T.R.
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE
DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

HAPLOID INDUCTION VIA UNFERTILIZED OVARY CULTURE IN CUCUMBER

Prepared By

Huma MAJEED

Supervisor

Assoc. Prof. Dr. Özhan ŞİMŞEK

Master's Thesis

September 2022

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COMPLIANCE WITH SCIENTIFIC ETHICS

I declare that all the information in this study was obtained following academic and ethical rules. I also state that I have fully cited and referenced all materials and results that are not inherent in this study, as these rules and behavior require.

Huma MAJEED

The MSc thesis entitled **“Haploid Induction Via Unfertilized Ovary Culture In Cucumber”** has been prepared in accordance with Erciyes University graduate school of natural and applied science thesis preparation and writing guide.

Prepared
Huma MAJEED

Supervisor
Assoc. Prof. Dr. Özhan ŞİMŞEK

Head of Department

Prof. Dr. Mehmet ARSLAN

Department of Agricultural Biotechnology

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HIYARDA DÖRELENMEMİŞ OVARYUM KÜLTÜRÜ İLE HAPLOİD UYARTIMI

Huma MAJEED

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ÖZET

Hıyar (*Cucumis sativus*), Cucurbitaceae familyasında yer alan, çoğunlukla sebze olarak kullanılan silindirik meyveye sahip, bir bitkidir. Meyve veya sebzelerde herhangi bir ıslah yönteminde, yetiştiricilerin temel amacı homozigotluğu elde edilmektedir. Geleneksel yöntemler kullanılarak, kendileme ile bitkilerde homozygotlaştirma işleme uzun yıllar sürmektedir. Ancak haploid bitkilerini elde edilmesi ile bu homozygotlaştirma isleme cok daha kisa sürelerde sağlanmaktadır. Herhangi bir türün haploid bitkileri, normal kromozom sayısının yarısına sahiptir. Bu tez çalışmasında Hıyarda haploid bitki elde etmek olanaklarının araştırılması için ovaryum kültürü kullanılmıştır. Bu çalışmada, gynogenesis (ovaryum kültürü) ile bazı hıyar genotiplerinde ('Kros' ve 'Silah') farklı ortam, çiçek aşamaları ve sıcaklık kombinasyonunun embriyo oluşumu ve haploid bitki elde etme üzerin etkileri araştırılmıştır. Kallus oluşumu için altı farklı bitki büyüme düzenleyicisi ve embriyo oluşumu için dört farklı bitki büyüme düzenleyicisi kullanılmıştır. Elde edilen bulgulara göre temel ortamının önemli etkilerini olduğu gözlenmiştir. Kallus oluşumunda en etkili bitki büyüme düzenleyicisi 0.12 mg/L TDZ ve 0.06 mg/L TDZ olarak belirlenmiştir. Embriyo benzer yapıların oluşumu için 1 mg/L NAA ve 3 mg/L BAP ortamının etkili olduğu sonucuna varılmıştır..

Anahtar Kelimeler: Hıyar, Ovaryum Kültür, Haploid, Kallus, Embriyo, Doku kültürü

HAPLOID INDUCTION VIA UNFERTILIZED OVARY CULTURE IN CUCUMBER

Huma MAJEED

Erciyes University, Graduate School of Natural and Applied Sciences
Master Thesis, September 2022
Supervisor: Assoc. Prof. Dr. Özhan ŞİMŞEK

ABSTRACT

Cucumber *(Cucumis sativus)* is a creeping vine plant in the family Cucurbitaceae, with cylindrical fruit mostly used as a vegetable. In any breeding method for fruit or vegetables, the main goal of breeders is to achieve homozygosity to develop new varieties. By using traditional methods of breeding, it normally takes many years in crossing and self-pollination. But it is possible to obtain homozygosity in shorter time with the use of technique like double haploid production. Haploid plants of any species have half the normal number of chromosomes as the original plant does. The ovary culture method has been used to obtain embryos and haploid plants in cucumber. Production of haploids by *in vitro* techniques allows breeders to release new lines more quickly and screen for resistance (diseases) more efficiently. In this work, the effect of the combination of different Plant Growth Regulators (PGRs), flower stages, and temperature in some cucumbers genotype ('Kros' and 'Silah') by gynogenesis on embryo formation and haploid plants were investigated. In this research, six combinations of different PGRs for callus induction and four combinations of different PGRs for embryo maturation was utilized. Effect of PGRs, genotype, flower stage and temperature were examined carefully. According to the findings the importance of medium became clear, the conclusion has reached that 12 mg/L Thidizuron (TDZ) and 0.06mg/L TDZ are most effective in callus induction and 1mg/L NAA and 3 mg/L 6-Benzylaminopurine (BAP) is most effective maturation medium.

Keywords: Cucumber, Ovary culture, Haploid, Callus, Embryo, Tissue Culture

CONTENT

HAPLOID INDUCTION VIA UNFERTILIZED OVARY CULTURE IN CUCUMBER

ABBREVIATIONS

2,4-D :2,4-Dichlorophenoxyacetic Acid

2ip : N6-(2-Isopentenyl) Adenine

60Co : Cobalt 60 Radioactive Beam Source

ABA : Abscisic acid

$AgNO_3$: Silver Nitrate

B5 : Gamborg Nutrient Media

BAP : Benzyl Amino Purine

BBD : Plant Growth Regulator

CAD : Cadaverine

CBM : Cucumber Basic nutrition Medium

CMV : Cucumber Mosaic Virus

CRISPR-Cas9 : A Genome Editing Tool

DH : Double Haploid

DM : plant Mildew Disease

DNA : Deoxyribose Nucleic Acid

D-pollen : Died pollen

E6 : Rescue et al. Nutrient medium

ELS : Embryo-Like Structure

EP : Chée and Cantliffe Nutrient Medium

F1 : 1. Generation of offspring

FAO : United Nations Food and Agriculture Organization

FCA : Flowering Control Gene

GA3 : Gibberellic Acid

IAA : Indole Acetic Acid

IMC : Domblides et al. Nutrient Medium

Kin : Kinetin

Kgy : Kilo Gray

LD : Long Day Conditions

MS : Murashige and Skoog Nutrient Media

N6 : Bing et al. nutrient medium

NAA : Naphthalene Acetic Acid

NaClO : Sodium Hypochlorite

NLN : Brassica napus Liquid Nutrient Medium

PM : Powdery Mildew Plant Disease

P-pollen : Pollen in Pre-Mitotic Division

Put : Putresin

QTL : Quantitative Trait Locus

RAPD Marker : Randomly Amplified Polymorphic DNA Marker

Scap : Plant Scabies

SD : Short Day Conditions

Spd : Spermidin

Spm : Spermin

SSR Marker : Simple Sequence Repetitive marker

STS : Silver Thiosulfate

TDZ : Thidiazuron

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INTRODUCTION

The increase in the world population brings along some problems. Radioactive and chemical wastes caused by technological activities carried out by developed and developing countries, unconscious use of natural resources, soil and water pollution caused by industrial and agricultural wastes, disposal of inorganic domestic wastes to nature, and destruction of agricultural lands are some of them. These events, which cause the deterioration of the natural ecosystem, cause the deterioration of the health standard of all living things on the earth and the extinction of some species. Water contamination with environmental pollution, pesticides, heavy metals, chemical, and radioactive materials cause the formation of free radicals in the human body (Kasnak and Palamutoğlu, 2015). Free radicals damage nucleic acids in the structure of DNA, lipids, and proteins in the cell membrane and cell structure, causing many diseases such as cancer, diabetes, coronary diseases, cataracts, and liver disorders (Velioğlu, 2000). To eliminate the negative effects of free radicals, natural food sources with antioxidants should be consumed (Kasnak and Palamutoğlu, 2015).

Human beings have included plant-based products in their lives since the birth of humanity and have developed these products to meet their basic needs such as food, shelter, and clothing. They searched for the formula of taking herbal products in their daily life and discovered that plants in wild form could be grown in different regions and that the yield and quality obtained from the plant increased when cultural processes such as irrigation and pruning were applied (Vavilov et al., 1992). This discovery caused the population, which will increase in the following years, to encounter less hunger problems. These plants, which we call cultivated plants, are grown with a special interest in the commercial scale and have superior characteristics compared to wild ones. However, not every plant that is consumed, due to various needs is a cultivated plant. Plants in wild forms can also be bred if necessary. Many of the cultivated plants have spread across the globe through migrations, wars, trade, and similar means (Karaağaç and Balkaya, 2017).

Today, the majority of vegetables with high antioxidant content are cultured varieties (Sarıkamış, 2016). The cultivars can be indigenous or new varieties developed by breeding methods. Plant breeding studies are carried out by many institutions and organizations in order to improve the characteristics of local or existing commercial varieties. Especially in recent years, the fact that hybrid varieties have superior qualities compared to other varieties has accelerated the studies in this field and new technologies have been developed in this regard. The main reason for such emphasis on hybrid varieties is the view that yield and quality that can respond to the demand of the world population can be achieved (Demir, 1990). However, the development of these varieties requires significant investment and time. The fact is that they reclaim lines as candidates for variety in a short time which can also reduce investment costs. This has also become possible with improved techniques such as haploid plant technology.

In vegetables such as cucumbers, tomatoes, peppers, eggplants, melons, and zucchini, which are the most consumed in the world, production with hybrid seeds is preferred. The obvious reasons for this are yield, simultaneous harvesting, controlled plant growth, and other quality factors. The hybrid seeds of these vegetables, which have a large share in production in our country, are also produced by Turkish companies, especially in recent years. In particular, the execution of state-supported projects by these companies has significantly reduced dependence on foreign seeds. When considered from other aspects, this developing seed sector creates employment in the agriculture field, increases the added value, causes foreign exchange savings, and contributes greatly to the country's economy by providing seed exports and foreign exchange income (Ayanoğlu and Yalvaç, 2002).

Cucumber (*Cucumis sativus* L.*)*, which is one of the most common vegetables in the world, is a very important plant in terms of human health and nutrition. 100 g of cucumber fruit contains only 12 calories. Although the low-calorie content causes it to be preferred in diet programs, its alkaline structure removes the acidity caused by eating meat and dairy products in humans. It is good for heart and vascular diseases. This fruit, which has a water content of 96%, contains 0.6 g of protein, 0.1 g of fat, and 2.2 g of carbohydrates. Although it is low in protein, fat, and carbohydrate content, Cucumber is also very rich in vitamins, enzymes, and

minerals. 100 g of fruit contains 45 mg vitamin A, 0.03 mg vitamin B1, 0.02 mg vitamin B2, 0.3 mg Niacin, 12 mg vitamin C, 12 mg calcium, 0.3 mg iron, 15 mg magnesium and 24 mg phosphorus (Sevgican, 1989).

The Cucurbitaceae is a large family consisting of approximately 118 genera and 825 species that can adapt to many regions (Skalova et al., 2010). The most commercially cultivated species of this family are cucumbers, watermelons, melons, and zucchini. The cucumber plant, which is used for human consumption, the cosmetic and pharmaceutical industry, and the canning industry, especially in pickle making, is one of the most popular species of this family. Cucumber production is carried out in 3,541,521 ha area in the world. The total amount of cucumber production in 2020 is 164 million tons. The country that produces the most cucumbers in the world is China with approximately 72 million tons. Türkiye is the second country that produces the most cucumbers with 1.92 million tons. While Russia ranks 3rd in this ranking followed by Iran and Mexico in terms of production amount in 2020 (FAO, 2020). When evaluated in terms of production amount in Türkiye, cucumber ranks third after tomatoes and watermelons among the most produced products (Tüik, 2019). In addition, the top three cities with the most production are Antalya, Mersin, and Izmir, respectively.

The cucumber is thought to have been discovered by scientists about 5,000 years ago in India, one of its primary gene centers located in Southeast Asia. The center of origin, India, is thought to have spread from the Himalayan mountains and the northern part of the Bay of Bengal to the west of Asia, China, North Africa, and Southern Europe. Although it is thought that mass migrations were effective in this spread (Günay, 1993), it was seen in the archaeological findings found in historical excavations that it was cultivated in India around 3000 BC and in Egypt in 2000 BC. Its passage to Europe took place in 600 BC by spreading from Anatolia to Greece. It is known that cucumbers were distributed to America by Europeans (Vural et al., 2000, Shoemaker, 1953; Thompson and Kelly, 1957).

The genus *Cucumis* L., which belongs to the family Cucurbitaceae (Pumpkins), has approximately 30 species that are grown in warm regions. In Türkiye, only two species are commercially cultivated, *Cucumis melo* L. (melon) and *Cucumis sativus* L. (cucumber) (Engin, 1991). The number of chromosomes is known as 2n=14 (Şeniz et al., 1995).

The scientific classification of the cucumber plant is as follows;

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Cucurbitales

Family: Cucurbitaceae

Genus: Cucumis

Species: sativus

Binomial name : *Cucumis sativus*

From an anatomic point of view, the root structure of the plant is the main root stake, the side roots have the appearance of a fringe root. It has an herbaceous body with a creeping and climbing character and performs the climb employing leeches located on the trunk and side arms. Its flowers are usually monoecious. Hermaphrodite and gynoecious flowering varieties are also encountered. However, commercially grown varieties are mostly developed only from female flower-giving lines. These plants bear parthenocarpic fruit. There is no need for pollination in commercial cultivation. If the plant is under stress, the plants in this structure may also begin to give male flowers. Preference for these varieties is the idea that parthenocarpic fruit structure facilitates consumption. However, if plants with female flower tendencies are to be included in the breeding, silver nitrate or GA3 (Gibberellic acid) is applied to the plants and male flowers are encouraged to form. The male and female flowers are yellow in color and have 5 sepals and 5 petals (Aybak and Carefree, 2004).

When evaluated in terms of fruit, many different types of cucumbers are grown. Varieties contain different genetic structures according to fruit color, fruit shape, fruit length, number of fruits in a knuckle, fruit thorn, the way the fruit stalk is attached to the fruit, fruit crispness, and the taste and aroma status. In terms of length, cucumbers are classified in three different

ways: short (maximum 15 cm), medium (16-25 cm), and long (25 cm and above). When the types are evaluated in terms of the number of fruits formed in a node, single (1 fruit), semi-multi (2-3 fruits) or multi (more than 3 fruits) varieties are available. According to the way the fruit can be evaluated, table sliceable, table cocktail, and pickled cucumber varieties are available (Demirel et al., 2016).

Health and nutrition, differences in consumer preferences, meeting market demands, criteria that will provide both economic and practicality in cultivation for the producer, suitability for transportation, etc. are important elements in terms of determining the main goals in the path to be followed by today's breeders. The obstacles encountered in the path to the development of a good variety of vegetables or fruits are all important clues. In the improvement of cucumber varieties, breeding programs are planned based on these obstacles. In cocktail-type cucumber (short) production, especially multi and semi-multi varieties are preferred. In line with consumer preferences and market demands, varieties with a frequent, neckless-round fruit structure and undesirable streaking at the fruit tip are being developed in the breeding of the prickly cucumber variety. Table sliceable, Beith-Alpha-type cucumbers are preferred for semi-multi-varieties with a dark color, and neckless fruit without a bitter feeling. In addition, it is absolutely desirable to have disease resistance such as powdery mildew (PM), cucumber mosaic virus (CMV), and zucchini mosaic virus (ZYMV) in this type of cucumbers. While long-fruit stemmed, frequent spiny, light green, short-type morphological features are required in cucumber types produced as pickles, scabies disease (Scap), powdery mildew (PM), and mildew (DM) resistance is required. Apart from the desired characteristics for the types, the market to be addressed by the developed variety is also a determining factor in determining the breeding targets. For example; In the domestic market, table cucumbers with medium fruit structure, called Beith-Alpha type, are more preferred, while in Asian countries, cucumber varieties with long type fruit structure are preferred. The desire of the Russian market is in favor of prickly cucumber varieties. European countries are more demanding varieties with a fruit peel color of dark green. It is possible to respond to all these different requests and demands with F1 hybrid breeding studies (Demirel et al., 2016).

