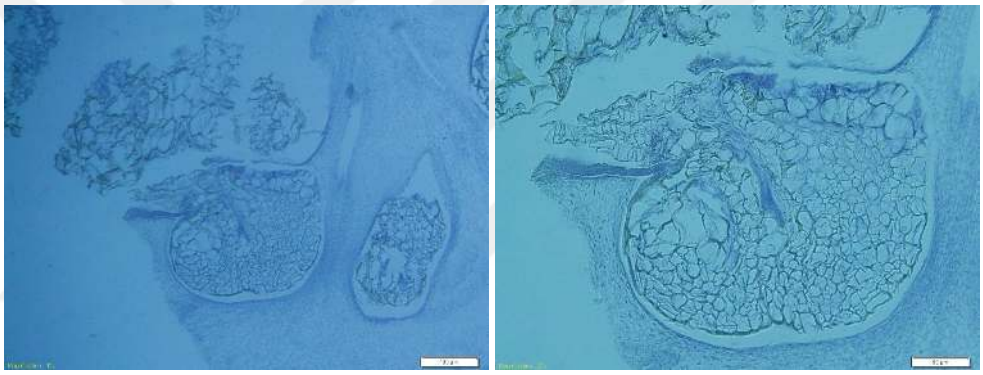

Figure 4.21. Callus tissue developing in ovules

From the sample taken from Beith Alpha, the hormone free nutrient media with (RM 1) was analysed and observed as albino development. So the histology analysis showed taht an embryo-like structure was observed in only one ovule, and the exact nature of this structure could not be determined (Figure 4.20.). In addition, embryo development was observed in external tissues of Beith Alpha.

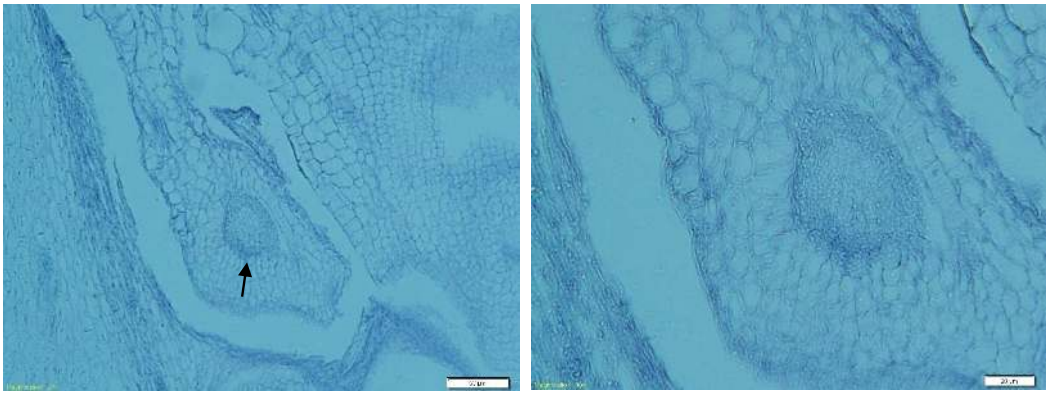

Figure 4.22. Left: Embryo-like structure in the ovule. On the right, a close-up view of the structure

4.2. Results and discussion of Cucumber Second Year Ovary Culture Experiment (II)

4.2.1. Plant Materials and Ovary culture process

The second year growing season's ovary culture experiment was performed in the year of 2022-2023. In this experiment also plant materials were the same as used in first year growing seasons' experiments which are Ptk40 F1, Sardes F1, Botanik F1, and Beith Alpha cucumber varieties haploid induction. The fresh unpollinated ovaries were taken one day before anthesis around 8 a.m to 9 a.m, and at this point, the color of the corolla is greenish-yellow Figure 3.16. The tested nutrient media combinations for embryo induction and plant regeneration were prepared and mentioned in Table 3.2 and 3.3. After cold (4°C) shock pre-treatment for 4 days, female flower buds were prepared and sterilized Figure 3.8. And then cucumber ovary culture process was performed i.e ovaries' slices inoculated on prepared induction media combinations (IM5-IM8) in 90 mm petri dishes Figure 3.9.

4.2.2. Embryo and Callus Formation

After the ovaries inoculated on induction media (IM), the cultures stayed in growing chamber at 25±1°C in light conditions and a photoperiod of 16/8 Figure 3.11. for 2 weeks. Followed by subculture stages. The embryo and callus formation were observed and recorded Figure 4.22. Subsequently, the fresh nutrient regeneration media combinations Table 3.3. were changed every 4 weeks Figure 4.21. Generally, plant growth regulators (PGRs), especially auxins, have widely been used for induction of gynogenesis and their optimum concentration varied considerably from species to species or variety to variety (Kumar et al., 2003). The plant regeneration rate was evaluated 3 months after ELS were observed.

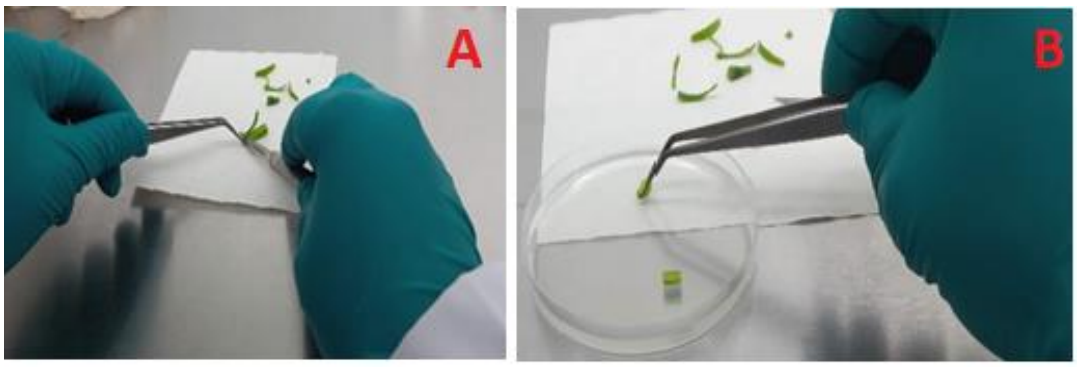

Figure 4.23. Ovary slices culture into plastic petri dishes
(a) inoculating ovary slices on induction nutrient medium combination, (b) ELS on cultured ovary slice to be transferred to the regeneration medium combination

4.2.3. Effect of distinct nutrient medium combination on Embryo and plant regeneration

Throughout the haploid plant production, the application of cold and heat stress pre-treatment has been an essential factor to increase the efficiency of gynogenesis in different species (Golabadi et al., 2017). In particular, the different rates of plant embryo induction were induced

from four days cold shock pre-treated female flower buds at 4℃. The highest embryo induction rate (80%) appeared within two weeks from the first day of culture in this experiment. Observations made in ovary culture during this trial are presented Figure 4.22.

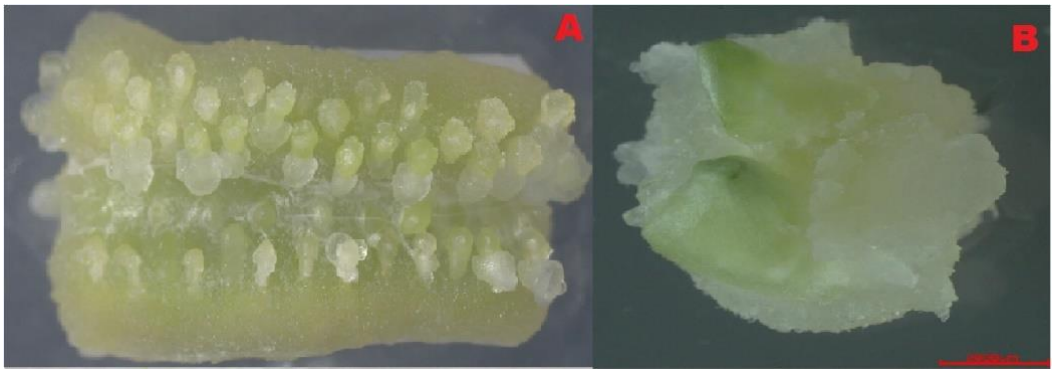

Figure 4.24. Embryo formation from cucumber ovary culture
(A) ovule enlargement (ELSs), (B) Embryogenic callus development

The result indicated that cold pre-treatment of ovaries was effective for the direct formation of haploid embryos (Figure 4.22.). Ovules enlargement was considered as embryo-like structures (ELS) formation observed within two weeks from the first day of culture. And callus formation appeared on the surface of cultured ovary slices at almost 2 to 3 weeks of initiation culture in this experiment. Embryo regeneration medium combinations were prepared as a result of seeing embryos sprouting on the cultured ovaries for 4-6 weeks in induction media and all ovaries' slices were transferred to these medium. All evaluation of tested nutrient media combinations and varieties are shown below.