Hybrid seed breeding is based on the hybridization of two individuals who have become homozygotes by themselves, resulting in individuals with superior characteristics to the parents. Hybrid seed technology is seen as the greatest success in plant genetic studies, especially because of its contribution to plant yield. Hybrid seed breeding studies are carried out together with classical breeding methods and modern breeding methods. Breeding work carried out using only classical breeding methods can take many years. The development of modern breeding techniques allows the newly developed variety to meet the market quickly, but also provides benefits in reducing the costs of breeding companies. But modern breeding methods alone are not enough in plant breeding work. Phenotypic observations made while applying classical breeding techniques are accepted as an important tool in distinguishing the characteristics of the lines and therefore in the easy execution of selection studies (Demir, 1990).

Modern breeding methods are carried out and supported by technology. These studies on plants are called the science of plant biotechnology. Plant biotechnology makes a great contribution to genetics and plant breeding with techniques such as DNA markers, polymerase chain reaction, somatic hybridizations, gene transfers, gene cloning, CRISPR-Cas9 gene editing, callus culture, suspension culture, meristem and shoot tip culture, embryo culture, haploid plant production (Demir et al., 2010; Açar and Kaçar, 2021). In particular, haploid plant production and the acquisition of double haploid (DH) plants allow the varieties that are tried to be homozygotes by self-pollination to be obtained in a shorter time. For this reason, haploid embryo and plant acquisition studies are among the topics that have been emphasized a lot in the fields of horticultural and field crops in recent years. Appropriate protocols developed in different plants help commercial companies working in this field to develop commercial varieties by providing convenience.

As a result of meiosis division (2n=2x), the acquisition of plants with monoploid cell structure by applying different techniques *in vitro* environment from the apex cells in a haploid structure consisting of diploid sex mother cells is called haploid plant technology. Plants formed in this way are also called haploid plants. These plants have n chromosomes in their somatic tissues. For sex mother cells to form gamete cells, they must have a number

of chromosomes in multiples of 2n and 2n, that is, homologous sets of chromosomes. However, since homologous chromosomes are not present in the sex parent cells, homologous chromosome pairing does not occur in the metaphase I phase of meiosis division. Pollen or egg cells are not formed. Therefore, these plants are infertile. In order to use these plants with a single set of genes as a scientific tool in the field of genetics, it is necessary to convert them to the 2n structure. Doubling the number of chromosomes is possible by copying the single existing set of genes (Germana, 2011).

Today, plants with haploid plant structures can be converted into double haploid (2n=2x) plants by means of some chemicals applied to plant somatic tissues. Somatic tissues with n chromosomes undergoing mitosis division double the presence of DNA by replicating during division. In the Prophase I stage, these chemicals prevent the formation of spindle threads, prevent the chromosomes lined up in the equatorial plane from being divided into two different cells in Anaphase I in the later stages, and enable the structure containing n chromosomes initially in a single cell to turn into 2n numbers. These new cells, which were formed as a result of copying the same DNA, have a homozygous structure. Therefore, pure lines of the homozygous structure are formed. Pure lines allow the homozygotic process, which takes years with classical breeding methods, to be carried out in a short time. It also increases the frequency with which recessive characters appear in individuals (Rao and Suprasanna, 1996). The advantages of double haploid plants to the science of genetics have been reported by Bal and Pal (2020) and Abak (1993) as following;

1. Haploid plants cause increased genetic variation. Since the recessive characters in the population cannot be suppressed by dominant genes, these plants turn into DH and the recessive genes take a dominant position. Therefore, they can be observed in the first generations from a phenotypic point of view.
2. They help with cytogenetic studies. They provide results in a short time in disease resistance studies.
3. They contribute to plant breeding studies by developing homozygous lines in a short time. Especially in plants that are pollinated foreign and have a long vegetation time, homozygous lines can be produced in a shorter time.

4. DH populations are effective in the rapid identification of the genes being investigated through molecular markers.
5. Pure male plants can be obtained in dioic species that cannot be purified by self-purification.
6. Helps inheritance of quantitative traits, QTL mapping, and gene identification. The DH population is also a good experimental material for targeting local lesions in genomes.
7. It also allows plants with cytoplasmic male infertility to transfer their sterile cytoplasm to the desired location in a single step without repeatedly crossbreeding.
8. In species that show self-incompatibility, dioecious, and self-pollinated depression, it may allow the generation of inbred lines.
9. Allows mutations in the recessive direction to be quickly noticed in mutation correction.

The organ structures of haploid plants are smaller than those of plants with diploid and polyploid structures. Haploid plants have a smaller cell volume than diploids. The small volume of cells has a direct relationship with the level of plant ploidy. Therefore, as the ploidy level decreases, shrinkage is generally observed in plant tissues and organs. The level of ploidy of plants can be determined by different methods. The most used methods are flow cytometry, a technique based on measuring DNA content, and chloroplast counting of stomatal guard cells (Dunwell, 1976).

It is also known that haploid plants in nature can occur spontaneously. The first haploid plants were identified in 1922 by Blakeslee et al. in the Datura stramonium plant. In the study published in 1964 and 1966, it is reported that Guha and Maheshwari obtained haploid plants in Datura plant with the technique of anther culture. It is thought that natural haploid plants usually form in the form of polyembryonic and that the embryos in the seed are co-existing as diploid and haploid (Abak, 1983).

Different techniques are applied in the studies carried out to obtain haploid plants. These are first examined under two main headings: *In vitro* (in a laboratory environment) and *in vivo* methods (Mishra and Goswami, 2014).

In vivo methods are spontaneous formation, hybridization, and parthenogenesis techniques. Parthenogenesis techniques include the most widely applied haploid production methods among *in vivo* techniques. The aim of this method is to start embryonic division by

misleading the egg cell as if fertilization has occurred with different triggers in field conditions. These triggering factors are pollination of pollen after it has lost its ability to fertilize, different chemical and hormone applications, temperature shocks and pollination with pollen that has lost the ability to be fertilized by means of X and Gamma rays (Irradiated pollen technique). Especially the irradiated pollen technique gives very successful results in some species belonging to the pumpkin family. In this technique, the ovary triggered by vicious pollen begins to grow and after a certain period of time, the seeds are removed under laboratory conditions and cultured in a specific environment. The development of the embryos in the seed is observed and the embryos determined to be at the heart stage are taken from the seed *in vitro* conditions and the growth and development of the embryos in the growth medium is encouraged (Mishra and Goswami, 2014).

In watermelon with the application of irradiated pollen technique (Gürsöz et al., 1991; Yellow et al., 1994; Flood et al., 2013), melon (Sauton and De Vaulx, 1987; Lotfi et al., 2003; Baktemur et al., 2013), cucumber (Truong-Andre, 1988; Niemirowicz-Szczytt and Dumas de Vaulx, 1989; Sauton, 1989; Çağlar and Abak, 1997; Lotfi et al., 1997; Dolcet-Sanjuan et al., 2004; Erol, 2018), zucchini (Kurtar et al., 2002; Baktemur et al., 2014), and in pumpkin (Kurtar et al., 2009) embryos and plants with haploid structure were obtained.

In vitro methods are applied in two different ways as Androgenesis (haploid production from the male gamete) and Gynogenesis (haploid production from the female gamete). In studies based on haploid embryo and plant acquisition, these techniques give more successful results. The Androgenesis method is applied in the form of culturing the anthers containing microspores that can turn into embryos in the special nutrient environment (anther culture) or by isolating the microspores from the anthers of this nature and culturing them in the liquid phase nutrient medium. Researchers who argue that androgenesis techniques are not effective in all species in the Cucurbitaceae family report that embryo and plant formation is generally limited (Galazka and Niemirowicz-Szczytt, 2013). With this technique, haploid embryo formation and plant formation in the Cucurbitaceae family were studied in the melon (Dryanovska and Ilieva, 1983), cucumber (Lazarte and Sasser, 1982; Gambling et al., 2003; Kumar and Murthy, 2004; Mohamed and Refaei, 2004; Suprunova and Shmykova, 2008; Hamidvand et al., 2013; Amiryan et al., 2020), zucchini (Metwally et al., 1998). There is

very limited research on haploid plant production by microspore culture method in the Cucurbitaceae family (Çetinkaya, 2015).

Gynogenesis is using female gametes to be cultured in the appropriate inducing environment under *in vitro* conditions and to obtain haploid embryos and plants. This technique is applied in the form of direct culturing of the ovarium containing the ovule (ovarian culture) or culture of the ovule isolated from the ovary (ovule culture). In the pumpkin family, it has been seen that the female gamete culture gives quite positive responses. While in the mature stage, the ovule in the ovaries taken from the donor plant is required to be capable of fertilization or close to reaching the ability to fertilize (Gemes-Juhasz et al., 2002). It has been argued that the origin of the developing embryo or embryogenic callus may be due to egg cells, synergies, and antipodal cells (Mukhambetzhanov, 1997).

Gynogenesis techniques are seen as a good alternative solution to the haploid production of species that do not respond to androgenesis, plants with high albino regeneration, male infertile plants, and plants with monoecious, dioecious flower structures (Pavlova, 1987; Campion and Alloni, 1990; Choob et al., 1994; Mukhambetzkhanov, 1997; Thomas et al., 1999; Bhat and Murthy, 2007). For this reason, gynogenesis techniques give more successful results in plants with monoecious flower structures such as the Cucurbitaceae family. It becomes quite easy to use commercial cucumber varieties with parthenocarpic female flower propensity as breeding material and to obtain haploid plants from these plants. Studies of the ovule and ovarian culture in the Cucurbitaceae family melon (Dryanovska and Ilieva, 1983; Sauton and De Vaulx, 1987; Ficcadenti et al., 1999; Lotfi et al., 2003) zucchini (Chambonnet and De Vaulx, 1985; Shail and Robinson, 1987; Kwack and Fujieda, 1988; Gemes-Juhasz et al., 1996; Metwally et al., 1996; Shalaby, 2007) and cucumber (Dirks, 1996; Gemes-Juhasz et al., 2002; Chen et al., 2005; Diao et al., 2009; Wei et al., 2010; Xiaoli, 2011; Li et al., 2013; Moqbeli et al., 2013; Cetinkaya, 2015; Sorntip et al., 2017; Özdemir Çelik et al., 2019; Erol, 2018).

Haploid plants, which are a tool in plant breeding work, are not easily obtained. The responses of plant species or even different kinds of plants within the same species may differ from the protocols applied. The acquisition of these plants is considered a great success. Many factors affect the production of double haploid plants. These are factors such as

appropriate donor plant selection, appropriate explant selection, determination of the appropriate gamete stage, appropriate sterilization protocol, appropriate nutrient environment content to initiate embryo formation in the determined explant, appropriate regeneration environment content to ensure the transformation of the embryos into plants, determination of pre-application protocols that can be applied to the explant for embryo formation, plant growth chamber conditions (light, temperature, humidity). In order to establish appropriate protocols, the doses and combinations of macro-microelements, vitamins, sugar, and plant growth regulators (PGRs) in the nutrient medium are generally emphasized, pre-application techniques, and the determination of the appropriate gamete stage for embryo formation. However, it has been seen in studies that other factors are also very important. In particular, it is highly likely that the donor plant affects haploid plant formation (Honkanen et al., 1990; Mukhambetzkhanov, 1997; Ata et al., 2019; Rivas-Sendra, et al. 2020).

When the donor plant factor is considered, there are many points to be considered. The most important of these is the genotype of the donor plant. Genotype is one of the most limiting factors in the production of DHs. A protocol can give different results in plants with different genetic makeups found within the same species. It has been proven that the success rate of haploid studies depends on genotype in species such as cucumber (Çetinkaya, 2015), zucchini (Shalaby, 2007), pepper (Şensoy, 2011), sweet potato (Kobayashi et al. 1993), and onion (Alan et al., 2004). However, apart from genotype, the age of the donor plant, the season in which the plant is grown, the growing environment (greenhouse, open land, etc.), the health status of the donor plant, the soil structure of the growing environment, the light of the growing environment (light intensity, photoperiod, etc.), the temperature and humidity of the growing environment, the cultural processes applied to the donor plant (irrigation, fertilization, harvest, etc.) are the factors that directly or indirectly affect the haploid embryo and plant formation. However, very limited studies have been carried out on these issues, and even no research has been done on some species. Genotype success rates have also led to the idea that the donor plant factor is an issue that needs to be emphasized (Çetinkaya, 2015). In the study of a number of researchers, it is argued that the age of the donor plant,

and the environmental conditions in which it is grown, are effective in the culture of the unfertilized ovary (Gibson, 1987; Tyrnov and Alatorceva, 1990).

For this reason, in this study, which we carried out in order to benefit the haploid plant acquisition studies, embryos and callus were obtained with the gynogenesis technique in cucumbers, the temperature exposed during the development of the ovary to be used as an explant, the genotype effect and effect of different flower stages were carefully examined.

CHAPTER 1

GENERAL INFORMATION and LITERATURE REVIEW

Stating that approximately 250 species have achieved successful results so far in the identification of appropriate protocol techniques in haploid plant production, researchers argue that the use of double haploid technology can be seen as an effective tool in reducing DNA methylation, removing unwanted alleles, and easily detecting trait and mutation analyzes (Maluszynski et al., 2003; Ren et al., 2017; Khan et al., 2020). It is also a very effective method for easy identification of the resistance gene in resistance studies against plant diseases (Hussain et al., 2012).

The first haploid plant studies in zucchini were by Swaminathan and Singh (1958) on obtaining improved haploid shoots from diploid watermelon seeds treated with X-ray rays and detecting advanced haploid seeds by spontaneous parthenogenesis in cucumber plants by Aalders (1958). Dumas de Vaulx, (1979), who applied a different technique in the following years, applied the interspecies pollination technique by pollinating *Cucumis melo* with *Cucumis ficifolius* pollen and caused the nuclei in the ovule to divide as if fertilized. Thus, haploid embryos were developed. However, the frequency of haploid embryo and plant formation in these studies has been quite limited and researchers have started to look for different techniques that may be effective in the Cucurbitaceae family. Different techniques that provide induction of gamete cells have also been applied in the pumpkin family and successful results have been achieved. The protocols that are effective in this family are generally determined as irradiated pollen technique (parthenogenesis), androgenesis, and gynogenesis. However, it is not yet clear which method is more effective according to the type and genotype.

In general, the genotype and growth medium of the donor plant, determination of the appropriate radiation dose and type in irradiation technique applications, determination of the explant type; optimizing the nutrient medium of embryo formation and plant

regeneration, chromosome folding, and determination of the level of ploidy (Sauton, 1988; Sari et al., 1994; Claveria et al., 2005; Li et al., 2013; Al-Maksoud et al., 2009; Taskin et al., 2013; Baktemur et al., 2014; Cetinkaya, 2015; Hazem and Golabadi, 2018).

1.1. Studies on Haploid Embryo and Plant Production in the Cucurbitaceae Family

1.1.1. Studies on Androgenesis

The androgenesis technique is a more preferred method due to the high number of gametes to be inducted from anthers in most species. Thus, the number of embryos and plants to be formed is expected to be high (Asadi et al., 2018).

Although it responds positively to many important commercial species, pumpkins can have different results among other family members. Lazarte and Sasser (1982), who used cucumber anthers to produce haploid plants *in vitro* culture, reported that embryos developed following callus development, but haploid plants could not be obtained.