For instance, thidiazuron (TDZ) hormone with cold shock (4°C) pretreatment affected embryo development in used four commercial cucumber varieties. In the application of 0.06 mg/l TDZ (IM5), the highest embryo formation rate (106%) of Ptk40 F1 variety, followed by Sardes F1 (60%). The lowest embryo formation rate (46%) was obtained in Botanik F1 variety. The results indicated that there was no callus formation happened durig the study Table 4.20., Figure 4.23

Table 4.19. Effect of thidiazuron induction medium on ovary culture of used cucumber varieties in this experiment

Medium Code	Variety	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN (%)	EC	EC/100 CSN (%)	EC/100 DSN (%)
IM5	Beith Alpha	50	47	50	50	106.382	0	0	0
	Ptk40	50	40	53	106	132.5	0	0	0
	Botanik	50	23	23	46	100	0	0	0
	Sardes	50	27	27	54	100	0	0	0

CSN cultured slice number, DSN developed slice number, EN embryo number; EC embryogenic callus; ECN Embryogenic cullus number

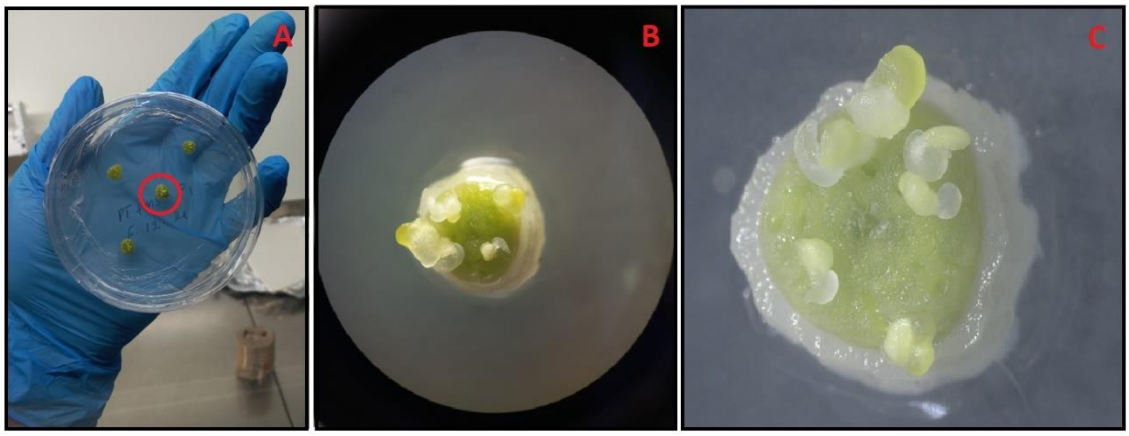

Figure 4.25. Embryo development from TDZ application
(A) Embryo formation on cucumber ovary slices in Petri dish, (B) Embryos development observation under Binocular microscope, (C) Embryo like structures observetion under stereo microscope.

The use of 2,4-D and KIN plant growth hormones combination and cold shock (4°C) pretreatment indicated the effect on embryo and callus development in used four commercial cucumber varieties. In the application of 0.15 mg/L 2,4-D, 1.5 mg/L KIN medium combination (IM6) produced the high callus formation rate (34%) of Ptk40 F1 variety, Beith Alapha and Botanik F1 has 7% each of them while Sardes F1 has no embryo or callus formed in this trial. The results indicated that there was no embryo formation observered durig the experiment Table 4.21., Figure 4.24.

Table 4.20. Effect of 2,4-D and Kinetin nutrient media combination on ovary culture of used cucumber varieties in this experiment

Medium Code	Variety	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN (%)	EC	EC/100 CSN (%)	EC/100 DSN (%)
IM6	Beith Alpha	50	7	0	0	0	7	14	100
	Ptk40	50	17	0	0	0	17	34	100
	Botanik	50	7	0	0	0	7	14	100
	Sardes	50	0	0	0	0	0	0	0

CSN cultured slice number, DSN developed slice number, EN embryo number; EC embryogenic callus; ECN Embryogenic cullus number

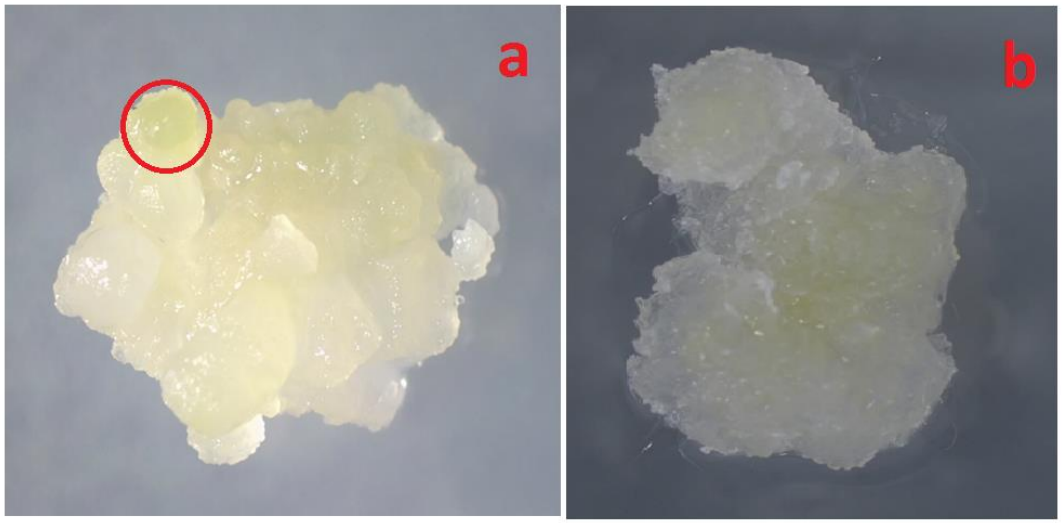

Figure 4.26. Observation of callus development under stereomicroscope; (a) Embryogenic callus, (b) normal callus

In this experiment, embryo induction medium combination supplimented with 2,4-D:BAP in a contration (0.1:1) mg/L (IM7) and cold shock (4°C) pretreatment was tested and had the effect on embryo and callus formation Figure 4.25. The results demonstarted that the best obtained embryo formation rate (14%) Ptk40 F1 variety, followed by (8%) Botanik F1 and 4% of Beith Alpha, whereas the callus development was not observed in all varieties Table 4.22.

Table 4.21. Effect of 2,4-D and BAP nutrient media combination on ovary culture of used cucumber varieties in this experiment

Medium Code	Variety	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN (%)	EC	EC/100 CSN	EC/100 DSN
IM7	Beith Alpha	50	2	2	4	100	0	0	0
	Ptk40	50	17	7	14	41.176	0	0	0
	Botanik	50	20	4	8	20	0	0	0
	Sardes	50	0	0	0	0	0	0	0

CSN cultured slice number, DSN developed slice number, EN embryo number; EC embryogenic callus; ECN Embryogenic cullus number

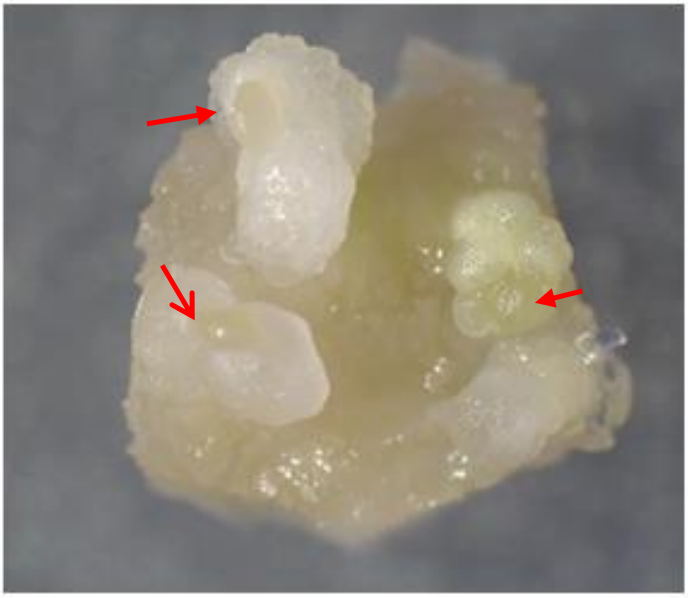

Figure 4.27. Observed embryo development
Red arrows show the embryo like structures on ovary slices

The use of 2,4-D and Putrescine (PUT) plant growth hormones combination and cold shock (4°C) pretreatment indicated the effect on embryo and callus development in used four commercial cucumber varieties. The concentration of 5 mg/L 2,4-D, 40 µM PUT medium combination (IM8) was applied to all used cucumber varieties in this study. The medium combination resulted the high embryo and callus formation rate (34%, 66% respctively) from Beith Alapha, Sardes F1 (30%, 66.66% respectively). However, Botanik F1 and Ptk40 did not produce embryo, the callus formation was 100% from Ptk40 and 50% of Botanik Table 4.23, Figure 4.26.