In the study conducted by Abdollahi et al. (2015) to examine the anther culture response of watermelon (*Citrullus lanatus* L.), they cultured the Charleston Grey and Crimson Sweet genotypes. Investigating the effect of different hormone doses and the presence of wheat ovaries in growing media in one branch of the study on the Charleston Grey genotype, the researchers erected explants in MS nutrient media (Murashige and Skoog, 1962) containing N6-benzylaminopurine (BAP) or Kinetin (Kin) combined with the auxin hormone 2,4-dichlorophenoxyacetic acid (2,4-D). They also transferred wheat ovarium to growing media containing different concentrations of hormones, including a control group. As a result of the study, it is reported that adding 10 wheat germs to the MS nutrient medium containing 2.0 µM 2,4-D, 1.5 µM BAP increases the embryo yield per antenna by 2.5-3 times. In another branch of the study, it is said that different pre-cooling and preheating degrees and times were tested on Crimson Sweet type anthers. Researchers who pre-treated flower buds for 2-5 days at 4 °C, cultured anthers for 4-8 days at 4 °C, and cultured anthers for 7 days at 30 °C

and 2 days at 32 °C report that the highest % of embryos were obtained from anthers who were pre-treated at 32 °C for 2 days. It is reported that the embryos formed are regenerated in the MS environment containing 2.22 μM BAP, 3% sucrose, and 0.8% agar, and 10 of the 12 plants obtained in total are haploid.

In a study examining the efficacy of anther culture on different watermelon genotypes and subtypes, 2 mg L-1 2,4-D + 90 g L-1 sucrose-supplemented MS medium was used and BAP concentrations were tested at different doses (1 and 1.5 mg L-1). In addition, spermidine (Spd) and putresin (Put) were added separately and together to the media contents in different doses (500 and 1000 μM L-1) to investigate the effect of polyamines. In the first study, which examined only the hormone dose effectiveness, they reported that the anthers were collected 2 days before the anthesis and cultured after waiting for 2 days at 32 °C in dark conditions. In the other study where the polyamine efficacy was examined, it is stated that the anthers were collected 2 days before the anthesis and not subjected to pre-treatment. In the study, the researchers concluded that they obtained different results according to genotype, that the use of polyamine had a positive effect on the anther culture, that the application of pre-temperature reacted depending on the genotype, and positively affected the formation of callus (Akbaş and Solmaz, 2019).

Sari et al., (2002) used the irradiated pollen method in melons to produce double haploid lines. Their studies showed a successful development of haploids without any inbreeding aggression. Kurtar et al., (2016) conducted a study of anther culture in these species with the idea that although the irradiated pollen technique in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) works successfully, it does not generate sufficient frequency for large-scale production. In the study, researchers using the MS growing medium they modified using different ratios of 2.4-D, BAP, Kin, NAA reported that they received the highest response from the 57S21 and G9 lines with a combination of 2.0 mg L-1 or 4.0 mg L-1 BAP + 0.05 mg L - 1 NAA (E6 nutrient site). In addition, it is said that the regenerated plants were rooted in an MS environment supported by 0.01 mg L-1 IAA (Indole-3-Acetic Acid), and 74 plants obtained were propagated by micro-propagation, and because of ploidy

analysis, 35 plants (47.3%) were haploid (n = 20) and the others (52.7%) were diploid (2n = 40).

Kumar et al. (2003) conducted anther culture studies on Calypso and Green Long cucumber genotypes and subjected cucumber buds to 4 °C pre-cooling ranging from 0-10 days and also preheating at 32 °C for 1 day. They added the hormones BAP, Kin and TDZ cytokinin modified by 2,4-D and NAA to the B5 nutrient medium (Gamborg, 1984) and sutured anthers of the culture varieties. Optimum embryogenic callus/embryos are reported to be obtained in B5 medium supported by 2.0 μM 2.4-D and 1.0 μM BAP and pre-cooling of flower buds at 4 °C for 2 days. It was determined that the obtained plants were 21 haploid in the Calypso variety and 17 in the Green Long variety. Aiming to establish an appropriate protocol for the production of haploid embryos and plants through androgenesis in cucumber, Kumar and Murthy (2004) cultured cucumber anthers in a B5 nutrient medium modified with different ratios of sugars (sucrose, maltose, glucose, and fructose) and amino acids (glutamine, glycine, arginine, aspargine, and cystine). The researchers, who identified Calypso and Green Long varieties as donor plants in their studies, report that the type and concentration of sugar and amino acids affect embryogenesis, 0.25 M sucrose is best for embryo formation, and B5 media with 2.0 μM 2.4-D and 1.0 μM BAP content causes the highest embryo formation in both varieties. In addition, the researchers claim that the addition of the amino acids glutamine, glycine, arginine, asparagine, and cysteine to a modified B5 nutrient medium with 25 μM NAA, 0.25 μM Kin and 0.09 M sucrose at a rate of 1.0 mM maximizes embryo formation. Kumar et al., (2004) examined the effect of polyamines (putresin and spermidine; 5-1000 μM) on the androgenesis technique in cucumber varieties and planted cucumber anthers in B5 embryo formation medium containing 2.0 μM 2.4-D, 1.0 μM BA and different doses of putresin or spermidine. They found that the addition of putrescin and spermidine at a rate of 5-200 μM increased embryogenesis in anthers, while spermidine caused the highest embryo formation in both varieties (90.66 embryos in Calypso and Green Long 100.33 embryos according to 60 anthers cultured). It has also been reported that the use of 500 or 1000 μM putresin and spermidine adversely affects embryo formation. Stating that the obtained embryos are sutured to a modified B5 nutrient medium with 25 μM NAA, 0.25 μM

Kin and 0.09 M sucrose for regeneration, the researchers recommend the use of a nutrient medium containing 200 μM spermidine for the highest embryo formation.

Researchers trying to develop appropriate cucumber anther culture protocols focused on appropriate microspore stage, pre-application temperature, and genotype effect (Xie et al., 2005). According to the research, it was determined that the bud lengths in the appropriate microspore stage changed according to the genotypes, and the most suitable bud intake stage for cucumber microspore culture was the bud stage containing microspores in the middle and late single-core stage. In general, in anther culture, the morphologically appropriate time of the stage of development of the appropriate microspore is indicated as when the buds are 0.90-1.5 cm (centimeters) long and green in color, the upper ends of the petals are closed, and the anthers are white-green or light green. In addition, researchers argue that low-temperature pre-treatment increases microspore viability and that soaking anthers at a low temperature of 4 °C for 48-72 hours in the dark is the most favorable pre-treatment technique that triggers callus formation.

To develop an effective cucumber anther culture protocol, Song et al. (2007), who conducted studies under six different headings (pre-treatment, embryogenic callus induction environment, preculture conditions, embryo formation environment, embryo regeneration environment and genotype effect), found that the donor plant could respond well when exposed to a pre-temperature stress suitable for the normal ecology in which it was grown. They report that a cucumber genotype adapted to the cold zone they used in their trials responded well to cold shock, while a genotype selected from temperate regions responded better to heat treatment. The best medium for embryogenic callus induction is said to be the MS environment supported by 4.44 μM BAP, 2.26 μM 2, 4-D, 4.64 μM KIN, 3% sucrose, and 0.8% agar, while for embryo formation, it is said to be the MS medium supported by 0.54 μM NAA, 13.32 μM BAP, 3% sucrose and 0.8% agar. For the transformation of the embryo into a plant, an MS medium containing 2.22 μM BAP, 6% sucrose, and 1.2% agar is recommended. It is stated that calculus was obtained from 16 of the 20 genotypes they cultured in the trial, plant regeneration occurred from 3 of them and 93% embryo formation was recorded in the trial.

Suprunova and Shmykova, (2008) cultured cucumber anthers, unfertilized ovaries, and microspores for embryo development in growing media supported by different hormone concentrations. Ovaries were cultured in a modified MS (Masuda et al., 1981) environment with different hormones containing 5% sucrose, while anthers were cultured with 8% sucrose content MS + 100 mg/L serine, 800 mg/L glutamine and different PGRs (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 BAP; 0.4 mg/L 2.4-D and 0.2 mg/L BAP) and microspores 2,0 mg 2,4-D, NLZ (Lichter, 1982) containing 10% sucrose were transferred to the liquid nutrient medium. They argue that microspore culture is more effective than anther culture and that the best embryo and plant frequency in ovarian culture is obtained from MS growth medium containing 0.2 mg/L thidiazurone (TDZ) and 0.2 mg/L BAP. They report that they obtained haploid plants from the 'Gordion' genotype only from the 10 genotypes cultured by gynogenesis and that the stage in which anther responds best was the late single-nucleated microspore and early bicellular pollen grains.

In the cucumber androgenesis study using six different genotypes, researchers who conducted studies on the appropriate hormone dose and appropriate hormone content (El-Maksoud et al., 2009) cultured cucumber anthers in an MS nutrient medium enriched with three different hormone concentrations. At the end of the study, they reported that very large differences were detected between genotypes, that the relationship between genotype and hormone level was an effective factor in terms of callus formation and green tissue formation. In Zhan et al. (2009) study on microspore culture in cucumbers, they used 10 different cucumber genotypes and tried growing media containing different hormone constructions. Researchers who reported that they obtained embryos and plants at the cotyledon stage from the "7447" and "Poinsett97" genotypes reported that determining the appropriate microspore stage and genotype was the key factor for embryogenesis. Stating that the multi-core microspore in the late stage is the best stage, the researchers argue that the response is better than microspores exposed to a temperature of 4 °C for 2-4 days. They also report that hormones at low concentrations (0.5 mg/L 2, 4-D, and 0.2 mg/L BA) are effective for embryo formation, that there is no significant difference in embryo activity between the NLN and B5

nutrient mediums used, that regenerated plants are obtained only from cotyledon stage embryos and that no plants develop from other developmental stage embryos.

In a study conducted by Nguyen and Chen (2012) based on protocols prepared by Kumar et al. (2003), Song et al. (2007), and Zhan et al. (2009), Kaluoer reports that they used anthers of three cucumber genotypes, HH1-8-57, and Jinlu Nongjiale, as experimental material to investigate the most appropriate pre-application techniques (temperature and duration). In addition, in the study where the effective environment components were investigated, it is stated that pre-treatment of anthers at 4 °C for 2 days significantly increases the embryogenic callus induction rate and the highest embryogenic callus rate (81.3%) is obtained from Zhan medium (MS + 1.0 mg/L 2,4-D + 0.5 mg/L 6-BAP + 3% sucrose + 0.8% agar). In addition, the highest embryo formation rate (40.0%) is reported to be obtained from the embryo formation medium of Song et al. (2007) (MS + 0.1 mg/L NAA + 3.0 mg/L AP + 3% sucrose + 0.8% agar). Stating that the effect of the genotype is also clearly seen, the researchers say that the highest embryogenic callus induction rate is in the Jinlu Nongjiale genotype (81.1%) and the highest embryo formation rate is in the HH1-8-57 (40.0%) genotype.

Hamidvand et al. (2013) used two different cucumber genotypes (Beta Alpha and Esfahani) in their study investigating the effect of different genotypes and combinations of PGRs on the formation of callogenesis and embryogenesis in cucumber (*Cucumis sativus* L.) anther culture. They report that they obtained the highest embryo formation in the combination of 2 μM 2,4-D and 1.0 μM BAP hormones and the highest callus formation in the combination of 1.5 μM Kin and 2 μM 2,4-D hormones from the anthers they took into the growing environment with different doses of 2,4-D, NAA, BAP and Kin hormones.

In a study in which one type of cucumber (Beith alpha) and three local Iranian cucumbers (Basmenj, Isfahani, and Korki) were identified as donor plants, cucumber anthers were transferred to the MS growing medium at different strengths (half strength, full strength, 1.5 times strong, 2 times strong) and these growing mediums were supplemented with different concentrations of agar (0, 3.5, 7 and 14 g/L (Abdollahi et al., 2016). Scientists who evaluate the embryo formation status of the developing calluses report that macronutrients and agar

concentrations applied at different powers greatly affect androgenesis. In addition, it was observed that the highest rate (100%) of callus was formed in the Isfahani genotype developed in the 2x strength MS growing environment, and the maximum (1.26 and 1.23 embryos/anther) embryos per anther developed in this local variety in the full and semi-strong MS environment. In the second trial, the appropriate agar dose; They report that the highest callus formation was achieved at a dose of 14 g/L and 7 g/L agar in the Basmenj local variety, in the Isfahani variety at a dose of 3.5 g/L agar or liquid culture, in the Beith alpha type at a dose of 7 g/L agar and in the Korki local variety at a dose of 14 g/L agar. When all genotypes are examined, it is argued that the highest embryo approval is obtained in the use of 14 g/L agar in Korki variety.

Asadi et al. (2018) aimed to create a more efficient protocol due to the fact that very high rates of haploid embryos and plants were not obtained in studies where cucumber anther and microspore culture protocols were applied. For this reason, in the study they conducted, they subjected the buds of two cucumber genotypes to a pre-temperature application of 4°C, and then cultured the anthers of the sterilized buds in the growing medium they supported with BAP and 2,4-D. In order to encourage the formation of callus, they first kept the anthers at 35°C in the dark in liquid culture, then transferred them to the solid medium, and their development was ensured in the bright environment. At the end of the study, the researchers argued that the differentiation of genotype, anther tissues, growing medium components, and induction medium hormone content had a significant effect on cucumber anther culture studies and reported that androgenesis studies in cucumber still needed to be developed and that they could not obtain haploid embryos and plants at such a high rate as gynogenesis or parthenogenesis.

Amirian et al. (2020) conducted a study on two Beith alpha F1 cucumber hybrids (BT1 and BT2, gynoecious) and Dastgerdi cucumber (DTG, monoecious) genotypes to evaluate male flower induction and their androgenesis abilities. The researchers state that for the induction of male flowers in gynoecious cucumber genotypes, after the first true leaf formation, they applied the process of spraying combined solutions formed with $AgNO_3$ (3 mM), GA3 (1.5 mM), and $CoCl_2$ (3 mM) 3 times at intervals of one week. The researchers, who calculated

that the number of male flowers in the genotypes was 42, 14.5 and 0 respectively 6 weeks after the application, applied male flower induction only in gynoecious varieties for male flower formation. The researchers, who cultured the anthers they received at the late stage with multi-core according to the nutrient media protocol of Song et al. (2007), kept the anthers at a temperature of 25ºC for 4 weeks for the development of callus. The developing embryogenic calluses were kept in the MS growing environment with 2.22 μM BAP and 6% sucrose content for 3 weeks at the same temperature and light conditions. The embryos that reached the cotyledon stage were transferred to the hormone-free growing environment and their transformation into plants was encouraged. After all; Among the three genotypes, the highest embryogenic callus formation rate (62.2%) was reported to occur in the DTG genotype, while the lowest (3.3%) was detected in the BT1 genotype with AgNO3 promoting male flower formation. It is reported that 11 double haploid plants were obtained from DTG genotype, 2 from BT2-AgNO3, and 3 from BT2-GA3.

1.1.2. Studies on Parthenogenesis

Although the reactions vary according to species and genotypes in members of the Cucurbitaceae family, the most positive responses are obtained from the *In situ* technique and gynogenesis techniques. Studies on anther culture have generally been on embryogenic callus production and indirect embryo formation. However, in the studies carried out, it is reported that a very limited number of haploid plants are produced from the callus obtained from the members of the Cucurbitaceae family who have undergone *In vitro* androgenesis method (Shail and Robinson, 1987; Galazka and Niemirowicz-Szczytt, 2013; Asadi et al., 2018).

The parthenogenesis technique is governed by the logic of initiating embryonic division by misleading the egg cell as if fertilization had occurred through different triggers (interspecies pollination, different chemical and hormone applications, temperature shocks, and X and Gamma rays) *in vivo* conditions. More irradiated pollen technique (*in situ*) studies are carried

out by researchers. Some of the studies in situ are carried out *in vivo* and some of them (embryo rescue) are carried out *in vitro* (Vijverberg et al., 2019).

Dumas de Vaulx (1979), which aimed to create parthenogenic haploid embryos and plants by using two different species of interspecies pollination techniques, determined one species as *Cucumis melo* L. (2n=24) and the other as *Cucumis ficifolius* (2n=4x=48). By superficially cutting the stigmas of melon female flowers, which were to stimulate the ovarian, Cucumis pollinated with ficifolius pollen and obtained fruits. The researcher, who germinated the seeds from the melon fruits, saw that the developing plants were smaller in size and as a result of the chromosome counts he made in these plants, the plants had a haploid structure; He found that the rate of haploid plant formation varied according to the season, 0.284% in spring and 0.07% in autumn.