Table 4.22. Effect of 2,4-D and Putrescine nutrient media combination on ovary culture of used cucumber varieties in this experiment

Medium Code	Variety	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN (%)	EC	EC/100 CSN	EC/100 DSN
IM8	Beith Alpha	50	50	17	34	34	33	66	66
	Ptk40	50	50	0	0	0	50	100	100
	Botanik	50	25	0	0	0	25	50	100
	Sardes	50	45	15	30	33.33	30	60	66.66

CSN cultured slice number, DSN developed slice number, EN embryo number; EC embryogenic callus; ECN Embryogenic cullus number

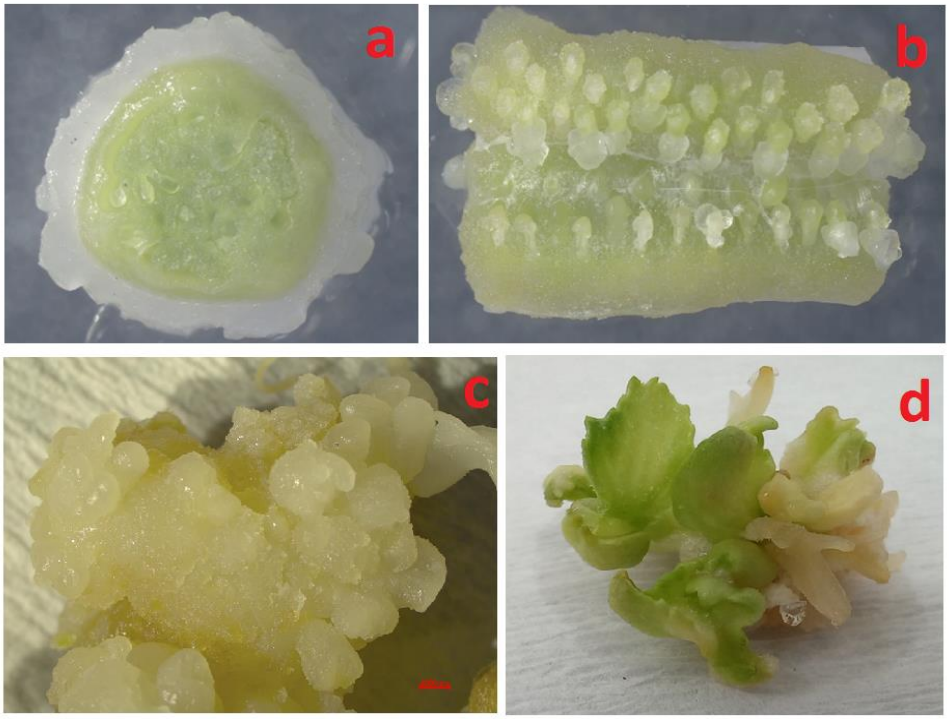

Figure 4.28. Negative and positive effect of 2,4-D combined with PUT on cucumber ovary culture (a) no embryo/ callus formed (negative), (b) Embryo like structures formed (positive), (c) embryo genic callus formation, (d) Embryogenic callus development and plant like sturucture started to form

In addition, part two of our research study was successfully performed and good results were obtained. Generally, the evaluation carried out during and after five months of ovary culture initiation day showed that embryo and callus formation were observed. During the haploid embryo and plant regeneration, the distinct regeneration nutrient media combination (Table 4.20.) was performed from the first subcultures in all used ovaries'slices of all varieties. Based the observation, we found out that cultured slices developed well in RM8 (0.1 mg/L IAA, 2 mg/L BAP) medium combination and increased the plantlet like structures production from several calli. The 0.1 mg/L IBA germiantion medium showed a very high performance in plant germination in this study and it was apllied to all cultured ovaries slices used in our study Table 4.24., Figure 4.27 and Figure 4.28.

Table 4.23. Evaluation of nutrient media and variety on haploid plant production via ovary culture in cucumber at five months of culture

Medium Code	Variety	CSN	DSN	EN	EN/100 CSN (%)	EN/100 DSN (%)	EC	EC/100 CSN (%)	EC/100 DSN (%)	PN-E	PN/100 EN (%)	PN-EC	PN/100 EC (%)	APN
IM5-	Beith Alpha	50	30	12	24	40	0	0	0	0	0	0	0	-
	Ptk 40	50	20	26	52	130	0	0	0	0	0	0	0	-
	Botanik	25	0	0	0	0	0	0	0	0	0	0	0	-
	Sardes	50	30	14	28	46.67	0	0	0	12	85.71	0	0	12
IM6-	Beith Alpha	50	0	0	0	0	0	0	0	0	0	0	0	-
	Ptk40	50	0	0	0	0	0	0	0	0	0	0	0	-
	Botanik	50	8	4	8	50	0	0	0	0	0	0	0	-
	Sardes	50	34	0	0	0	0	0	0	0	0	0	0	-
IM7-	Beith Alpha	25	0	0	0	0	0	0	0	0	0	0	0	-
	Pkt 40	50	16	12	24	75	8	16	50	2	16.67	0	0	-
	Botanik	50	0	0	0	0	0	0	0	0	0	0	0	-
	Sardes	50	0	0	0	0	0	0	0	0	0	0	0	-
IM8-	Beith Alpha	50	16	10	20	62.5	4	8	25	0	0	0	0	-
	Ptk 40	50	34	16	32	47.05	12	24	35.29	0	0	0	0	-
	Botanik	50	40	44	88	110	136	272	340	22	50	108	79.41	10
	Sardes	50	20	30	60	150	34	68	170	0	0	4	11.76	4

CSN: Cultured slice number, EN: Embryo number; ECN: Embryogenic cullus number; PN-EN: Plant number from embryo, PN-EC: Plant number from embbryogenic callus, APN: Albino plant number

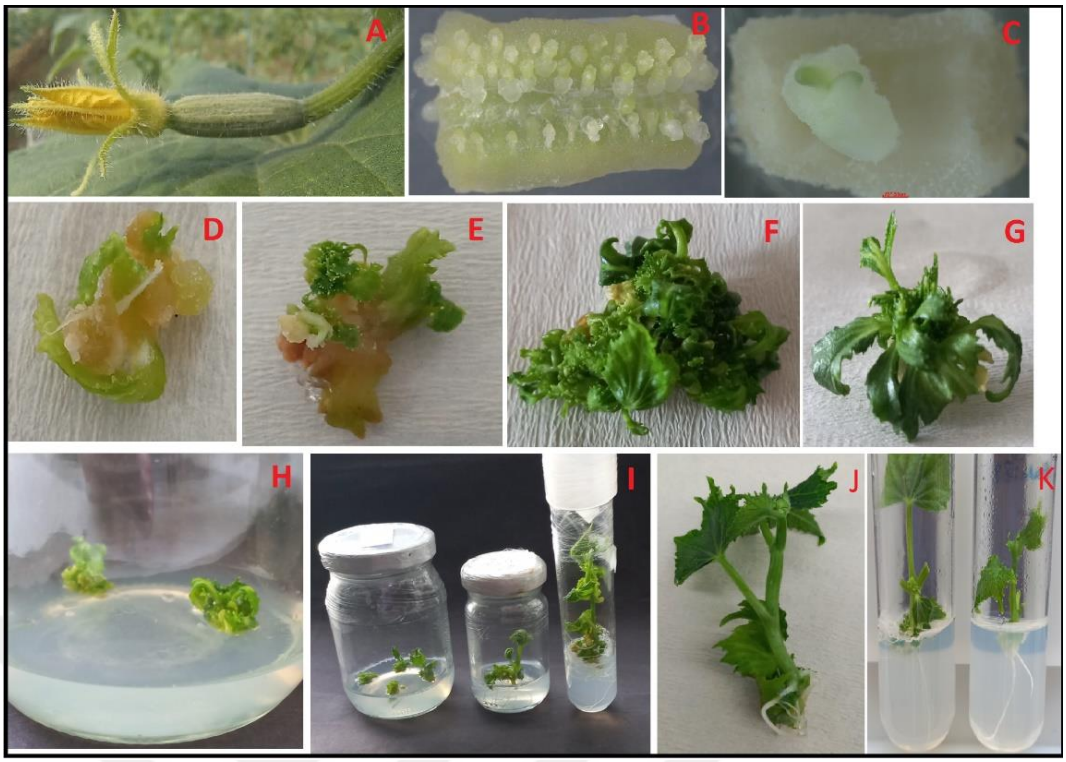

Figure 4. 29. Evaluation of nutrient media and variety on haploid plant production via ovary culture in cucumber at five months of culture

(A) Female flower bud, (B) Embryo like structure development, (C) Embryogenic callus, (D), Root like structure development, (E) to (H) Plantlet regeneration, (I) to (K) Plant development.

Figure 4.30. A summary of cucumber ovary culture growing chamber

(a) to (f) From ovaries' slices to the plant

4.2.4. Formation of albino and abnormal structures

Furthermore, based on results of this study Table 4.25. the greenest plantlets were obtained in Botanik F1, Sardes F1. However, the calli were not developed in ELS or plantlets from the varieties of Ptk40 F1 and Beith Alpha. Throughout the formation of plant like structure, the

plantlets from Sardes became albino and perished at early stage of their life Figure 4.31. Haploid and doubled haploid plants production is limited due to the occurrence of albino plants formation (Ankele et al., 2005). Albinism is a prominent challenge in interspecific crosses and tissue culture research, such as gynogenesis and androgenesis cultures, as well as the formation of doubled haploids. It is distinguished by the loss of chlorophyll pigments and the inadequate differentiation of chloroplast membranes. This, in turn, impedes photosynthesis, and the plants eventually perish before reaching maturity (Kumari et al., 2009). Molecular analysis has been performed for albino plants and the findings of the study indicated that there is aberrant form of plastids and very often deletions in their platid DNA and there were the insufficiencies in plastid transcription and translation (Ankele et al., 2005). Recently, researchers have been demonstrated albino plant formation is genotype-dependent event (Khatun et al., 2010; Żur et al., 2021). The variant phenotypes' structures were observed (Freytag et al., 1989), Figure 4.32. demonstrate the abnormal structures obtained during this research study.

Figure 4.31. Albino plantlet like structures

Figure 4.32. Abnormal structures

Many embryo and plantlet forms were obtained from ovaries taken into embryo developmental nutrient media. Compared to our study, during liquid medium application, the callus formation was observed approximately 2 weeks after the transfer of cultured to the regeneration medium. Number of ovaries developed (%): In the ovule culture study, the number of ovules developed by weekly observations following ovaries culture was recorded and evaluated collectively. Plant growth regulators such as auxin (2,4-D, NAA, IAA, etc.) and cytokinin (BAP, KIN, etc.) are used in ovule-ovary studies in cucurbits (Kara and Sari, 2019).

The results of this study in terms of embryo formation. According to experiment number two, this study resulted that embryo and callus formation rate was high in induction media of IM8 which was successful in all varieties [Botanik F1 (88% embryo per cultured slice, 110% embryo per developed slice), Sardes F1 (60% embryo per cultured slice, 150% embryo per developed slice), Ptk40 (32% embryo per cultured slice, 47.05% embryo per developed slice), Beith alpha (20% embryo per cultured slice, 62.5% embryo per developed slice)] respectively whereas IM7 was better in Ptk40 F1 (24% embryo per cultured slice, 75% embryo per developed slices), IM6 (8% embryo per cultured slices, 50% embryo per developed slice) Botanik F1, MI5 (28% embryo per cultured slices, 46.67% embryo per developed slice Sardes F1, 52% embryo per cultured slices, 130% embryo per developed slice; Ptk40 F1, 22% embryo per cultured slice, 40% embryo per developed slice Beith Alpha) Table 4.23. According to the results of this study, the embryo formation into induction nutrient medium combination, IM8 (2,4-D and PUT) was successfully noticed in all verities followed by IM6 (2,4-D, KIN). Based on embryo development to the plant like structure, botanic was successful variety in medium combination (IM8) with the application of cold pre-treatment (4°C).

Thus, Erol and Sari (2019) examined nutrient media supplemented with polyamines as well as putrescine to induce haploids from ovule-ovary culture in cucumber varieties. The results of their study demonstrated that the medium with putrescine (SPD and PUT) produce embryos in varieties of Sardes F1, Cemre F1 and Altay F1 but no plant obtained. Compared to our study, the medium containing putrescine (2,4-D and PUT) was better for embryos and embryogenic callus formation in Botanik F1 and Sardes F1 varieties. And the plants were obtained in our study. In brief According to combinations of culture media tested in the first experiment, 0.5 mg/L 2,4-D with 1 mg/L KIN the induction (IM2) and 0.5 mg/L NAA and 2 mg/L BAP regeneration media (RM2) combinations, from the second year's experiment the induction of 5 mg/L 2,4-D with 40 µM PUT (IM8) induction and 0.1 mg/L IAA and 1 mg/L BAP regeneration promised better results. The temperature (heat and cold shocks) pre-treatment showed the effluence on haploid embryo and callus formation from cucumber ovary culture. In terms of cucumber variety, Demirel and Onus (2021) carried out a study based on the effects of cucumber types and genotypes on haploid embryo formation and obtaining plants in Çengel F1, Ufuk F1, Sedir F1, Amisos F1, Ptk40. By appling 2,4-D and Kinetin fro induction, with regeneration medium (NAA and BAP) they produced Çengel F1

with 494.44% embryo- and 302.78% plant-formation, respectively on the other hand cultivar Ptk40 had lowest respond on embryo formation. While in our study the successful variety was Ptk40 (20%) of embryo formation in the application of 2,4-D and Kinetin for induction, NAA and BAP regeneration medium.

Additionally, due to the temperature pre-treatments, nutrient medium, and variety interaction the results demonstrated that Botanik F1 (88%) Sardes F1 (60%), Ptk40 (32%), Beith alpha (20%), whereas IM7 was better in Ptk40 F1 (24%), IM6 (8%) Botanik F1, MI5 (28%) Sardes F1, (52%) Ptk40 F1, (22%) Beith Alpha. Based on embryo development to the plant structure, Botanik F1 variety was successful variety in medium combination (IM8) with the application of cold pre-treatment (4°C). By heat shock (35°C/3days) and (IM2), the best results were found in Botanik F1(20%), Ptk40 F1 (20%), Beith Alpha (16%), and Sardes F1 (13.3%) respectively compared to other induction medium combination used in this study. Owing to the female flower buds' collection, some studies have been revealed that collection time may affect the haploid induction. For instance, Demirel and Onus (2021) they took flower buds at 1 day or 6 hours before flowering. However, Pradeepkumara et al (2023) analyised effect of developmental stage on gynogenesis efficiency. Used female flower buds were collected at four different developmental stages (two days before anthesis, one day before anthesis, on the day of anthesis and one day after anthesis) during early morning hours between 08:00 to 10:00 am. And the best result obtained in one day before anthesis ovaries taken in the early morning. The female flower buds utilized in our study were collected one day prior to anthesis around 8 a.m to 9 a.m, and the best results were obtained.

Zou et al (2020), in the utilization of thirteen *Cucurbi*ta lines (nine winter squash, three pumpkins, and one winter squash/pumpkin hybrid) the effects of genotype and induction media on haploid production via unfertilized ovary culture analysis was carried out. The study resulted the highest ELS and plantlet induction rate (39.67 ELSs and 10.18 plantlets per 100 ovary slices) respectively and were harvested from 1.0 mg/L 6-BA medium in the L-13 genotype. By flow cytometry, the ploidy level of 59 regenerated plants was detected, and 18.64 % haploid, 3.40 % haploid-diploid, 32.20 % diploid, and 45.76 % tetraploid were observed.Contrarily, in the application of anther culture to induce haploid plant in watermelon (Smile and Sugar Baby) genotypes, Silva et al (2021) tested nutrient media combinations of 2,4-dichlorophenoxyacetic (2,4-D) at 0.0, 0.5, 1.0, 2.0 or 5.0 μM or with 6-benzylaminopurine at 0.0, 0.5, 1.0, 1.5, or 2.0 μM, in combination with 2.0 μM of 2,4-dichlorophenoxyacetic with cold shock pre-treatment at 4°C to collected male flower buds. The highest callus formation rate (64% and 52%) obtained 3.78 μM and 4.17 μM 2,4-D medium composition respectively. The combination of 2.0 μM of 2,4-D and 6-benzylaminopurine did not increase callus formation. This study demonstrated that callus induction was related to the cold pre-treatment of male flower buds at 4°C, the anther response was depended on to genotype.

According to many studies demonstrated that it is very important to select appropriate genotype for haploid plant induction for ovary culture technique. In the total of 131 plantlets regenerated, Botanik F1 variety produced 117 plants 57 of the germinated from 0.1 mg/L IBA and 12 plantlets were from Sardes F1 and 2 of Ptk40 F1 varieties. Plantlets of Sardes F1 were albino and Ptk40 died at early stage of their life. Randomly selected twenty of germinated plants from Botanik variety were selected for ploidy level determination by flow cytometry and 19 of them diploids and 1 tetraploid were obtained. The using of simple sequence repeats (SSR) to determine haploid plants formation as pure doubled haploid is one of the simplest and most effective methods (Deng et al., 2020). Some studies have indicated that the obtained homozygous doubled haploids plant by colchicine-induced or spontaneous doubling could be used in breeding (Bhat and Murthy, 2007; Diao et al., 2009; Amirian et al., 2020).

5. CONCLUSIONS

Haploids and double haploids (DH) are with potential in plant breeding programs to elaborate new crop cultivars and varieties. The basis of vegetable breeding is the inbred pure lines production. Due to the traditional breeding of the vegetable species which belong to the cucurbit plants, breeding studies take a very lengthy time. The generation of haploids and DHs plants is an especial appealing biotechnological method, which known as haploidization. It is very useful developing 100% pure homozygous plants from a single generation. Gynogenesis is a pivotal approach used to produce haploid and DHs as homozygous plants.

The application of the in vitro ovary culture during is a modern and advanced biotechnological approach to enhance haploid plants of economically significant crops as well as cucumber, to fulfil the preferences and demands of consumers. Cucumber (*Cucumis sativus* L.) is a diploid (2n = 14) which belongs to the *Cucurbitaceae* family. Cucumber is aforethought as a pivotal horticultural crop. Generally, cucurbits are recalcitrant crops to produce haploid plant. Numerous variables have been reported to affect ovary culture in haploid studies of cucumber genotypes, including plant donor genotype, growth regulators, low and high temperature shocks, female gametophyte developmental stages, and other environmental and culture conditions. Plant growth regulators such as auxin (2,4-D, NAA, IAA, etc.) and cytokinin (BAP, KIN, TDZ, etc.) and some of polyamines as well as putrescine (PUT) are used in ovary culture studies in cucurbits.