Gürsöz et al. (1991), who conducted studies on stimulation with irradiated pollen culture in watermelon, identified one F1 hybrid variety (Panonia) and three openly pollinated varieties (Crimson sweet, Sugar baby, and Aleppo Black) as plant material. In their study, Sauton and De Vaulx, (1987) applied the protocol and denatured the pollen with 300 Gy (Gray) rays. They investigated the effect of this protocol on the growing season and genotype of the donor plant. While it is recommended that the pollination period should be done during the period when plant development is active, they report that the most appropriate pollination period is the period between 31 May and 13 June in their own studies.

In addition, it is reported that 761 embryos were obtained, 72% of which were globular and 28% of which were at heart stage, and that only 17 of these embryos turned into plants and that all of them were haploid. Przyborowski and Niemirowicz-Szczytt (1994), who aimed to obtain haploid embryos and plants in cucumbers with the irradiated pollen technique, pollinated four different F1 varieties (Sweet Salad, Polan, Fremont, WPR 187) with the pollen of the ‘B’ ingrown line they neutered with 300 Gy gamma rays. Then, after 3-5 weeks, they planted the seeds they received from the harvested fruits in the E20A (Sauton and De Vaulx 1987) environment. The researchers, who reported that they transferred the rooted plants to the MS environment, found that they obtained the most embryos in the Polan F1

variety (3.1 embryos per fruit), 50% of them turned into plants, and although the number of embryos per fruit in the Fremont F1 variety was 1.2, the conversion rate to the plant was 67.5%. This research, which was studied for an average of three years, also focused on the seasonal effect and examined the reaction of donor plants to irradiated pollen culture developed in summer and spring. At the end of the study, it was found that the number of embryos developing per fruit (2.51 pieces) was better in summer than in spring (0.69 pieces). Caglar and Abak (1997) aimed to obtain haploid embryos and plants by parthenogenic stimulation using the *in situ* technique. In their study, they focused on issues such as gamma ray doses and determination of pollination time. Researchers studying 27 different genotypes reported that low doses of radiation (200, 300 Gy) increased the frequency of haploid embryo formation regardless of genotype. It is argued that the best pollination time for haploid plant production for the climatic conditions of the Mediterranean Coastal Region of Türkiye is May-September.

Faris et al. (1999) conducted haploidization studies using irradiated pollen technique in cucumber lines of different genotypes. The researchers, who examined the study in four different stages, tried it on five pure lines and 3 hybrid lines where they pollinated with sterile pollen that had been administered 0.2 kGy (Kilo Gray) and 0.3 kGy radiation dose in the first stages and aimed to find the most effective radiation dose. In the second phase of the study, the genotype, which is a hybrid of the two most efficient pure lines and one pure line, was selected and these genotypes were pollinated with neutered pollen with a beam dose of 0.05 kGy, 0.1 kGy, 0.2 kGy, and 0.3 kGy. In the last 3rd and 4th trials, a genotype known to have high embryo yield was pollinated with 0.1 kGy and 0.3 kGy ray-treated pollen. In the first trial, it is reported that the highest embryo yield was obtained from the Gy3xM and BxOg hybrids and that the 0.2 kGy radiation dose worked well in almost both genotypes, but when all genotypes were evaluated, the effective beam dose varied according to the genotype. In this experiment, a total of 717 embryos were obtained and 3.3% of them are said to have turned into plants. When all radiation doses were examined, it was seen that the most effective radiation dose was 0.3 kGy. In the next trial, it is reported that the highest embryo yield in all doses administered from 3 different cultured genotypes is obtained from the

Gy3xM hybrid, and the highest embryo yield for all genotypes is observed by stimulation of neutered pollen with a beam dose of 0.1 kGy. In the application of beam dose 0.05 kGy, it was seen that all the embryos developed were diploid. In the 3rd and 4th applications, where the most effective radiation dose was determined according to the results of the first two trials, 0.1 kGy and 0.3 kGy were applied to a fertile genotype and it was determined that the most effective radiation dose for embryo yield was 0.1 kGy in both. However, it is stated by researchers that the conversion to the plant remains at a very limited rate.

Caglar and Abak (1999) focused on the donor plant genotype and donor plant growing season, which are the factors for the formation of haploid embryos and plants developed by applying the In situ technique in cucumber, the effect of ray doses on fruit set, and parthenogenetic embryo formation rate. The researcher, who dusted Qamar F1, Seraset F1, Dere, and Çengelköy varieties with pollen exposed to gamma rays at 300, 450, and 600 Gy, concluded that the most efficient embryo-forming radiation dose was 300 Gy. In addition, the researcher who states that genotype and season have a great effect on production with irradiated pollen culture in cucumber, reports that he obtained more haploid plants from embryos cultured *in vitro* between May and September, and that hybrid genotypes formed more haploid plants than in Dere and Çengelköy, which pollinated in the open. For haploid embryo formation, it is reported that the warm period between April and October is the best season, while the highest seed production occurs in June. At the end of the two-year study, a total of 190 haploid plants from four varieties were obtained.

Dolcet et al. (2004) studied parthenogenetic haploid embryo formation using pollen denatured by irradiation at doses of 250 and 500 Gy with gamma rays originating from 60Co (Cobalt 60) in cucumbers. The researchers, who regenerated the embryos *in vitro* culture by detecting them by X-ray radiography, transplanted the embryos into the E20H8 (7.9 mM $CaCl_2.2H_2O$, 0.17 mM $CoCl_2.6H_2O$, 0.10 mM FeEDTA, 20 g/L sucrose, 8 g/L Difco Bacto agar) growing medium they created by modifying the E20 medium. In addition, it is said that 0.06 µM indole acetic acid (IAA) and 15 µM silver thiosulfate (STS) are added to the growing medium of plants in order to support root growth and prevent vitrification. It is reported that

the ploidy level of the developing plants is determined by the "Flow cytometry method" and that 500 μM Colchicine is added to the growing environment of the plants determined to be haploid and the plants are made DH. At the end of the study, it is stated that the most effective irradiation dose is 500 Gy, a total of 587 fruits are formed from all genotypes, 116 embryos are detected and saved from them, and 38 haploid plants are obtained with this study.

Smiech et al. (2008), in their irradiated pollen culture study on the use of RAPD markers in the production of haploid and double haploid cucumbers, pollinated four different genotypes (Krak F1, Frykas F1, Izyd F1 and Polan F1) with pollen they neutered with gamma ray doses of 300 Gy and 800 Gy. It is stated that embryo recovery technique is applied with the method of opening one by one 3-5 weeks after dusting and the embryos are transferred to the E20A environment for development. The study was conducted in two seasons: May-June (spring) and August-September (summer). At the end of this research, scientists obtained a total of 54 plants that obtained 15.2% plant regeneration from all varieties in the spring season. It is reported that the lowest embryo regeneration in this season is obtained from the Polan F1 variety (7.8%) and the highest from the Krak F1 variety (21.1%). In the season shifting from summer to autumn, the highest embryo regeneration is said to occur in the Krak F1 variety (41.9%) and the lowest in the Izyd variety (9.3%). The researchers, who obtained a total of 333 embryos and 85 plants from all varieties in this season, report that they received better responses in the summer period in the three varieties (25.5%) than in the spring period and that they detected more embryo-to-plant transformation in the spring period in the Izyd variety.

Kurtar et al. (2009) investigated the effects of gamma rays and 60Co radiation doses (50, 100, 200, and 300 Gray) administered on different dates (9, 11, 15, 21, and 28 July) on the acquisition of haploid embryos and plants using irradiated pollen technique in pumpkins (*Cucurbita moschata* Duchesne ex Poir.). At the end of the study, it was seen that low-dose (50 and 100 Gy) and early (9 and 11 July) irradiation increased haploid embryo and plant formation, as well as haploid embryo formation and haploid embryo form significantly affected the variety and fruit harvest time. Researchers who determine that the best fruit

harvest time is 3 weeks after pollination report that the necrotic embryos increase as the harvest time is delayed, and the highest rate of haploid embryos and plants are obtained from the fruits with irradiation dates on 9 and 11 July. In this study, 213 embryos were obtained from 13 fruits in total, and it is said that 34 of them (15.96%) turned into plants.

Ari et al. (2010), who identified the disease-resistant melon (*Cucumis melo* L.) F1 hybrid varieties as a donor plant, used the pollen they denatured with a gamma ray source of 300 Gy in pollination one day later. The researchers, who observed 94% conversion from pollinated ovaries to fruit, harvested the fruits 3-4 weeks after pollination. Three different methods for the extraction of seeds (M1; Individual extraction of seeds, M2; Sowing the seeds all in petri dishes, M3; It is reported that 280 haploid, 44 diploid, and 8 myxoploid embryos were obtained from 204 harvested fruits. According to the three different techniques applied, it is stated that the time to detect haploid embryos from fruits is 162, 125, and 49 minutes respectively and that the application of the correct technique is important both in terms of time and economy. Therefore, they advocate the necessity of applying the M3 technique.

Godbole and Murty (2012), who aim to obtain haploid embryos and plants from the female flowers they pollinate with melon pollen to which they apply various doses of gamma-ray (150, 200, 250, 300, and 350 Gray), report that they harvest the fruits that develop parthenogenic after 21 days. The researchers, who cultured the extracted seeds in an E20A growing medium, say that the best radiation dose is 250 Gy and that the effects of doses as low as 150 and 200 Gy and high doses such as 300 Gy on the formation of haploid embryos are determined to be quite low. They found that from the cultured embryos, only those at the cotyledon stage turned into plants. The fact that the formed plants are haploid has been confirmed by the "Flow Cytometry" method.

Taskin et al. (2013) pollinated two different watermelon genotypes with neutered pollen by administering five different doses (60Co 50, 150, 200, 275, and 300 Gy) to develop a beneficial haploidization protocol in watermelon. They saved the embryos by opening the seeds they extracted from the harvested fruits one by one 25 days after pollination and

transferred them to the CP growing medium containing 30 g/L sucrose, 8 g/L agar, 0.08 mg/L B12, and 0.02 mg/L IAA. They stated that 60 haploid embryos were obtained from 43 watermelon fruits harvested in total and that the most successful dose among the irradiation doses administered was 275 Gy (5.26% haploid embryo), while the genotype with the best response was Genotype 1.

Baktemur et al. (2014), who tried the irradiated pollen technique with 3 different doses (150, 200, 300 Gy) in fourteen different pumpkin genotypes, report that they obtained 1851 embryos from 219 fruits in the first year. In addition, the researchers reported that 9.12% haploid embryos developed from 150 Gy beam doses and 3.53% haploid embryos developed at 200 Gy beam doses and that the most successful genotype in terms of embryo yield in the study was Genotype 3 and the most successful genotype was Genotype 4. In the second-year trials, 8 genotypes were tried and the highest embryo formation was obtained from the application of 150 Gy beam dose; They stated that 2625 haploid embryos were obtained from 217 harvested fruits and that the most successful genotype in this year was genotype 6.

Çelikli (2016), in his master's thesis, examined the genotypes of Galia and Fortywood melon (*Cucumis melo* L.) (Galia; K-7, K-12 and K-13, Kirkagac; K-17) in the study of haploid embryo and plant retrieval by means of parthenogenesis, embryos developed from melons stimulated by pollen irradiated by three different beam doses were cultured *in vitro* culture in three different nutrient mediums. At the end of the study, it is said that the number of seeds is higher in Galia genotypes than in Fortywood genotypes, that 350 Gy radiation dose reduces the number of haploid embryos in all genotypes, and that the optimal radiation dose in both types of melon is 300 Gy. In addition, while haploid embryo acquisition and plant regeneration are reported to vary depending on genotype, radiation dose, and growing medium, the researcher recommends that 300 Gy beam dose be applied to denature pollen and cultured in MS growing medium for embryo transformation into plants.

In order to produce grafted seedlings, Kurtar et al. (2017) aimed to convert zucchini (Cucurbita spp.) lines with high potential to be rootstock to cucumber into homozygous lines

by the irradiated pollen technique method. In the study, 17 rootstock candidate lines irradiated the pollen with a radiation source of 60Co with a radiation dose of 150 Gy and pollinated the female flowers with neutered pollen. As a result of the observations, it was determined that the genotype had a significant effect on the frequency of haploids, and it was reported that 24 haploid plants were obtained from 821 embryos in H genotypes, and 233 embryos and 3 haploid plants were obtained from I genotypes. The frequency rate of haploid regeneration per embryo was found to be 1.29% in I genotypes and 2.92% in H genotypes.
Hazem and Golabadi (2018) received the embryos they obtained with the embryo recovery technique in their studies in which they stimulated the formation of embryos with the pollen they received from cucumber plants that they deactivated through chemical mutagens. In their study, they investigated the effects of genotype, male smallpox age, receptor plant, and chemical mutagens (NaN3 and colchicine) on seed production (total number, full, half-full, and empty seeds) and developing plant morphology by enabling deactivation in pollen grains. Also touching on the effects of different plant growth regulators on embryo cultures, the scientists first planted and cultivated donor plant seeds that they kept in NaN3 (0.0012 mg/L, 0.002 mg/L, and 0.005 mg/L), colchicine (0.005 mg/L) and water (control) for 24 h. The researchers, who performed embryo recovery in different structured seeds taken from haploid donor plants pollinated by mutant plants, stated that they received the highest total and semi-filled seed number from donor plants pollinated by mutant plants using 0.0012 mg/L and 0.005 mg/L NaN3 and colchicine. The recovered embryos were germinated in MS growing environments containing different combinations of hormones. When the success rates of hormone concentration effect were evaluated, the highest regeneration rate (66.67%) was found in growing environments with 2 mg/L BAP + 1 mg/L Kin + 0.5 mg/L IBA and the lowest regeneration rate (26.67%) in growing environments with 2 mg/L BAP + 1 mg/L Kin + 0.5 mg/L BIA.

Aiming to obtain cucumber double haploid plants from donor plants with the irradiated pollen technique, which is a parthenogenesis method, the researchers applied different doses of putresin, spermidine, and cycocel (0.50 mg/L, 500 mg/L, and 5000 mg/L) to the mother plants and investigated the effectiveness of these polyamines in producing double haploid

plants. Researchers who apply the polyamines mentioned as spraying at intervals of 3 days at intervals of 9 days before and 9 days after the anthesis period of female flowers report that they denatured the anthers they received from male flowers with a beam dose of 300 Gy at 60Co ray source and then stimulated the female flowers with vicious pollen dust. It is said that the seeds taken in the resulting fruits are transferred to 25 mL liquid E20 medium supported by 3% sucrose and 0.01 mg/L IAA and the germination of the embryos is stimulated. At the end of the study, it was seen that polyamines had a great effect on embryo formation. In donor plants without polyamine, the frequency of embryo formation was 3.2 embryos/fruit, whereas in polyamine applications, embryo frequencies were 5.2 embryos/fruits in 500 mg/L putresine, 5.2 embryos/fruits in 50 mg/L spermidine, 4.8 mg/L and 50 mg/L cycoceles. It is said that the lowest embryo formation is observed in the application of 5000 mg/L spermidine (0.4 embryos/fruit), that the effect rates of different doses in different polyamine derivatives also change, and that the formation of embryos at the highest dose in general decreases (Ebrahimzadeh et al. 2018).

Shariatpanahi and Ramezanpour, (2019) stated that they used 7 different cucumber genotypes in their study in which they tried the effectiveness of 300 Gy and 500 Gy radiation doses and that they conducted studies on pollen viability and pollen tube behavior after irradiation, genotype effect, fruit set rate, number of seeds obtained, parthenogenic embryo formation rate. They found that different doses of radiation affected the number of seeds and embryos depending on the genotype and that the highest embryo frequencies were found in the Extreme genotype (2.25 embryos/fruit) irradiated with a 300 Gy dose and the Karim genotype irradiated with a 500 Gy dose (1.75 embryos/fruit). The researchers suggest that an appropriate irradiation dose should be administered in each genotype so that parthenogenic embryo formation is higher.