For the case of genotype most the cucurbit species are recalcitrant. The analysis and evaluation of the influence of variety and culture nutrient media combination on haploid production through ovary culture in cucumber was carried out. And seventeen nutrient media combinations (eight induction and nine plant regeneration) were tested on four commercial cucumber varieties. The response of varieties was feasible. Some varieties performed splendidly to the applied culture media. While some do not respond. According to combinations of culture media tested in the first experiment, 0.5 mg/L 2,4-D with 1 mg/L KIN the induction (IM2) and 0.5 mg/L NAA and 2 mg/L BAP regeneration media (RM2) combinations, from the second year's experiment the induction of 5 mg/L 2,4-D with 40 µM PUT (IM8) induction and 0.1 mg/L IAA and 1 mg/L BAP regeneration promised better results. The temperature (heat and cold shocks) pre-treatment showed the effluence on haploid embryo and callus formation from cucumber ovary culture. In terms of cucumber variety, temperature, and nutrient medium interaction the results demonstrated that Botanik F1 (88%) Sardes F1 (60%), Ptk40 (32%), Beith alpha (20%), whereas IM7 was better in Ptk40 F1 (24%), IM6 (8%) Botanik F1, MI5 (28%) Sardes F1, (52%) Ptk40 F1, (22%) Beith Alpha. Based on embryo development to the plant structure, Botanik F1 variety was successful variety in medium combination (IM8) with the application of cold pre-treatment (4°C). By heat shock (35°C) and (IM2), the successful variety was Ptk40 (20%), Botanik (20%), Beith Alpha (16%), and Sardes (13.3%) respectively compared to other induction medium combination used in this study.

According to many studies demonstrated that it is very important to select appropriate genotype for haploid plant induction for ovary culture technique. In the total of 131 plantlets regenerated, Botanik F1 variety produced 117 plants 57 of the germinated from 0.1 mg/L IBA and 12 plantlets were from Sardes F1 and 2 of Ptk40 F1 varieties. Plantlets of Sardes F1 were albino and one of Ptk40 perished at early stage of their life. Twenty of obtained plantlets from cucumber ovary culture were selected for ploidy level determination by flow cytometry and nineteen were found diploids and 1 tetraploid was observed in this study.

Molecular analyses are needed for detecting haploid plants at an early stage. The analysis of gene expression associated with gynogenesis mechanisms in cucumber should be performed. Currently, haploid and double haploids (DHLs) production in plant breeding is on the good point whereas the horticulture crop production increases day per day. This shows that the performance haploid production techniques as well as in vitro ovule/ovary culture, anther culture, pollination with irradiated pollen provide 100% homozygous cucumber lines within short time compared to the classical breeding techniques. This method may help in development, improvement, and elaboration of new cultivars with high/good quality/quantity needed to the consumers. Owing the earned information and findings of numerous studies on cucumber genotypes and varietes, the application of high and advanced biotechnological approaches, such as transcriptome/ RNA-sequencing analysis for genes related to the cucumber haploid induction over ovary culture technique, it is strongly recommended in terms of gaining more and new ideas to follow in the haploid plant production in cucurbits especially cucumber crop. The haploid inducer-mediated genome-editing system is a breakthrough technology for producing haploids and doubled haploids. Various reseachers have been stated that the use of CRISPR/Cas9 application in cucurbit plants and even if it has several bottlenecks, the targeted knock-out of CENH3 gene would allow breeders to produce haploid inducer lines which can used to obtaine embryos. According to this statement many studies about genome editing to obtain haploids plants are required

Further studies are required to optimise the embryo regeneration media composition due to the cucumber growing seasons, genotypes, and nutrient medium. More research is needed for efficient protocols in almost cucurbit haploid induction through gynogenesis for supporting and reducing the costs that have been found in using irradiated pollen methods. The genotypes independent new protocols should be designed and developed through different research by improving existed ones particularly on modification of culture media, cold, and heat shock pre-treatment's applications.

REFERENCES

Aalders , L. E., 1958. Monoploidy in cucumbers. Journal of Heredity, 49: 41-44.

Abak, K., 2004. Outlook on vegetable production in Turkey. In III Balkan Symposium on Vegetables and Potatoes 729: 25-31. https://doi.org/10.17660/ActaHortic.2007.729.1

Abd El-Maskoud, M. M., El-Gendy S. E. A., and El-Kady M. M., 2009. Genotypes and genotype×medium composition interaction effects on androgenic haploid production in cucumber (*Cucumis sativus* L.). J. Agric. Sci. Mansoura University. 34: 10305-10312

Akbaş, F. C., and Solmaz, İ., 2019. Obtention of haploid plant in *Citrullus lanatus* var. *lanatus* and *Citrullus lanatus* var. *citroides* species by anther culture method. International Journal of Environmental Research and Technology, 2: 25-36.

Amirian, R., Hojati, Z., and Azadi, P., 2020. Male flower induction significantly affects androgenesis in cucumber (*Cucumis sativus* L.). The Journal of Horticultural Science and Biotechnology, 95: 183-191. https://doi.org/10.1080/14620316.2019.1655488

Anonymous 1: https://www.haifa-group.com/cucumber-0/crop-guide-cucumber [Last accessed date: 21.08.2023]

Anonymous 2: https://www.vegetables.cornell.edu/pest-management/disease-factsheets/disease-resistant-vegetable-varieties/disease-resistant-cucurbit-varieties/ [Last accessed date: 21.08.2023]

Anonymous 3: https://www.google.com/search?q=fruit+images+of+beith+alpha&source=lmns&bih=568&biw=1366&rlz=1C1CHBF_enTR728TR728&hl=enGB&sa=X&ved=2ahUKEwj74JnNmZWBAxWK-qQKHSuPAQoQ_AUoAHoECAEQAA [Last accessed date: 06.09.2023]

Ashok Kumar, H. G., and Murthy, H. N., 2004. Effect of sugars and amino acids on androgenesis of *Cucumis sativus* L. Plant Cell, Tissue and Organ Culture, 78: 201-208. https://doi.org/10.1023/B:TICU.0000025637.56693.68

Ashok Kumar, H. G., Murthy, H. N., and Paek, K. Y., 2003. Embryogenesis and plant regeneration from anther cultures of *Cucumis sativus* L. Scientia horticulturae, 98: 213-222. https://doi.org/10.1016/S0304-4238(03)00003-7

Baktemur, G., Keleş, D., Kara, E., Yıldız, S., and Taşkın, H., 2022. Effects of genotype and nutrient medium on obtaining haploid plants through ovary culture in cucumber. Molecular Biology Reports, 49: 5451-5458. https://doi.org/10.1007/s11033-022-07238-y

Balkaya, A., Kandemir, D., and Sarıbaş, Ş., 2015. Türkiye sebze fidesi üretimindeki son gelişmeler. TÜRKTOB Türkiye Tohumcular Birliği Dergisi, 4: 4-8.

Bhat, J. G., Murthy, H. N., 2007. Factors afecting in-vitro gynogenic haploid production in niger (*Guizotiaabyssinica* (L. f.) Cass.). Plant Growth Regulator, 52: 241-248

Blakeslee, A. F., Belling, J., Farnham, M. E., and Bergner, A. D., 1922. A haploid mutant in the jimson weed, (*Datura stramonium*). Science, 55: 46-647. https://doi.org/10.1126/science.55.1433.646

Çağlar, G., and Abak, K., 1996. Gama Işınının Hıyar Polenlerinin Canlılık ve Çimlenmesi Üzerine Etkileri. PIV. Ulusal Nükleer Tarım ve Hayvancılık Kongresi, 7.

Çağlar, G., and Abak, K., 1997. Progress in the production of haploid embryos, plants and doubled haploids in cucumber (*Cucumis sativus* L.) by gamma irradiated pollen, in Turkey. In I International Symposium on Cucurbits, 492: 317-22. https://doi.org/10.17660/ActaHortic.1999.492.43

Çağlar, G., and Abak, K., 1999. In situ haploid embryo induction in cucumber (*Cucumis sativus* L.) after pollination by irradiated pollen. Turkish Journal of Agriculture and Forestry, 23: 63-72. https://journals.tubitak.gov.tr/agriculture/vol23/iss7/8

Campbell, A. W., Campbell, P. J., Griffin, W. B., Burritt, D. J., and Conner, A. J., 2001. Prediction of sample size to maintain genetic variation in doubled-haploid populations following marker selection. Theoretical and Applied Genetics, 103: 1028-36. https://doi.org/10.1007/s001220100639

Çetinkaya, E., 2015. Farklı besi ortamı kombinasyonlarının bazı hıyar (*Cucumis sativus* L.) genotiplerinde gynogenesis yolu ile embriyo ve haploid bitki oluşumu üzerine etkisi. Yüksek Lisans Tezi, Akdeniz Üniversitesi, Antalya, 61s. http://acikerisim.akdeniz.edu.tr/xmlui/handle/123456789/2693

Chambonnet, D., and De Vaulx, R. D., 1985. Obtention of embryos and plants from *in vitro* culture of unfertilized ovules of *Cucurbita pepo*. Cucurbit Genet Coop, 8: 66.