The researcher, who applied the irradiated pollen technique in order to give haploid embryos and plants to commercial melon varieties with high pathological resistance to commercially high-quality fruit, reported that they focused on fruit set after pollination, haploid embryo formation, *in vitro* germination, and growth of embryos, and the effectiveness of colchicine

used in increasing the level of ploidy in haploid plants (Hooghvorst et al. 2020). The researchers, who evaluated the 6 spontaneous lines developed from the "Piel de Sapo" genotype and the "Melito" variety, found that the genotype effect of the genotypes developed from Piel de Sapo was greater in haploid production and that 15.78% (178 pieces) of the female flowers they pollinated turned into fruit. In addition, the researcher who detects haploid embryos with three different methods (individual embryo opening, X-ray radiography and germination in a liquid environment) reports that results are obtained 4-5 times faster than individual opening with X-ray radiography, and that embryo germination in liquid environment adversely affects the development of haploid embryos. It was observed that approximately 59 of the harvested fruits carried haploid embryos, 50.94% of the existing embryos did not turn into plants, only 26 plants were formed, 73.08% of them were haploid, 23.08% were spontaneous double haploid (DH) and 3.84% were myxoploid. It has been determined that immersion of apical meristem in colchicine solution *in vivo* is the best method of increasing chromosome levels. At the end of the study, the researchers suggest that more work needs to be done to improve parthenogenetic efficiency.

1.1.3. Studies on Gynogenesis

The stimulation of female gametes by means of different indicators to form plants containing a single set of genes is an effective method that can be applied especially in species where anther culture technique cannot be applied, morphological male gamete infertility and monoecious or dioic flower structure that is difficult to homozygotic. In addition, the fact that the most effective radiation dose in the irradiated pollen culture technique, which is one of the parthenogenesis methods, could not be determined in the studies of scientists working on some pumpkin species to focus on ovule-ovarian culture methods.

In studies where ovule or ovary culture is applied, the gamete maturity level is generally expressed as the period when the egg cell reaches or is close to reaching full maturity (Wu, 2003). It is argued that the haploid plant that develops in this method can develop from synergy or antipodal cells other than the egg nucleus. However, the molecular mechanism

that triggers embryo formation through gynogenesis cannot be clearly explained (Palmer and Keller, 2005; Bohanec, 2009). However, it is known that plants can develop from the nucellus and other somatic tissues surrounding the embryo sac in the gynogenesis method and that the plants obtained will be diploid. For this reason, some researchers have tried to isolate other tissues from the embryo sac and have tried the method of culturing and have achieved successful results (Wagner et al., 1989; Kalinin et al., 1991). However, applying this method to plants with a small ovarian structure can be a bit challenging. In cucumbers, it was seen that the frequency of haploid embryo formation was lower from ovaries taken at an early stage, and haploid embryo and plant formation obtained from mature and near-mature ovaries would be higher (Gemes-Juhasz et al., 2002).

The first study on "Ovule-Ovarian culture" in the Cucurbitaceae family was the study of Chambonnet and Dumas de Vaulx (1985) on the pumpkin plant. In this study, researchers who take the ovaries at different stages of flowering into the growing medium report that the ovules they isolated from the pumpkin ovaries they received 1 or 2 days before the anthesis period gave the most successful result, and the plant formation frequency was 4-7% plant/ovule. This study has been an exciting study for the pumpkin family and has offered the opportunity to researchers who will work in the relevant field to focus on this subject.

Kwack and Fujieda (1988) collected unfertilized ovaries of two different pumpkin genotypes (*Cucurbita moschata*) 1-2 days before anthesis and at the anthesis stage, pre-cooling the ovaries to 2-5 days 5 °C and 2 to 5 days 10 °C. Following this procedure, the ovules isolated from the ovaries were transferred to MS environment containing 10 g/L , 30 g/L , 60 g/L, and 100 g/L sucrose with a power of 1, 3/4, 1/2 and 1/4. At the end of the research, the researchers observed that the most effective ovary intake time for embryo yield was the flowering time and that the most effective pre-application technique was to soak at 5 ° C for 2 days, and observed that half power MS and 30 g/L sugar dose constituted the most effective nutrient medium combination. They state that the developing embryos form callus in general and that plants with abnormal structure often develop. Two of the 3 plants obtained were tetraploid, and one was diploid (2n=40).

Gemes-Juhasz et al. (1996), in their studies on gynogenesis in zucchini and cucumber, say that they cultured 2-3 cm long unfertilized zucchini ovaries and 0.5-1 cm long unfertilized cucumber ovaries from donor plants. They cultured the ovaries sterilized in a 5.25% sodium hypochlorite (NaClO) solution in EP growing medium containing 0.02 mg L-1 TDZ and 4% sucrose (Chee et al., 1992) for 4-10 days. Then, the development of ovaries transferred to the EP growing environment with NAA and BA content in various ratios was achieved. Researchers have reported that embryos develop after 6 weeks in zucchini and about 10-15 embryos are formed from each ovarium, while only a few embryos develop in cucumbers, and that donor plant genotype is a very important factor for the response to gynogenesis. It is reported that 70% of the pumpkin plants whose ploidy level is determined by chromosome counting method are haploid, 30% are aneuploid and double haploid.

Yılmaz (2005) aimed to develop a suitable regeneration protocol for the acquisition of haploid embryos and developing embryos by the gynogenesis method in Cucurbita pepo. The researcher, who collected the ovaries of Gum and Zeybek F1 varieties 1 day before the anthesis, during the anthesis period, and 1 day after the anthesis at the stage of flower development, performed surface sterilization by soaking the ovaries in 70% ethanol for 40 seconds and then soaking them in 1% sodium hypochlorite solution for 20 minutes. The explants then report that they were cultured for embryo formation in CBM growing medium (Gemes-Juhasz et al., 2002) with 0.01 mg/L, 0.1 mg/L, and 1 mg/L TDZ added. After 7 – 14 days, the developing embryos were transferred to modified growing media formed from different doses of 0.01 mg/L, 0.05 mg/L, 0.1 mg/L and 0.5 mg/L NAA doses and 0.1 mg/L BA after 7 – 14 days. The study reports that at the end of the study, callus formation occurs, but no conversion to plant occurs in any embryo.

Researchers who used the technique of producing haploid plants by gynogenesis method in order to obtain homozygous individuals in a short time that could be genetic material for pumpkin breeding studies (Bing et al., 2006) supported the N6 growing medium they determined as the culture medium with 2,4-D, NAA and BAP plant growth regulators. The scientists, who reported that they transferred the developed embryos to the hormone-free N6

environment by induction, stated that there were 120 plants developed from the embryo sac and that they were able to transfer 71 of them to the soil. Also at the end of the study, it is argued that the stage of development of the ovules, the nutrient medium, the donor genotype, and the date of cultivation of the donor plant are the factors that affect the gynogenesis studies.

In Shalaby (2007) studies on haploid embryo and haploid plant retrieval developed by ovule culture method to produce homozygous breeding lines in summer squash, the variety focused on the position of the ovary on the plant, the effect of pre-temperature application, and the effect of the sucrose rate in the induction growing medium. At the end of the study, he says that genotype is a very effective factor in pumpkin gynogenesis studies and that the highest response is obtained from Raad F1 variety of the 12 genotypes cultured. The researcher, who also evaluates the position of the ovary on the plant, states that the second female flower in which the ovary receives the highest response is the second female flower, while the highest response is obtained as a result of 32 °C 4 days of waiting for 4 days from the ovaries subjected to 0, 4, 7, 12 days of pre-temperature application at 4 °C and 32 °C. He says that the MS nutrient mediums modified with three different doses (30 g/L, 60 g/L, 90 g/L) were obtained from 30 g/L sucrose content. At the end of this study, it was determined that 65% of the plants that developed were haploid and 35% were double haploid.

"Cucumber mosaic virus (CMV)", which is one of the deadliest viruses in cucumbers, is among the issues that breeders work on the most in order to gain genetic resistance. Plapung et al. (2014), aiming to obtain these virus-resistant strains in a short time with haploid plant extraction applications, applied the gynogenesis technique in 68 cucumber lines. They added BAP:IAA hormones to the MS environment in a ratio of 2:1 for embryo formation and cultured the ovules in this growing medium. They report that they used MS + 2:1 (IAA: BAP) + 5 ppm (one millionth) $AgNO_3$ or MS + 5 :1 (2ip (6-(gamma, gammadimethylallylamino) purine): IAA) + 5 ppm AgNO3 nutrient medium for plant regeneration, that 42 clones and plants were propagated from 14 plants, and that 14 plants were haploid. From the homozygous lines obtained, they indicate that there are genotypes with virus resistance.

Plapung et al. (2014b), who kept the cucumber ovaries collected 1 day before the anthesis in 10% calcium hypochlorite and 3% sodium hypochlorite solution for 20 minutes, kept the ovules of the ovaries in 5 ppm $AgNO_3$ + CBM nutrient medium for 1 month to obtain haploid embryos and plants. They then transferred the developing ovules to the individually modified nutrient medium with Kin, BAP, 2ip: IAA in ratios of 2:1, 3:1, and 4:1 ppm. Scientists who converted incubated embryos into plants in a nutrient medium with BAP, 2ip: IAA content in the ratio of 2:1, 3:1, 4:1, and 5:1 ppm concluded that the number of plants obtained and the effective hormone doses varied according to the genotype.

Kurtar et al. (2018) said that *Cucurbita maxima* and *Cucurbita moschata* were better produced through parthenogenesis and androgenesis, but that genotype dependence affects the result at a high rate. For this reason, they applied the ovarian culture technique, which they saw as an alternative technique, in their study. The researchers, who collected the female flowers of four different winter squash and two different pumpkin lines one day before and in the anthesis for calculus development, used the MS growing medium they modified with 2,4-D and BAP for callus development. For plant development, they state that they perform it in growing environments modified with BAP, NAA, TDZ and IAA hormones. At the end of the study, they found that the donor plant genotype influenced gynogenesis studies with different culture periods. It was determined that the highest embryo formation occurred in the environment containing 23 4.0 mg /1 BAP + 0.05 mg/L NAA + 0.1 mg/L TDZ, and the most appropriate flower intake time was the anthesis period. In addition, when the ploidy levels of 122 plants were examined, they reported that 70 plants were determined as haploid, 46 plants as diploid and the others as mixoploid.

In the gynogenesis study conducted by Zou et al. (2018) with the idea that the studies on ovary culture in watermelon were inadequate and ineffective, they tried to create a combination of growing medium and embryo regeneration medium for an effective haploid embryo formation. In addition to these, they also conducted experiments on topics such as genotype effect, retention time in induction environment, and appropriate ovary stage to be explanted. Researchers evaluated the gynogenesis efficacy of 8 different genotypes and noted

that donor plant genotype is the key factor for embryogenesis. In the study, 50 plants were obtained and when the ploidy level of these was examined, it was determined that 48 of them were haploid and 2 of them were diploid. According to genotypes, the embryo formation rate ranged from 0% to 15.14%. The most effective embryo formation and embryo regeneration mediums are reported to be MS + 3 mg/L 2.4 D + 2 mg/L BAP, 0.5 mg/L NAA and MS + 0.8 mg/L BAP + 0.2 mg/L NAA, respectively. In addition, it is recommended to keep the embryo formation environment for 13 days and to collect the ovaries from the donor plant during the full anthesis period.

Nitwatthanakul and Tiraumphon (2018), who conducted research on some factors affecting gynogenesis, such as the callus formation environment and donor plant genotype in melon ovule culture, report that they conducted trials on three Thai melon varieties (Green Net, Honeydew and Pot Orange). In addition, the researchers who specified three different nutrient mediums for callus differentiation found that the most callus development (75.54%) occurred in MS growing medium with 0.20 mg/L BAP and 0.40 mg/L NAA. They state that there is no significant difference between the growing medium and genotypes.

Zhu et al. (2019) aimed to establish an effective protocol for obtaining haploid embryos and plants in watermelon (*Citrullus lanatus*) with the unfertilized ovarium culture technique. For this purpose, they focused on the appropriate sterilization technique, the duration of the cultured ovules in the dark, and the combination of embryo formation and regeneration growing environment. At the end of the study, it is reported that soaking unfertilized watermelon ovaries in 10% NaClO solution for 10 minutes is suitable for the prevention of contamination, and that soaking ovaries in dark conditions for 14 days after culturing promotes ovarian growth. In addition, it is stated that MS medium containing 0.5 mg/L NAA, 1.0 mg/L BAP and 0.5 mg/L Kin is effective in embryo differentiation, while M2 medium containing 0.02 mg L-1 TDZ, 0.5 mg L-1 NAA, 0.5 mg L-1 BAP is suitable for plant regeneration. When the ploidy levels of the obtained plants were examined, it was determined that they were haploid, diploid, and tetraploid. SSR marker analyses report that diploid and tetraploid plants have a homozygous structure and that they are double haploid and tetra haploid.

Black and Yellow (2019) investigated the ability of donor plants to form haploid embryos and plants through gynogenesis and the effects of polyamines (spermidine (Spd), putresin (Put)) on ovule culture in three different gum squash genotypes and three different dark green squash genotypes. Stating that they use MS + 5 mg L -1 2,4-D containing growing medium for embryo stimulation, the researchers state that they add Spd and Put to the nutrient medium in different doses (40 μM L -1, 80 μM L -1 and 160 μM L -1). The researchers also evaluated different doses (80 μM L-1, 160 μM L-1, and 320 μM L-1) in which Spd and Putt were used together in a 1:1 ratio. According to the results of the research, in terms of egg development and callus formations, they state that the response from different Spd and Put applications vary according to genotype and polyamine administration is more effective on ovule development than the control group. They argue that 40 μM L-1 Put is the best polyamine application for callus formation, but no plants are formed at this rate, and it would be beneficial to use higher doses of polyamine for plant formation.

Considering that the studies on gynogenesis for winter squash (*Cucurbita maxima*) and pumpkin (*Cucurbita moschata*) are quite limited, researchers applied the technique of unfertilized ovarian culture on a hybrid of 9 winter zucchini, 3 pumpkins, and 1 winter squash (Zou et al., 2020). In the study, where it is aimed to determine the optimum induction medium content, it is stated that the most effective nutrient medium for embryo formation is the I3 medium containing 1 mg L-1 BAP. It is said that the highest embryo-like structures (ELS) are formed in the L-13 (hybrid) genotype at a rate of 39.67% and the conversion to plant is 10.18%. Of the 59 plants whose ploidy level was determined by the flow cytometry method, 18.64% were haploid, 3.40% were myxoploid, 32.20% were diploid and 45.76% were tetraploid.

Yıldız and Solmaz (2020) tried to stimulate female gametes for embryo formation by ovule-ovary culture method in watermelon. The researchers report that they used 4 different genotypes in their study, Kar 23, Kar 37, Kar 116, and Kar 147, and that they planted the ovaries they collected one day before anthesis in different doses of 2.4-D (11.36 μM/L and

22.72 μM/L) and TDZ (Thidiazuron) (12.5 μM/L, 25 μM/L). Scientists who also investigated the effectiveness of polyamine by adding 500 μM L-1 Put or Spd and Put + Spd polyamines to these nutrient mediums stated that the genotypes respond to the ovule-ovary technique at different levels. They report that embryo formation and plant development occur in MS nutrient media with 11.36 μM/L 2.4-D + 25 μM/L TDZ + 500 μM Spd, 11.36 μM/L 2.4- D + 25 μM/L TDZ + 500 μM PUT and 22.72 μM/L 2.4-D + 12.5 μM/L TDZ in Kar 37 and Kar 147 genotypes.