Chen, J. F., Cui, L., Malik, A. A., and Mbira, K. G., 2011. In vitro haploid and dihaploid production via unfertilized ovule culture. Plant Cell, Tissue and Organ Culture, 104: 311-319. https://doi.org/10.1007/s11240-010-9874-6

Claveria, E., Garcia-Mas, J., and Dolcet-Sanjuan, R., 2005. Optimization of cucumber doubled haploid line production using *in vitro* rescue of *in vivo* induced parthenogenic embryos. Journal of the American Society for Horticultural Science jashs, 130: 555-560. https://doi.org/10.21273/JASHS.130.4.555

Deepa, S. K., Hadimani, H. P., Hanchinamani, C. N., Shet, R., Koulgi, S., and Ashok, O., 2018. Studies on character association in cucumber (*Cucumis sativus* L.). International Journal of Current Microbiology and Applied Sciences, 7: 1977-1982. https://doi.org/10.20546/ijcmas.2018.711.224

Demirel, E., and Onus, A. N., 2021. Bazı hıyar (*Cucumis sativus* L.) çeşit ve tiplerinde gynogenesis yolu ile haploid embriyo ve bitki eldesi. Uluslararası Tarım ve Yaban Hayatı Bilimleri Dergisi, 7: 360-367. https://doi.org/10.24180/ijaws.969741

Deng, Y., Fu, W., Tang, B., Tao, L., Zhang, L., Zhou, X., Wang, Q., Li, J., and Chen, J., 2021. Transcriptome analysis of ovary culture-induced embryogenesis in cucumber (*Cucumis sativus* L.), PeerJ, 9, p.e12145; https://doi.org/10.7717/peerj.12145

Deng, Y., Tang, B., Zhou, X., Fu, W., Tao, L., Zhang, L., and Chen, J., 2020. Direct regeneration of haploid or doubled haploid plantlets in cucumber (*Cucumis sativus* L.) through ovary culture. Plant Cell, Tissue and Organ Culture (PCTOC), 142: 253-268. https://doi.org/10.1007/s11240-020-01839-w

Diao, W. P., Jia, Y.Y., Song, H., Zhang, X. Q., Lou, Q. F., and Chen, J. F., 2009. Efficient embryo induction in cucumber ovary culture and homozygous identification of the regenetants using SSR markers. Scientia horticulturae, 119: 246-251. https://doi.org/10.1016/j.scienta.2008.08.016

Domblides, E., Shmykova, N., Khimich, G., Korotseva, I., Kan, L., Domblides, A., Pivovarov, V., and Soldatenko, A., 2019. Production of doubled haploid plants of *Cucurbitaceae* family crops through unpollinated ovule culture in vitro. In VI International Symposium on Cucurbits, 1294: 19-28. https://doi.org./10.17660/ActaHortic.2020.1294.4

Domblides, E. A., Shmykova, N. A., Belov, S. N., Korottseva, I. B. and Soldatenko, A.V., 2019. DH-plant production in culture of unpollinated ovules of cucumber (*Cucumis sativus* L.). Vegetable crops of Russia, 18: 3-9. https://doi.org/10.18619/2072-9146-2019-6-3-9

Dong, Y. Q., Zhao, W. X., Li, X. H., Liu, X. C., Gao, N. N., Huang, J. H., Wang, W.Y., Xu, X. L., and Tang, Z. H., 2016. Androgenesis, gynogenesis, and parthenogenesis haploids in cucurbit species. Plant cell reports, 35: 1991-2019. https://doi.org/10.1007/s00299-016-2018-7

Dunwell, J. M., 2010. Haploids in flowering plants: origins and exploitation. Plant biotechnology journal, 8(4): 377-424. https://doi.org/10.1111/j.1467-7652.2009.00498.x

Emiroğlu, Ü., and Gürel, A., 1993. Bitki ıslahında modern biyoteknoloji. The Biotechnology Revolution Short Course, 8-12.

Erol, M. H., and Sarı, N., 2019. The effect of ovule-ovary culture and spermidine-putrescine applications on haploid embryo induction of cucumber (*Cucumis sativus* L.). Alatarim, 18: 108-117.

Faris, N. M., Nikolova, V., and Niemirowicz-Szczytt, K., 1999. The effect of gamma irradiation dose on cucumber (*Cucumis sativus* L.) haploid embryo production. Acta Physiologiae Plantarum, 21: 391-396. https://doi.org/10.1007/s11738-999-0011-2

Freytag, A. H., Rao-Arelli, A. P., Anand, S. C., Wrather, J. A. and Owens, L. D., 1989. Somaclonal variation in soybean plants regenerated from tissue culture. Plant Cell Reports, 8: 199-202.

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

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

Gałązka, J., Słomnicka, R., Góral-Radziszewska, K., and Niemirowicz-Szczytt, K., 2015. From pollination to DH lines- verification and optimization of protocol for production of doubled haploids in cucumber. Acta Scientiarum Polonorum. Hortorum Cultus, 14: 81-92.

Gémes-Juhász, A., Balogh, P., Ferenczy, A., and Kristóf, Z., 2002. Effect of optimal stage of female gametophyte and heat treatment on in vitro gynogenesis induction in cucumber (*Cucumis sativus* L.). Plant Cell Reports, 21: 105-111. https://doi.org/10.1007/s00299-002-0482-8

Gémesné, J. A., Venczel, G., and Balogh, P., 1996. Haploid plant induction in zucchini (*Cucurbita pepo* L. *convar. giromontiina* Duch) and in cucumber (*Cucumis sativus* L.) lines through in vitro gynogenesis. In III International symposium on in vitro culture and horticultural breeding 447: 623-626. https://doi.org./10.17660/ActaHortic.1997.447.124

Germana, M. A., 2011. Anther culture for haploid and doubled haploid production. Plant Cell, Tissue and Organ Culture (PCTOC), 104: 283-300. https://doi.org/10.1007/s11240-010-9852-z

Golabadi, M., Ghanbari, Y., Keighobadi, K., and Ercisli, S., 2017. Embryo and callus induction by different factors in ovary culture of cucumber. Journal of Applied Botany and Food Quality, 90: 68-75. https://doi.org./10.5073/JABFQ.2017.090.010

Gu, H. H., Zhou, W. J., and Hagberg, P., 2003. High frequency spontaneous production of doubled haploid plants in microspore cultures of *Brassica rapa* ssp. chinensis. Euphytica, 134: 239-245. https://doi.org/10.1023/B:EUPH.0000004945.01455.6d

Guan, W., Maynard, E.T., Aly, B., Zakes, J., Egel, D. S., and Ingwell, L. L., 2019. Parthenocarpic cucumber cultivar evaluation in high-tunnel production. HortTechnology, 29: 634-642. https://doi.org/10.21273/HORTTECH04370-19

Günay, A., 1970. Marmara bölgesi önemli hıyar çeşitlerinin sitolojik, biyolojik ve morfolojik hususiyetleri üzerinde araştırmalar. Ankara Üniversitesi, Ankara, 395.

Gürsoy, I., Solmaz, I., Deliboran, S. and Sari, N., 2012. In vitro ovule and ovarium culture in watermelon. In *Cucurbitaceae* 2012. Proceedings of the Xth EUCARPIA Meeting on Genetics and Breeding of Cucurbitaceae, Antalya, Turkey.

Gürsöz, N., 1990. Kavun (*Cucumis melo var. inodorus ve reticulatus*) ve karpuzda *(Citrullus lanatus* (thunb.) mansf) ışınlanmış polenle uyartılan in situ partenogenetik embriyolardan in vitro kültürü ile haploid bitki eldesi. Yüksek Lisans Tezi, Çukurova Üniversitesi, Adana, 60 s.

Hooghvorst, I., and Nogués, S., 2020. Opportunities and challenges in doubled haploids and haploid inducer-mediated genome-editing systems in cucurbits. Agronomy, 10: 1441. https://doi.org/10.3390/agronomy10091441.

Hooghvorst, I., and Nogués, S., 2021. Chromosome doubling methods in doubled haploid and haploid inducer-mediated genome-editing systems in major crops. Plant Cell Reports, 40: 255-270. https://doi.org/10.1007/s00299-020-02605-0

Hooghvorst, I., Torrico, O., Hooghvorst, S., and Nogués, S., 2020. In situ parthenogenetic doubled haploid production in melon "Piel de Sapo" for breeding purposes. Frontiers in Plant Science, 11: 378. https://doi.org/10.3389/fpls.2020.00378

Isak, M. A., Majeed, H., Yetişir, H., and Şimşek, Ö., 2022. Evaluation of factors affecting embryo-like structure and callus formation in unpollinated ovary culture of watermelon (*Citrullus lanatus*), International Journal of Agricultural and Natural Sciences, 15: 319-35.

Ishii, T., Karimi-Ashtiyani, R., and Houben, A., 2016. Haploidization via chromosome elimination: means and mechanisms. Annual review of plant biology, 67: 421-438. https://doi.org/10.1146/annurev-arplant-043014-114714

Kara, B., and Sari, N., 2019. Effects of spermidine and putrescine applications on haploid plant obtention via ovule culture in sakız and dark green squashes (*Cucurbita pepo* L.). Türkiye Tarımsal Araştırmalar Dergisi, 6: 206-13.