Gemes-Juhasz et al. (2002), who aimed to create an effective protocol in gynogenesis technique by using the parthenocarpic 5 cucumber breeding line, researched to improve the stage of ovary removal from the donor plant, pre-temperature applications, and embryo formation environment. Stating that they cultured the ovaries they received at different times (3 days before anthesis, 6 days before anthesis and anthesis) from donor plants developed in greenhouse conditions, the scientists report that they used 150 explants from each genotype in their research. In addition, the cultured ovaries were subjected to pre-temperature application at 24 °C, 28 °C, and 35 °C for 2-10 days and it was determined that the most effective pre-application process was 35 °C and that the waiting time changed according to the genotype. Ovaries cultured for embryo formation in CBM + 0,02 mg/L TDZ + 4% sucrose growing medium were kept in dark conditions for 2-5 days from the time of culture. They report that they added 0.05 mg/L NAA and 0.2 mg/L BAP to the CBM nutrient medium in order to turn the embryo into a plant and that the maximum embryo formation rate on the basis of genotype was 18.4% and the plant formation rate was 7.1%.

Sagi et al., (2004), who carried out gynogenesis studies in different plants, aimed to obtain haploid embryos and plants by applying the same protocol to zucchini and cucumber plants. Stating that they use ovaries from donor plants for 3-4 weeks from the first flowering, the researchers state that they collect the unfertilized ovaries 6 hours before the anthesis and subject them to the pre-temperature application at different degrees (24 °C, 28 °C, or 35 °C) and durations (1-5 days) in the dark after culture. They state that for the formation of embryos in the ovary, 0.02 mg/L TDZ and 4% sucrose content CBM nutrient medium is used, and for

the developing embryos to turn into plants, ovaries are transferred to the nutrient medium with 0.05 mg/L NAA, 0.2 mg/L BA and 3% sucrose content. At the end of the study, they report that embryo development occurs within 4-6 weeks from the cultured pumpkin ovaries, a maximum of 12 embryos and 2-3 embryos, in general, are formed from the ovaries, and about half of the embryos are transformed into plants. It is reported that embryos are formed by direct embryogenesis 6-12 weeks after cucumber ovaries, but the majority of embryos stop developing or turn into plants with abnormal structures. They report that a one-unit increase in pre-application temperature affects embryo formation.

Suprunova and Shmykova, (2008), who tried ovule culture in their study on haploid plant production techniques in cucumbers, sterilized the ovaries they collected in anthesis, 6 hours before anthesis and 2 days before anthesis, and then removed their ovules and planted them in a modified MS nutrient medium with different hormone derivatives and hormone doses with 5% sucrose content. The ovules, which developed under 22°C conditions for two weeks in dark conditions, were then kept for the formation of callus under the conditions of 14 hours of illumination in MS growing environment with 0.05 mg/L NAA, 0.2 mg/L BA, 3% sucrose content. Developing calluses were transferred to MS with 0.02 mg/L NAA, 0.4 mg/L BAP and 3% sucrose content for exile incentive. At the end of this study, they report that embryos were formed in the highest Gordian genotype and that 0.5% of the plants were obtained as a result of taking the calluses developing from the ovules to the shooting environment.

Diao et al., (2009) aimed to create haploid plants using the gynogenesis technique in six different cucumber varieties and tried to improve the haploidization technique by conducting research on different subjects. They aimed to determine the most effective duration by subjecting unfertilized ovaries of all genotypes to 2, 3, and 4 days of pre-temperature stress at 35 °C. The researchers added TDZ hormone at different doses (0.01 mg/L, 0.02 mg/L, 0.04 mg/L) to the embryo formation medium and measured the responses of 6 different genotypes to these doses. In addition, they report that the embryo-forming efficacy of 10 mg/L AgNO3, which was added to the growing medium on two different genotypes, was also tested. At the end of the study, they generally state that the pre-temperature stress of 35 °C 3 days in the dark increases the embryo rate for all genotypes, and that the addition of

0.04 mg/L TDZ hormone to the induction medium is the appropriate dose for creating embryos at the highest frequency (72.2%), that AgNO3 has no clear effect on embryo formation, but causes the embryo to have a green and more developed structure. In addition, the researchers say that only 2 of the 40 plants they obtained were detected as haploid, and according to the SSR marker analysis applied in the remaining 33 plants with diploid structure, 17 of them were double haploid.

Wei et al., (2010) who examined the effects of different polyamine derivatives (Spermidine (Spd), Spermin (Spm), Putresin (Put), and Cad) and different hormone derivatives on gynogenesis in cucumber, identified 5 genotypes with high gynogenesis response and 4 genotypes with low gynogenesis response as donor plants. Researchers evaluated the embryo formation frequencies by keeping the ovaries for different periods of time (3, 6, 9, and 16 days) in the embryo formation medium containing ZR (zeatin-riboside), IAA, ABA (Abscisic acid), and GA3 (Gibberellic acid) reported that IAA/ZR, ABA/GA3, IAA/GA3 hormone combinations promoted gynogenesis to a small extent. It is reported that 3 days of soaking in the IAA/ABA combination increases the response to gynogenesis, while varieties with high gynogenesis responses interact with environments with low IAA and high ZR and GA3. They found that genotypes with high embryo formation responded to environments with a high content of Spd + Spm and low Put content. However, they report that the addition of Spd to the induction medium did not significantly increase the gynogenesis response.

Li et al., (2012) aimed to create an optimal nutrient medium for embryo formation and plant regeneration of embryos in their ovarian culture studies on three different varieties in Cucumis sativus L. The ovaries were subjected to temperature stress for 3 days after being cultured and at the same time, they provided embryo stimulation in growing environments containing TDZ at different rates. According to the findings obtained in the study, ovaries report that the combination of regeneration medium with 0.05 mg/L NAA and 0.4~0.6 mg/L BAP content should be preferred for the differentiation of the embryos that form the highest embryo in induction environments with 0.06~0.08 mg/L TDZ content. They report that plants were obtained from all genotypes used in the trial and that genotype, temperature pre-

administration time, stage of development of the ovary before culturing, and hormone concentrations affected gynogenesis.

Researchers who examined the effects of different TDZ hormone concentrations added to the CBM nutrient medium to form haploid embryos and plants in Chinese cucumbers (Li et al., 2013) also examined the embryo regeneration ability of AgNO3 at different concentrations. According to the study, it was determined that the best induction media were CBM + 0.03 mg/L and CBM + 0.07 mg/L TDZ content growing mediums, and it was found that it was beneficial to add 5-10 mg/L AgNO3 to the regeneration environment in order to transform the embryos developing in the induction environment into plants. While the highest embryo formation rates were 7.85-12.14% in the cultured genotypes, the highest plant regeneration rate was in CBM nutrient medium with 0.05 mg/L NAA, 0.2 mg/L BA, and 5 - 10 mg/L AgNO3 content. According to the flow cytometry analysis of the obtained plants, it was determined that 80% of them were haploid. The researchers also report in this study that ovaries should be taken during the anthesis period.

Tantasawat et al., (2015) researched the acquisition of effective induction and embryo differentiation environments, ELS (embryo-like structures), and the application of pre-temperature effective to callus formation (14 days dark at 25 °C, 35 °C 3 days after soaking 35 °C and then 11 days in darkness) in ovarian culture studies conducted in cucumber (*Cucumis sativus*) and 35 °C 3 days of soaking. After sterilizing the ovaries they collected 1 day before the anthesis, they planted 5 cultured media containing different hormone derivatives and different sugar doses modified with MS growing medium. They also reported that they added putresine to one of the mediums. Ovaries that completed the pre-temperature stress period were transferred to growing mediums (D1, D2, D3) with 3 different contents combined with MS growing medium. At the end of the research, ELS and callus were formed in all genotypes, the highest ELS formation occurred in 1 mg L -1 TDZ, 1 mg L -1 BAP, 3% sucrose content in I2 environment (60.4%), and all three embryo differentiation environments acted equally. They say that the % of callus formation increases in the I5 environment with polyamine content. In addition to these, it is stated that callus is formed at close rates at two degrees of temperature, which is a pre-abiotic stress factor, and that it is

slightly more effective to leave ELS formation at a temperature of 25 ° C for 14 days. They found that the genotype had a great effect on ELS and callus formation.

Çetinkaya, (2015) develop an effective nutrient medium for gynogenesis in cucumber, she used three different basic nutrient mediums (MS, Miller (Dirks, 1996), and CBM) modified with 2,4-D and Kinetin in a 1:10 ratio for embryo formation. 1, 2 days, and 6 hours before anthesis, the ovaries were collected and left in a 20% commercial NaClO solution for 20 minutes, then peeled off their surfaces and transferred to the embryo formation medium. The cultured ovaries were kept in dark conditions of 35 °C for 2 days and 24 °C for 3 days and then in a bright environment for 9 days and transferred to embryo differentiation environments. For the differentiation of the embryos, the three basic growing mediums were modified with NAA and BAP in a ratio of 1:4. In the three different F1-level cucumber breeding cultivars used in the study, embryos and plants were obtained at varying rates according to genotypes. It is stated that the genotype that responds best in terms of embryo and plant formation is genotype no. 583, that the developing embryos and plants are directly stimulated, but that some plants that form turn into embryogenic callus in the regeneration environment and form new plants. According to the results of the trials established every week, it is reported that plant age is an effective factor in embryo formation in cucumbers and that the % of embryo formation decreases with the increase in plant age. According to the number of ovaries cultured, 84.26% embryos develop and 42.28% plants are obtained. The genetic level of 114 of the 137 plants obtained was determined by the chloroplast counting method in the stoma, indicating that 87 of the plants were haploid, 9 were diploid and 18 were myxoploid.

Chen et al., (2016) tried to develop a different gynogenesis protocol in seven different cucumber genotypes, aiming to obtain plants that have a direct double haploid structure. In the study, they planned to keep the ovaries they collected two days before anthesis in a B5 medium containing 10, 20, 30, 40, and 50 mg/L colchicine and to simultaneously conduct callus development, plant regeneration, and chromosome folding events. As a result, the highest calculus development was found in 20.19% of A3 genotypes, and it is reported that

only A3 (10.47%) and A11 (16.67%) genotypes determined plant formation. The double haploid % of plants whose ploidy levels were determined by flow cytometry analysis was 55.56% in A3 genotype and 69.23% in the A11 genotype. It is also argued that it is appropriate to apply colchicine at a dose of 30 mg L -1 to the growing medium for direct double haploid plant retrieval and that values above this dose will be fatal in ovaries.

Golabadi et al., (2017), in their three-stage study, first wanted to try the effect of pre-temperature stress on cucumber gynogenesis, stating that while some of the cultured ovaries were tested with 35 °C 4 days of high-temperature stress, no application was made to the other part. They also cultured the ovaries in a semi-solid MS nutrient medium supported by various auxin and cytokinin hormones (2,4-D, Kin, IAA, IBA, NAA, BAP) during this process. According to the results obtained from this, they transferred the ovaries of successful genotypes to the growing medium containing different combinations of 2,4-D, Kin, IAA, IBA, NAA, and BAP hormones at varying rates for embryo formation. In the second trial, the study was conducted to compare it with the best genotype and a local variety, and the ovaries of these genotypes were cultured in growing environments that varied according to their AgNO3 content. Researchers report that in the first trial, the highest embryo was formed from the growing medium with a hormone content of 0.5 mg/L NAA + 0.7 mg/L 2.4-D + 1 mg/L KIN + 1.8 mg/L BAP applied thermal shock and the highest callus formation occurred in the growing medium with 1.5 mg L-1 NAA + 0.7 mg L-1 2.4-D + 1 mg/L KIN + 1.8 mg/L BAP content. After subjecting the two genotypes that were determined to be the best in this trial to the second experiment, they found that 4 mg/L BAP and 1.5 mg/L 2.4-D were the optimum hormone content for embryogenesis. In the last trial, they determined that the NBDC6*6/32441 genotype, which is compared with the local Iranian cult, did not have the ability to create embryos and calluses as much as the local cult. In addition, they report that AgNO3 increases the rate of embryo formation, on the contrary, it decreases the formation of callus.

Sorntip et al. (2017), in their gynogenesis study on double-haploid response stubborn cucumber genotypes, aimed to develop the best embryo formation, embryo differentiation and plant regeneration environments. Stating that they kept the ovaries in the dark for 1 week,

2 weeks and 3 weeks according to the order of transfer in the growing environments they created with different hormone and vitamin contents, the researchers state that ELS formation is significantly affected by embryo differentiation and plant regeneration environment. It is reported that the ovaries transferred from D2 to the plant regeneration medium MST3+ of the differentiation medium constitute ELS at the highest rate (59.89%), that the ELS turn into shoots in plant regeneration environments and that the resulting plants provide root growth in the MS environment. It was also emphasized that the genotype effect did not change the outcome of this study too much. In the study, they determined that of the 10 plants that survived after being transferred to the soil, 3 were haploid, 1 was triploid, and 6 were diploid. They confirmed that the 6 diploids that developed were in the double haploid structure by means of simple sequence repetition analysis.

Asadi et al. (2019), who conducted gynogenesis study in cucumber, investigated the effect of different TDZ concentrations (0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08 mg/L) added to the growing medium on haploid embryo and plant formation. In the study, researchers who conducted experiments on Esfahani and Beith Alpha genotypes preferred MS growing medium as the basic growing medium. They report that the ovaries collected 1 day before the anthesis are kept at 35 ° C for 3 days after being cultured and embryo formations occur after 2 weeks. Then, they state that the ovaries containing the embryos are subcultured in a growing medium with 1.5 mg/L GA3 content for the maturation of the embryos and that the growing medium with 0.05 mg/L NAA and 1.5 mg/L BAP content is preferred for organogenesis. Scientists who report that the highest rate of embryos was obtained in TDZ 0.08 mg/L content say that 23.33% embryos were formed from Esfahani genotype and 20.66% from Beith Alpha genotype. In addition, they found that all of the plants that developed in the research had a diploid structure and as a result of the analysis, these plants had a double haploid structure.

Özdemir et al., (2019) investigated the effect of different thermal shock applications and different doses of sucrose in growing environments in their cucumber ovary culture study to develop an effective double haploid protocol. The researchers, who also examined the

haploid embryo and plant-forming efficacy of three commercial cucumber genotypes, Horizon F1, Meriç F1, and Cedar F1, collected female flowers that were at the stage 1 day before the anthesis and cultured them after applying sterilization procedures and kept the ovaries in the dark at a temperature of 35 °C or 27 °C for 1 week. They also aimed to provide embryo stimulation in MS nutrient environments containing sugar doses of 30 g/L, 60 g/L, 90 g/L, and 120 g/L. Researchers who observe haploid embryo and plant formation that vary according to genotypes state that heat shock applications and different sugar concentrations generally promote the formation of callus. In the study, it was seen that the application of thermal shock suitable for embryo formation varied according to genotypes, and it was determined that I2A (30 g/L sucrose) and I2D (120 g/L sucrose) application encouraged embryo formation and that I2B (60 g/L sucrose) environment formed more callus.

Domblides et al., (2019a) conducted a comprehensive gynogenesis study in the Cucurbitaceae family and focused on issues such as genotype affecting double haploid technology, growth status of the donor plant, embryo sac status suitable for embryo formation, culture media components (embryo formation, plant regeneration), *in vivo* adaptation. They stated that cucumbers in 12 different genotypes, winter squash in 5 different genotypes, summer squash in 9 different genotypes, 1 paty pan squash, and 1 Styrian squash were used in the study, and they say that the appropriate explant stage for embryo development should be ovaries containing mature ovules just before unfertilized anthesis. The IMC induction medium produced by the researchers who conducted the study, which included NLN nutrient medium vitamins, MS nutrient medium amino acids, 30 g/L sucrose, 200 mg/Lampicillin and 7 g/L agar, is claimed to be the best medium for developing DHs. It is underlined that TDZ (0.2 mg/L, 0.02 mg/L) and 2.4-D (1 mg/L, 2 mg/L) added to the culture medium in summer squash and cucumber positively affect the development of ovules, cucumber ovaries should be subjected to thermal shock at a temperature of 32 °C for 7-10 days and the culture medium should be refreshed every 7 days. They report that 55 embryos were formed in summer gourds, 20 in cucumbers, 9 in winter gourds, 2 in patty pan gourds, and 1 in Styrian gourd per ovary cultured.

Domblides et al., (2019b), who conducted studies on unfertilized ovary culture in cucumbers, subjected 8 promising cucumber genotypes to trial. The researchers, who reported that they carried out their studies in accordance with a previously developed protocol in the trial, added 30 g/L sucrose supplemented with 200 mg/L ampicillin and 0.2 mg/L TDZ to the IMC (Domblides, 2019a) growing medium. They report that the highest embryo was obtained from female flowers 2.1-2.6 cm tall, and that callus and embryo formation were observed in all 8 genotypes used in the study, but plant regeneration occurred in only 6 genotypes. The researchers, who produced a total of 26 plants, stated that the maximum induction of gynogenesis was 63.1% in genotype 1810, but the highest plant growth was in genotype 1763 (12 pieces).

Erol and Sarı, (2019) conducted their studies on 5 different hybrid varieties in their study where they examined the effect of polyamines on embryo formation with the gynogenesis technique in cucumbers. They report that the embryo formation environment is created by modifying the MS nutrient medium with 0.18 Mm/L TDZ. They also added putresin and spermidine (Spd) polyamines to their nutrient medium by combining these polyamines together (1:1, v:v) at concentrations of 40, 80, 120, 160, and 200 μM/L and 80, 160, 240, 320 and 400 μM/L separately. As a result of the study, they saw that different Spd and Put contents gave different results according to the types and reported that Cemre F1 genotype in Spd-containing environments, Altay F1 type in Spd + 2,4D containing environments, Altay F1 type in Put containing Put, Sardes F1 type in Spd + Put containing environments responded more positively to ovule development. In addition to the solid media culture study they conducted, the researchers who tried to develop the ovules in liquid culture also used the same hormone and basic nutrient media content as the solid media culture and supported the liquid nutrient medium with polyamines with concentrations of Spd 160 μM/L, Put 160 μM/L and Spd + Put 320 μM/L (1:1). At the end of this study, they observed that the use of polyamines stimulated embryo development and callus development and that two haploid plants developed from the ovaries of Cemre and Altay varieties cultured.

CHAPTER 2

MATERIAL AND METHOD

For studying haploid plant production through ovary culture in cucumber, the plants were grown in the open field at Agriculture research fields, Faculty of Agriculture, Erciyes University. The work was done at the Tissue Culture Laboratory at Betül Ziya Erden Genome and Stem Cell Research Center (GENKOK), Erciyes University, Kayseri, Türkiye. The duration of the research is from May 2021 to July 2022.

2.1. Material

Two different cucumber cultivars had been used in this study and the seedlings were bought from Anamas Tohum in Antalya, Türkiye. Two cucumber varieties were 'Kros' and 'Silah'. 32–37 days old cucumber plants were selected as donor parents for picking female flowers for ovary culture. The flower samples were taken after the second female flower started appearing.

2.2. Method

2.2.1. Preparation Of Field And Growing Cucumber Plants

The elemental part of any field experiment is field preparation. After ploughing with the help of tractor, rows were made and then mulching was done as shown in Fig. 2.1. Mulch is a black sheet of plastic used to resist weed growth and water evaporation. Before mulching, pipes were set for drip irrigation and then seedlings were sown alternatively and ½ to 1 inch deep in the soil. The fence was made around the field to make sure that no animal could set foot into our field and eat or destroy small plants. Plants were irrigated and examined regularly.

Fig. 2.1 Mulch And Drip Irrigation Establishment In The Field

2.2.2. Collecting Female Flowers As Sample

For studying haploidy, unpollinated flowers were used and pollination was controoled with clips.Flower samples were taken at three stages 1) 12 hours before anthesis, 2) 12 at the time of anthesis, it was a stage when flowers were just about to open or open so we needed to extra care for this stage. For this we closed the flowers earlier like day before the anthesis and then collect them at the time of anthesis. 3) 12 hours after anthesis, it was a time when flowers were done with pollination normally, however, we closed flowers with the help of clips as shown in Fig. 2.2 to restrict unnecessary pollination. All three flower stages are shown in Fig. 2.3. We used an ice box (Fig.2.4) to transfer flowers from the field to the tissue culture laboratory to keep them fresh and also chilling temperature to guarantee the quality of flowers.

*Fig. 2.2.*Clipping Of Flowers To Avoid Pollination

Fig.2.3. Flower Stages; A (12 Hours Before Anthesis), B (At The Time Of Anthesis) And C (12 Hours After Anthesis)

Fig. 2.4. Ice Box

2.2.3. Preparation Of Growth Mediums

The primary focus in haploid studies is to find the appropriate hormone concentration and nutrient medium for callus and embryo stimulation. For callus induction, 6 different types of hormonal combinations were used having MS medium in common. 4 different hormonal combinations were prepared for embryo maturation along with the MS kept in common. Both combinations of hormones i.e., for callus and embryo, have been listed in Table 2.1.

In each medium, 30g/L Sugar and 4.4g/L Agar, and 7.5g/L MS were also kept in common.

Table 2.1. Combinations Of Plant Growth Regulators Used In The Experiment

	Medium	M.S. (g/L)	Agar (g/L)	Sugar (g/L)	2, 4-D (mg/L)	Kn (mg/L)	TDZ (mg/L)	NAA (mg/L)	BAP (mg/L)
Induction Medium	I1	4.4	7	30	0	-	-	-	-
	I2	4.4	7	30	0.5	1	-	-	-
	I3	4.4	7	30	1.0	5	-	-	-
	I4	4.4	7	30	1.5	10	-	-	-
	I5	4.4	7	30	-	-	0.06	-	-
	I6	4.4	7	30	-	-	0.12	-	-
Maturation Medium	M1	4.4	7	30	-	-	-	0.5	2
	M2	4.4	7	30	-	-	-	1	3
	M3	4.4	7	30	-	-	-	1	4
	M4	4.4	7	30	-	-	-	0.05	-

All the chemicals and hormones were measured accurately on a weighing instrument as shown in Fig. 2.5. Mediums were mixed in the conical flask and then pH is maintained to 5.7-5.8 with the help of a pH meter by adding acid or base if required.

Fig.2.5. Preparation Of Mediums (Taring (Left);Measuring On Weighing Scale (Middle) And Pouring Into The Flask (Right)).

After the prepared nutrient media were adjusted to pH 5.8. It was sterilized in an autoclave for 20 minutes at 121°C. The media removed from the autoclave was kept inside a sterile cabinet cool down to room temperature. Then It was poured in equal amounts into 60 mm Petri dishes and left to solidify. Then plates were wrapped with stretch film to be used during the experiment and kept at 4°C as shown in Fig. 2.6.

Fig. 2.6. Autoclave Machine (Left) And Autoclaving Prepared Mediums (Right)

2.2.4. Sterilization Of Ovaries

The petals of the flowers have been removed from the ovary. The ovaries were first washed with soap. After washing, they were rinsed with tap water (2.7 a) and taken into a sterile cabinet. Then, the ovaries were soaked in 75% ethanol solution for 5 minutes (2.7.b), then 1-2 drops of Tween-20 were dripped in 20% sodium hypochlorite (NaClO) solution for 15 minutes (2.7.c). After washing with chemicals, they were again rinsed with sterile distilled water for 3 times (2.7.d) as shown in Fig.2.7.

2.2.5. Culture Of Ovaries Into Induction Medium

The outer parts of the sterilized ovaries were cut with the help of sterile forceps and scalpels. Then it was peeled off in a way that would not damage the ovules. Thus, to get more benefit from the nutrient medium, the damaged parts during sterilization were also removed from the ovaries. Ovaries having ovules were cut into 2-3mm slices without damaging the ovules. The slices were then planted in petri dishes of diameter 60 mm as shown in Fig.2.8. Ovaries were kept in callus induction medium for 4-6 weeks and then transferred to embryo maturation medium.

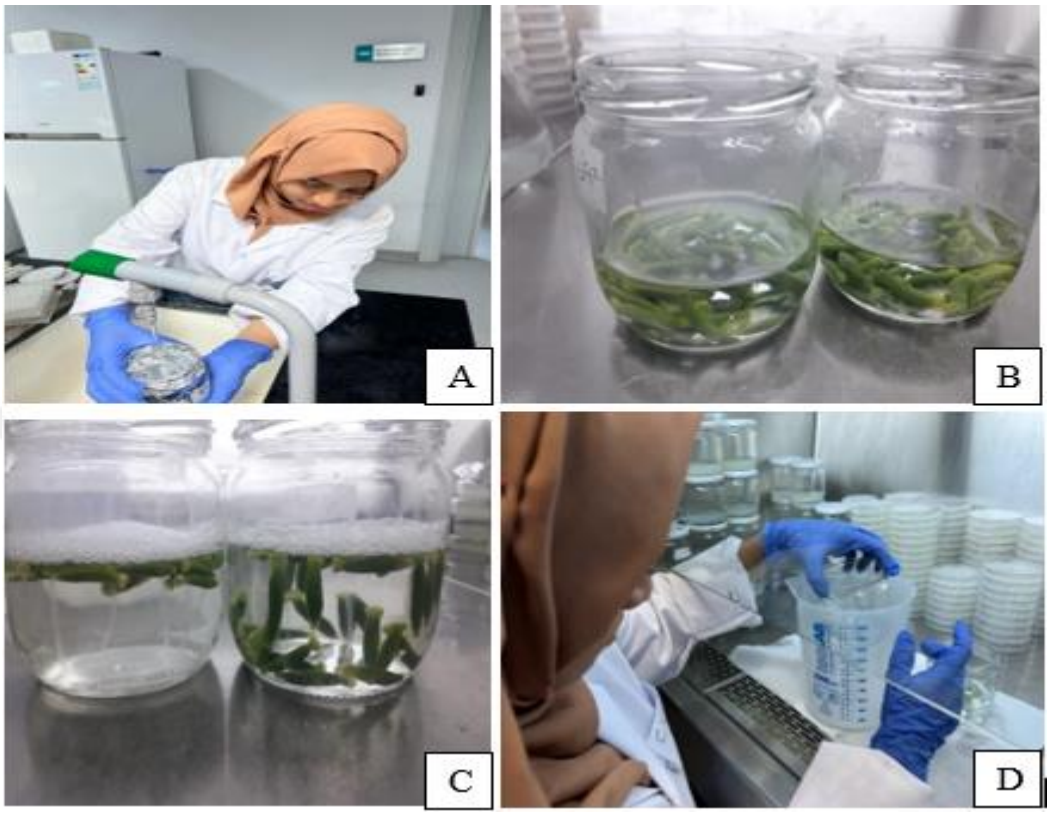

Fig.2.7. Sterilization Of Ovaries

Fig. 2.8. Culturing Of Ovaries Into Induction Medium

2.2.6. Application Of Preliminary Heat And Cold Stress And *In Vitro* Culture Conditions

The ovaries were subjected to heat and cold stress for 3 days. For each hormonal combination, total 15 petri plates were made. Out of 15 plates, 5 were kept at 35°C, the other 5 plates were kept at 4°C and the remaining 5 were directly placed at 25°C. After 3 days of heat and cold treatment, all 10 plates were shifted to 25°C growth chamber with 16/8 hours of light and dark photoperiod conditions, as shown in Fig.2.9.

Fig.2.9. Using Growth Incubators

2.2.7. Transfer Of Callus To Maturation Medium

After 4-6 weeks, the callus was transferred to the embryo maturation medium(the list of mediums is shown in Table.2.1). Callus was cut carefully inside a sterile cabin with the help of sterilized forceps and scalpels. İt was made sure to separate infected callus Petri plates from non-infected plates.

2.2.8. Statistical Analyses

When calculating the callus development, the number of calli developed in each petri dish is calculated as a %. Calculations were performed based on variety, medium, and flower stages. LSD student's test was applied to the data using JMP® software (SAS Institute, Cary, NC)

version. 8.00 and tables were created for callus and embryo production. All the data were transformed into %s before analyses.

CHAPTER 3

RESULTS

3.1. Parameters Emphasized In This Research

Callus and ELS (Embryo Like Structure) development is achieved through ovary culture by focusing on some parameters described below. Statistical results have been discussed in the following section.

The first parameter is the genotype, two different genotypes i.e., 'Kros' and 'Silah' were used in this experiment so genetipic difference was compared the overall success rate in both genotypes. Genotype plays a significant role in determining the result and thus having more one than 1 variety is the basic need of any scientific experiment. Both genotypes were treated equally from day one to day end, thus we got fair results without discrimination. Sometimes it is normal that one genotype gives different results than other under the same conditions.

The second important parameter was the Flower stage. Three different stages were chosen for this experiment which were 12 hours before anthesis (12h B/A), At the time of anthesis (A T/A), and 12 hours after anthesis(12h A/A) as they were considered significant from the literature. To make sure about timings, a proper plan was made and followed perfectly to get flawless results. Sterile clips were used each time to close the flower.

The third essential parameter was the PGRs which was planned after a careful review of the literature. 6 different types of combinations for callus induction and 4 different types of combinations for embryo maturation has been used. Each PGRs has specific effects and outcomes. To study the variety of consequences and to get desired results, the best possible combinations of hormones were manipulated.

The fourth basic parameter was temperature. According to literature heat and cold stress showed significant results in the embryo and haploid regeneration. 25°C, 35°C, and 4°C incubators were used in this scrutiny to compare and evaluate the results.

The fourth parameters are the combination of the previous 3 factors i.e., Flower stage, Hormonal combinations, and Temperature. We did a statistical analysis based on these 3 parameters and 4 combinations made by combining these 3 parameters. Overall statistical analysis was done based on 7 factors which are discussed later.

3.2. Results Of Callus Formation In 'Kros'

In genotype 'Kros', callus development was observed after 4-6 weeks of ovary culture. Calli were taken out from the growth chamber and counted watchfully for absolute results. Petri plates were kept separated on the basis of genotype, flower stage, hormone, and temperature. The number of callus per 5 explants were noted down and then all values were converted to % and angle transformation. Data was analyzed using JMP software.

Table 3.1. shows callus formation in % in three parameters i.e., PGRs *Flower stage, and PGRs*Flower stage*Temperature. In this table, values are written for callus %, value written outside parenthesis shows % value and value inside parenthesis shows transformation value. Letters after number shows difference at statistical significance level . At the end of table LSD values are mentioned for each parameter in the table.

For PGRs combination, highest callus formation is 80%, that is for 5th combination PGRs (0. /L TDZ) however, lowest value is for 1.5mg/L 2,4D+10mg/L Kn+ combination of hormone and that is 46%. In the case of hormone*flower stage, the lowest value which is 40 g obtained for 1.5mg/L 2,4D+10mg/L Kn+ 12 hour before anthesis combination while the highest number of 86 a is observed in 3 cases; 0.06 mg/L TDZ + at the time of anthesis, 0.12mg/L TDZ+ at the time of anthesis, 0.12 mg/L TDZ+ 12 hours before anthesis. In case of hormone+temperature*flower stage, highest value is observed in 3 cases; 0.06mg/L TDZ +MS+at the time of anthesis+25°C, 0.06 mg/L TDZ +MS +at the time of anthesis+ 35°C, 0.12 mg/L TDZ +12 hours before anthesis+35°C. By looking at table 3.1, we can conclude the best PGRs, best PGRs*flower stage, and best PGRs*flower stage* temperature for callus induction. TDZ hormone appeared as best PGRs in inducing callus in cucumber ovaries. In this experiment, we used TDZ in two different concentrations i.e.,0.06mg/L and 0.12mg/L and both gave almost the same results in callus formation in terms of % and angle transformation. LSD value also shows the significance of PGRs application in callus induction.

LSD value of PGRs *flower stage means it can cause a significant difference in callus induction. By looking at table 3.1, we can conclude the best combination of hormone and flower stage which is again TDZ PGR and flower stage is 12 hours before anthesis and at the time of anthesis. Statistical analysis helps us to the derived best possible combination which is very useful in future studies. LSD value for PGRs *stage*temperature is non-significant which means callus induction is independent of this combination.