Kaur, H. A., Sharma, S. P., Kaur, N., and Kalia, A., 2017. Gynogenic response of muskmelon genotypes with temperature and MS medium supplementations. In International Symposium on Horticulture: Priorities and Emerging Trends 1255: 13-18.

Koli, S. P., and Murthy, H. N., 2013. Haploid plant regeneration from unpollinated ovules of *Cucumis melo* L. var. *conomon* cv. Mudicode. British Biotechnology Journal, 3(4): 605-613.

Kumari, M., Clarke, H. J., Small, I et al., 2009. Albinism in plants: a major bottleneck in wide hybridization, androgenesis and doubled haploid culture. Crit Rev Plant Sci 28: 393-409. https://doi.org/10.1080/07352680903133252

Kurtar, E.S., Balkaya, A., and Ozbakir, M., 2018. Production of callus mediated gynogenic haploids in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.). Czech J. Genet. Plant Breed, 54: 9-16.

Kurtar, E. S., 1999. Kabakta (*Cucurbita pepo* L.) haploid embriyo uyartımı ve bitki oluşturma üzerinde araştırmalar. Doktora Tezi, Çukurova Üniversitesi, Adana, 202 s.

Kurtar, E. S., and Balkaya, A., 2010. Production of in vitro haploid plants from in situ induced haploid embryos in winter squash (*Cucurbita maxima* Duchesne ex Lam.) via irradiated pollen. Plant Cell, Tissue and Organ Culture (PCTOC), 102: 267-277. https://doi.org/10.1007/s11240-010-9729-1

Kurtar, E. S., Balkaya, A., and Kandemir, D., 2016. Evaluation of haploidization efficiency in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) through anther culture. Plant Cell, Tissue and Organ Culture (PCTOC), 127: 497-511. https://doi.org/10.1007/s11240-016-1074-6

Kurtar, E. S., Sarı, N., and Abak, K., 2002. Obtention of haploid embryos and plants through irradiated pollen technique in squash (*Cucurbita pepo* L.). Euphytica, 127: 335-344. https://doi.org/10.1023/A:1020343900419

Li, J. W., Si, S. W., Cheng, J. Y., Li, J. X., and Liu, J. Q., 2013. Thidiazuron and silver nitrate enhanced gynogenesis of unfertilized ovule cultures of *Cucumis sativus* L. Biologia plantarum, 57: 164-168. https://doi.org/10.1007/s10535-012-0269-x.

Li, J. X., Ge, G. M., Pang, S. M., Fang, G. N., Wu, X. B. and Zhou, H. X., 2012. Study on in vitro Culture and Plantlet Regeneration from Unpollinated Ovary of *Cucumis sativus* L. J. Northern Horticulture, 23: 047.

Lotfi, M., and Salehi, S., 2008. Detection of cucumber parthenogenic haploid embryos by floating of immature seeds in liquid medium. In: Pitrat M. (eds), *Cucurbitaceae* 2008, Proceedings of the IXth EUCARPIA meeting on genetics and breeding of *Cucurbitaceae*, INRA, Avignon, France.

Lotfi, M., Kashi, A., and Onsinejad, R., 1997, May. Induction of parthenogenetic embryos by irradiated pollen in cucumber. In I International Symposium on Cucurbits 492: 323-328. https://doi.org./10.17660/ActaHortic.1999.492.44

Lower, R. L., and Edwards, M. D., 1986. Cucumber breeding, Breeding vegetable crops. In: (Basset M. J, ed.). Breeding vegetables crops, 173-203.

Lower, R. L., Edwards, M. D., and Basset, M. E., 1986. Breeding vegetable crops. Vegetable crops department, University of Florida, Gainesville, Florida.

Malik, A. A., Li, C., Shuxia, Z., and Jin-feng, C., 2011. Efficiency of SSR markers for determining the origin of melon plantlets derived through unfertilized ovary culture. Horticultural Science, 38: 27-34. https://doi.org/10.17221/47/2010-HORTSCI

Mibus, H., and Tatlioglu, T., 2004. Molecular characterization and isolation of the F/f gene for femaleness in cucumber (*Cucumis sativus* L.). Theoretical and Applied Genetics, 109: 1669-1676. https://doi.org/10.1007/s00122-004-1793-7

Moqbeli, E., Peyvast, G. H., Hamidoghli, Y., and Olfati, J. A., 2013. In vitro cucumber haploid line generation in several new cultivars. AsPac J Mol Biol Biotechnol, 21: 18-25.

Nguyen, M. L., and Huyen, T. N. B. T., 2023. Effects of culture conditions on isolated microspore culture of melon (*Cucumis melo* L.). Vietnam Journal of Science, Technology and Engineering, 65: 30-36. https://doi.org/10.31276/VJSTE.

Nguyen, M. L., Ta, T. H. T., Huyen, T. N. B. T., and 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, 54: 140-148.

Nguyen, T. T. V., and Chen, J., 2012. Cucumber (*Cucumis sativus* L.) anther culture. China Cucurbits and Vegetables, 25: 1-5.

Niemirowicz-Szczytt, K., and Dumas de Vaulx, R., 1989. Preliminary data on haploid cucumber (*Cucumis sativus* L.) induction. Cucurbit Genet Coop, 12: 24-25.

Nitwatthanakul, N., and Tiraumphon, A., 2018. Effect of induction media on callus formation in un-pollinated ovule culture of three melon cultivars. In XXX International Horticultural Congress IHC2018: II International Symposium on Micropropagation and In Vitro Techniques 1285: 103-08.

Nyirahabimana, F., and Solmaz, I., 2022. Haploid Plant Production Technology in Cucumber (*Cucumis Sativus* L.). In Tutuncu M. (eds) Proceeding Book: In 10th International Molecular Biology and Biotechnology Congress-Molbiotech Online, 28-37.

Ozsan, T., Gozen, V., and Onus, N. A., 2017. Cucumber gynogenesis: effects of 8 different media on embryo and plant formation. International Journal of Agriculture Innovations and Research, 6: 419-422.

Plapung, P., Khumsukdee, S., and Smitamana, P., 2014. Development of cucumber lines resistant to cucumber mosaic virus by ovule culture. Journal of Agricultural Technology, 10: 733-741.

Pochard, E., and Dumas de Vaulx, R., 1971. La monoploidie chez le piment (*Capsicum annuum* L.). Z Pflanzenzüchtg, 65: 23-46.

Pradeepkumara, N., Dey, S. S., Munshi, A. D., Behera, T. K., Bhatia, R., Kumari, K., Prakash, P., Bhattacharya, R. C., and Talukdar, A., 2023. Cucumber F1 hybrid derived from two contrasting inbreds ensures high frequency gynogenesis for induction of haploids through a modified in vitro based protocol. South African Journal of Botany, 157: 314-324. https://doi.org/10.1016/j.sajb.2023.03.066

Przyborowski, J. and Nlemirowicz-Szgzytt, K., 1994. Main factors affecting cucumber (*Cucumis sativus* L.) haploid embryo development and haploid plant characteristics. Plant breeding, 112: 70-75. https://doi.org/10.1111/j.1439-0523.1994.tb01278.x

Ren, J., Wu, P., Trampe, B., Tian, X., Lübberstedt, T., and Chen, S., 2017. Novel technologies in doubled haploid line development. Plant biotechnology journal, 15: 1361-1370. https://doi.org/10.1111/pbi.12805

Robinson, R.W., 1999. Cucurbits., CAB International, New York, USA, 221.

Rolnik, A., and Olas, B., 2020. Vegetables from the *Cucurbitaceae* family and their products: Positive effect on human health. Nutrition, 78: 110788. https://doi.org/10.1016/j.nut.2020.110788

Salehi, B., Quispe, C., Sharifi-Rad, J., Giri, L., Suyal, R., Jugran, A.K., Zucca, P., Rescigno, A., Peddio, S., Bobiş, O., and Moise, A.R., 2021. Antioxidant potential of family Cucurbitaceae with special emphasis on *Cucurbita* genus: a key to alleviate oxidative stress-mediated disorders. Phytotherapy Research, 35: 3533-3557. https://doi.org/10.1002/ptr.7045

Salehian, H., Shahnazi, S. and Nazari, M., 2023. Production of doubled haploid plants in cucumber (*Cucumis sativus* L.) via parthenogenesis. In Vitro Cellular & Developmental Biology-Plant, 1-8. https://doi.org/10.1007/s11627-023-10368-y

San Noeum L. H, Ahmadi N., 1982. Variability of doubled haploids from in vitro androgenesis and gynogenesis in *Hordeum vulgare* L. In: Earle ED, Demarly Y (eds) Variability in plants regenerated from tissue culture. Praeger, New York, 273-283

Sari, N., 1994. Karpuzlarda ışınlanmış polen uyartımıyla haploid bitki eldesi üzerine genotipin ve mevsimin etkisi ile ışınlama yerine geçebilecek uygulamalar üzerinde araştırmalar. Doktora Tezi, Çukurova Üniversitesi, Adana, 244 s.

Sari, N., Solmaz, I., 2021. Doubled Haploid Production in Watermelon. In: Segui-Simarro, J. M. (eds) Doubled Haploid Technology. Methods in Molecular Biology, vol 2289. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1331-3_6

Sauton, A., 1989. Haploid gynogenesis in *Cucumis sativus* L. induced by irradiated pollen. Cucurbit Genetics Coop, 12: 22-23.

Sauton, A., and Dumas de Vaulx, R., 1987. Production of Haploid Plants in Melon (*Cucumis melo* L.) as a result of Gynogenesis Induced by Irradiated Pollen. Agronomie, 7: 141-147.

Savin, F., Decomble, V., Le Couvioir, M., and Hallard, J., 1988. The X ray detection of haploid embryos arisen in muskmelon (*Cucumis melo* L.) seeds and resulting from a parthenogenetic development induced by irradiated pollen. Cucurbit Genetic Cooperative, 11: 39-42.

Silva, C. M. D. J., Dias, R. D. C. S., and Melo, N. F. D., 2021. The effect of temperature and growth regulators on callus induction in watermelon anthers. Brazilian Archives of Biology and Technology, 64. https://doi.org/10.1590/1678-4324-2021180505

Smiech, M., Sztangret-Wisniewska, J., Galecka, T., Korzeniewska, A., Marzec, L., Kolakowska, G., and Niemirowicz-Szczytt, K., 2008. Potential use of RAPD markers in characteristics of cucumber (*Cucumis sativus* L.) Haploids and Double-Haploids. Acta Societatis Botanicorum Poloniae, 77: 29-34.

Snape, J.W., 1989. Doubled haploid breeding: Theoretical basis and practical applications. Review of advances in plant biotechnology, 2: 19-31.

Song, H., Lou, Q. F., Luo, X. D., Wolukau, J. N., Diao, W. P., Qian, C.T., and Chen, J. F., 2007. Regeneration of doubled haploid plants by androgenesis of cucumber (*Cucumis sativus* L.). Plant cell, tissue and organ culture, 90: 245-254. https://doi.org/10.1007/s11240-007-9263-y

Sorntip, A., Poolsawat, O., Kativat, C., and Tantasawat, P. A., 2017. Gynogenesis and doubled haploid production from unpollinated ovary culture of cucumber (*Cucumis sativus* L.). Canadian journal of plant science, 98: 353-361. https://doi.org/10.1139/cjps-2017-0112

Suprunova, T., and Shmykova, N., 2008. In vitro induction of haploid plants in unpollinated ovules, anther and microspore culture of *Cucumis sativus* L. In *Cucurbitaceae* 2008. Proceedings of the IXth EUCARPIA meeting on genetics and breeding of cucurbitaceae, Avignon, France, 371-374.

Sun, S. R., Zhai, Q. H., Hu J. B., Chen, J. F., and Zhang, P., 2009. Effects of several physiological factors on embryo formation in unpollinated ovary culture of pumpkin (*Cucurbita moschata* Duch. ex Poiret). Plant Physiology Journal, 45: 977–980.

Swaminathan, M. S., Singh, M. P., 1958. X-ray induced somatic haploidy in watermelon. Curr. Sci., India 27: 63-64

Tan, J., Lin, L., Luo, H., Zhou, S., Zhu, Y., Wang, X., Miao, L., Wang, H., and Zhang, P., 2022. Recent progress in the regeneration and genetic transformation system of cucumber. Applied Sciences, 12: 7180. https://doi.org/10.3390/app12147180

Tantasawat, P.A., Sorntip, A., Poolsawat, O., Chaowiset, W. and Pornbungkerd, P., 2015. Evaluation of factors affecting embryo-like structure and callus formation in unpollinated ovary culture of cucumber (*Cucumis sativus* L.). International Journal of Agriculture and Biology, 17: 613-18. https://doi.org./10.17957/IJAB.17.3.14.257

Taşkın, H., Kemal Yücel, N., Baktemur, G., Çömlekçioğlu, S., and Büyükalaca, S., 2013. Effects of different genotypes and gamma ray doses on haploidization with irradiated pollen technique in watermelon (*Citrullus lanatus* L.). Canadian journal of plant science, 93: 1165-1168. https://doi.org/10.4141/cjps2013-059.

Tatlioglu, T., 1993. Cucumber: *Cucumis sativus* L. Genetic improvement of vegetable crops, Pergamon, 197-234. https://doi.org/10.1016/B978-0-08-040826-2.50017-5

Truong-Andre, I., 1988. *In vitro* Haploid plants derived from pollination by irradiated pollen on cucumber. In Eucarpia Meeting on Cucurbit Genetics and Breeding, Montfavet, France, INRA.

Ugwu, C., and Suru, S., 2021. Cosmetic, culinary and therapeutic uses of cucumber (*Cucumis sativus* L.). Wang H.(eds). Cucumber Economic Values and Its Cultivation and Breeding, IntechOpen: Rijeka, Croatia, 39.

Vasudevan, A., Selvaraj, N., Ganapathi, A., Kasthurirengan, S., Ramesh Anbazhagan, V., Manickavasagam, M., and Choi, C. W., 2008. Leucine and spermidine enhance shoot differentiation in cucumber (*Cucumis sativus* L.). In Vitro Cellular & Developmental Biology-Plant, 44: 300-306. https://doi.org/10.1007/s11627-008-9135-0

Veilleux, R. E., 1994. Development of new cultivars via anther culture. HortScience, 29: 1238-1240.

Wei, A., Du, S., Han, Y., Liu, N., and Zhang, G., 2010. A study on the relationship between cucumber gynogenesis and content of ovary hormones and polyamines. Acta horticulturae, 871: 625. https://doi.org./10.17660/ActaHortic.2010.871.86

Xia, Z., Lu, Z., Junguo, Z., Ying, D. E., Yi, W. A., Qunfeng, L. O., and Jinfeng, C. H., 2020. Embryonary sac regenerated plants were obtained in cucumber via *in vitro* unpollinated ovary culture. Acta Horticulturae Sinica, 47: 455. https://doi.org./10.16420/j.issn.0513-353x.2019-0393

Xu, Z., and Chang, L., 2017. *Cucurbitaceae*. In: Identification and Control of Common Weeds: Volume 3. Springer, Singapore. https://doi.org/10.1007/978-981-10-5403-7_18

Xue, G. R., Yu, W. Y., Fei, K. W., Cui, H. N., and Sun, R. X., 1983. Watermelon plants derived by in vitro anther culture. Plant Physiol Commun, 4: 40-42.

Yang, H.Y., and Zhou, C., 1990. *In vitro* gynogenesis. In Developments in crop science , Elsevier, 19: 242-258. https://doi.org/10.1016/B978-0-444-88883-9.50014-5

Yi, G., Lee, H. S., and Kim, K. M., 2015. Improved marker-assisted selection efficiency of multi-resistance in doubled haploid rice plants. Euphytica, 203: 421-428. https://doi.org/10.1007/s10681-014-1303-1

Zhan, Y., Chen, J., and Malik, A. A., 2009. Embryoid induction and plant regeneration of cucumber (*Cucumis sativus* L.) through microspore culture. Acta Horticulturae Sinica, 36: 221-226.

Zheng, Y., Wu, S., Bai, Y., Sun, H., Jiao, C., Guo, S., Zhao, K., Blanca, J., Zhang, Z., Huang, S., and Xu, Y., 2019. Cucurbit Genomics Database (CuGenDB): a central portal for comparative and functional genomics of cucurbit crops. Nucleic acids research, 47: D1128-D1136. https://doi.org/10.1093/nar/gky944

Zou, T., Su, H.N., Wu, Q., and Sun, X. W., 2018. Haploid induction via unfertilized ovary culture in watermelon. Plant Cell, Tissue and Organ Culture (PCTOC), 135: 179-187. https://doi.org/10.1007/s11240-018-1454-1

Zou, T., Song, H., Chu, X., Tong, L., Liang, S., Gong, S., Yang, H., and Sun, X., 2020. Efficient induction of gynogenesis through unfertilized ovary culture with winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.). Scientia Horticulturae, 264: 109152. https://doi.org/10.1016/j.scienta.2019.109152.

Żur, I., Gajecka, M., Dubas, E., Krzewska, M. and Szarejko, I., 2021. Albino plant formation in androgenic cultures: An old problem and new facts. Doubled Haploid Technology : Hot Topics, Apiaceae, Brassicaceae, Solanaceae, 2: 3-23.

CURRICULUM VITAE

Fildaus NYIRAHABIMANA, completed her primary and secondary education in Rwanda. In 2013, I was awarded a Bachelor of Science Degree in Applied Chemistry-Biochemistry Option by the University of Rwanda-College of Science and Technology (former Kigali Institute of Science and Technology, KIST). In 2014, I won Turkish scholarships for a Masters of Science Degree in the Biotechnology Depertment under the Presidency for Turks Abroad and Related Communities (YTB). And again in 2019, the Turkish government awarded me a Doctorate program scholarship through YTB in the Biotechnology Department of the Institute of Natural and Applied Sciences at Cukurova University.