Table 3.1. Results Of Callus Formation In 'Kros' Cultivar In 3 Parameters PGRS + PGRS *Stage + PGRS *Stage*Temprature

	Application			Callus formation%		
	PGRs	Flower Stage	Temperature (C)	PGRs *Stage* Temperature	PGRs *Stage	PGRs
1	I1	12 h B/A	25	40(39)	48efg(44,33)	51c(45,66)
			35	60(51)		
			4	46(43)		
		A T/A	25	53(47)	51efg(45,66)	
			35	46(43)		
			4	53(47)		
		12 h A/A	25	53(47)	53def(47)	
			35	53(47)		
			4	53(47)		
2	I2	12 h B/A	25	60(51)	60cde(51)	60b(51,44)
			35	6(51)		
			4	60(51)		
		A T/A	25	66(55)	68bc(56,3)	
			35	66(55)		
			4	73(59)		
		12 h A/A	25	60(51)	53def(47)	
			35	60(51)		
			4	40(39)		
3	I3	12 h B/A	25	53(47)	60cde(51)	53c(47)
			35	60(51)		

			4	66(55)		
		A T/A	25	46(43)	53def(47)	
			35	60(51)		
			4	53(47)		
		12 h	25	40(39)	46fg(43)	
		A/A	35	53(47)		
			4	46(43)		
4	I4	12 h B/A	25	40(39)	40g(39)	46c(43,00)
			35	40(39)		
			4	40(39)		
		A T/A	25	53(47)	55def(48,\.3)	
			35	60(51)		
			4	53(47)		
		12 h	25	46(43)	44fg(41.66)	
		A/A	35	53(47)		
			4	30(35)		
5	I5	12 h B/A	25	86(72)	77ab(63.3)	80a(67,33)
			35	73(59)		
			4	73(59)		
		A T/A	25	93(81)	86a(75.3)	
			35	93(81)		
			4	73(64)		
		12 h	25	80(63)	77ab(63.3)	
		A/A	35	80(68)		
			4	73(59)		
6	I6	12 h B/A	25	80(68)	86a(73.66)	79a(66,44)
			35	93(81)		
			4	86(72)		
		A T/A	25	86(72)	86a(72)	
			35	86(72)		
			4	86(72)		
		12 h	25	66(55)	64cd(53.66)	
		A/A	35	53(47)		
			4	73(59)		

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

In Fig. 3.1. we can see a graph describing callus formation in % in genotype ‘Kros’ versus flower stages. LSD value of flower stage spells out that it is the prime factor in callus development. 67 percent is for at the time of anthesis, 62% is for 12 hours before anthesis and 57% is for 12 hours after anthesis. The highest % was observed at the second stage which is at the time of anthesis. Flowers must be closed before the time of anthesis to avoid unnecessary pollination otherwise it destroys the whole experiment. 67% is for at the time of anthesis, that means collecting flower samples at the time of anthesis give rise to the virtuous amount of callus at the end.

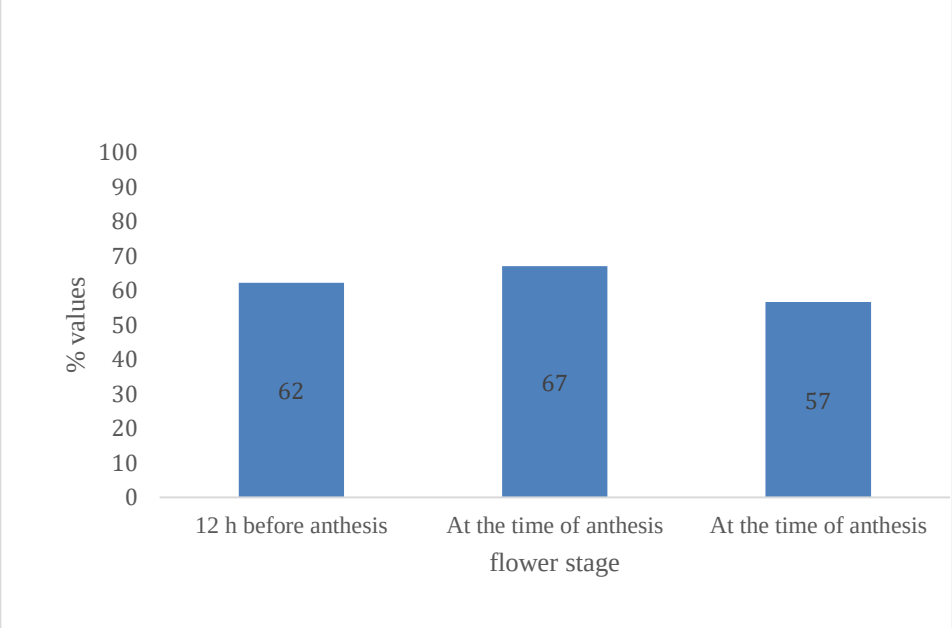

Fig. 3.1. Results Of Of Callus Formation By Flower Stage In ‘Kros’ Cultivar

In Fig. 3.2., the graph is illustrating callus formation in % in genotype ‘Kros’ versus temperature. 25°C, 35°C, and 4°C are prime temperatures in this experiment. Ovaries were placed at 2 different temperatures for preliminary stress treatment which may or may not prove effective. 64 % callus formation is given at 35C , 61% callus formation is given at 25C and 60% is given at 4C. LSD value for temperature is not significant. At all three

temperatures, callus % is almost same which makes it not significant in this experiment. Callus might show up with similar results with or without the temperature factor.

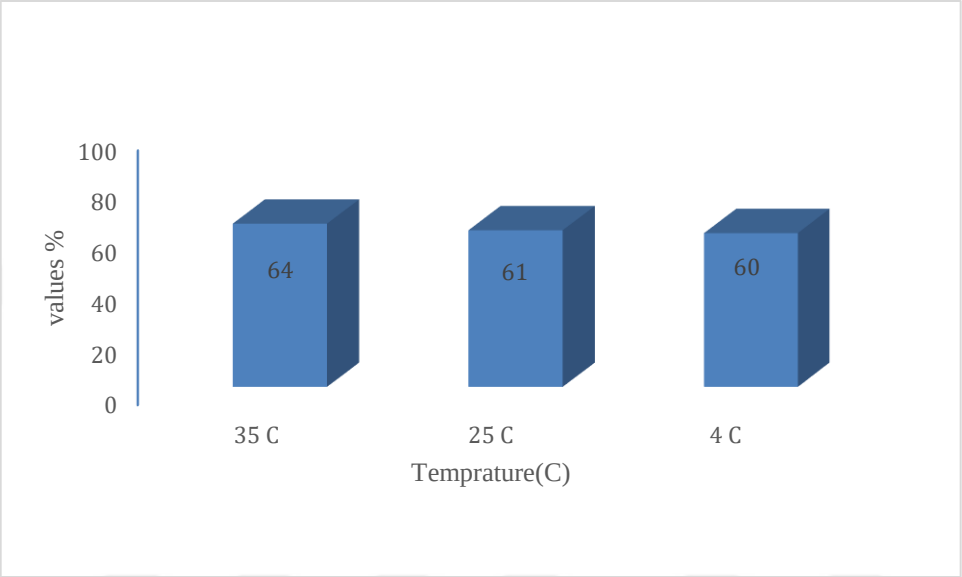

Fig. 3.2. Results Of Callus Formation By Temperature In 'Kros'

In Fig. 3.3, graph describes the relation between callus formation in % and flower stage*temperature in genotype 'Kros'. The highest value is 69% for" at the time of anthesis+ 35°C " and the lowest value is 53% for " 12 hours after anthesis+4°C. LSD value for flower stage*temperature is not significant which means it does not affect callus formation. This parameter did not worked efficiently in this experiment.

In Fig. 3.4, graph depicts callus formation % in genotype 'Kros' corresponding to hormone*temperature. Highest value of 86% is observed for 0.06 mg/L TDZ+ 25°C and lowest value of 42 % is observed for 1.5mg/L 2,4D, 10mg/L Kn+ 4°C. LSD test value for hormone*temperature is not significant which means that it seems to be unimportant in callus development.

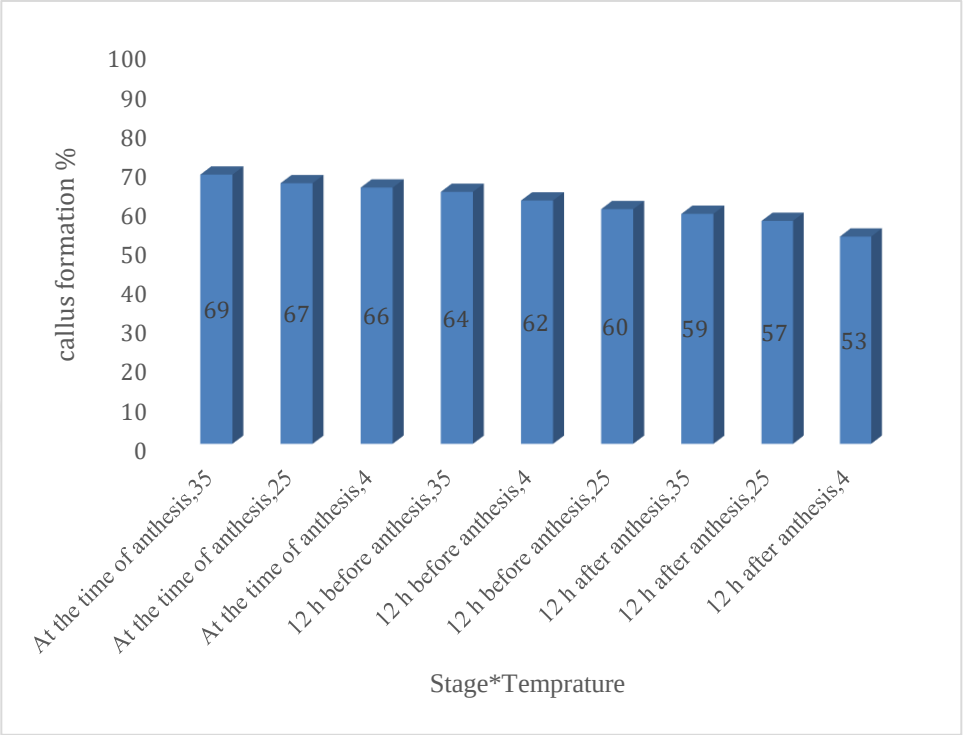

Fig. 3.3. Results Of Callus Formation By Stage*Temperature In ‘Kros’

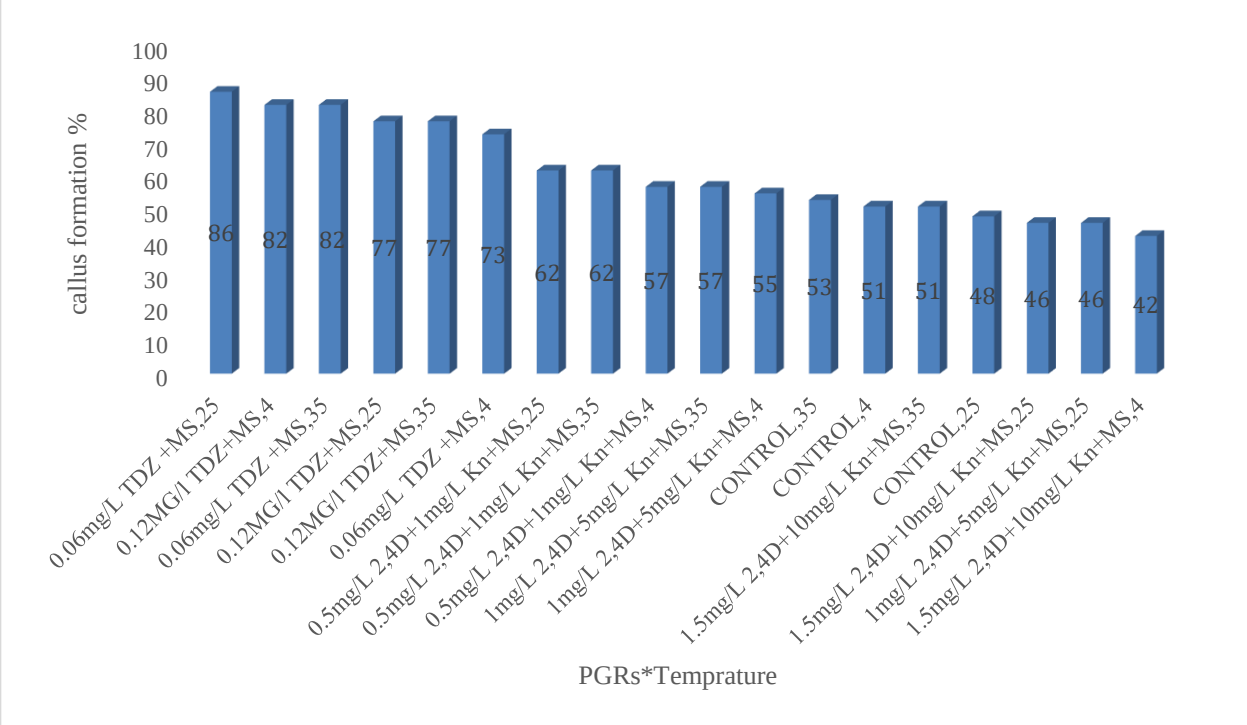

Fig. 3.4. Results Of Callus Formation By PGRs*Temperature In 'Kros'

3.3. Results Of Callus Formation In 'Silah'

İn genotype 'Silah', Callus development was seen after 4-6 weeks of ovary culture. Calli were taken out from the growth chamber and counted watchfully for absolute results. Petri plates were kept separated on the basis of genotype, flower stage, hormone, and temperature. The number of callus per 5 explants were noted down and then all values were converted to a % and angle transformation. Data was analyzed using JMP software.

Table 3.2. shows callus formation in % in three parameters i.e., PGRs PGRs *Flower stage, and PGRs *Flower stage*Temperature. In this table values are written for callus %, the value written outside the parenthesis shows % value, and the value inside the parenthesis shows the angle transformation value. Letters like a, b, c depict differences. At the end of table, LSD values are mentioned for each parameter in the table.

For parameter PGRs*temperature*flower stage, highest value is 93 which is observed for 3 combinations that are 1) at the time of anthesis+25°C+ 0.06mg/L TDZ , 2) 12 hours before

anthesis+35°C+0.12mg/L TDZ, 3) 12 hours before anthesis+4°C+ 0.12mg/L TDZ. However , lowest value of 26.66 is observed in two combination 1) at the time of anthesis+35°C+ 1.5mg/L 2,4D 10mg/L Kn., 2) at the time of anthesis+4°C+ 1.5mg/L 2,4D 10mg/L Kn. LSD value is not significant which means this parameter is not effectively involved and does not cause virtuous results.

For parameter, hormone*stage , the lowest value of callus formation is 33.33% observed for the combination; at the time of anthesis+1.5mg/L 2,4D 10mg/L Kn. The highest value is 88.88 and it is seen in the case of two combinations 1)at the time of anthesis+0.06mg/L TDZ 2)12 hours before anthesis+ 0.12mg/L TDZ. LSD test value is 12.27 which indicates its significance in callus formation. If we compare results with the table 3.1, TDZ hormone got more importance. Its performance is superior in both genotypes.

For parameter hormone, highest value is 82.22% for 0.12mg/L TDZ hormone and lowest value for callus formation is 42.96e % for 1.5mg/L 2,4D 10mg/L Kn. Hormone. LSD test value is 6.86 which is quite significant.

. Table 3.2. Results Of Callus Formation In ‘Silah’ Cultivar

	Applications			Callus formation %		
	PGRs	Flower stage	Temprature (°C)	PGRs *Stage* Temperature	PGRs * Stage	PGRs
1	I1	12 h B/A	25	73.33(59.21)	62.22bcd(52.42)	62.96c(54.52)
			35	46.66(43.07)		
			4	66.66(54.99)		
		A T/A	25	60(56.15)	57.77def(51.40)	
			35	60(50.77)		
			4	53.33(47.30)		
		12 h A/A	25	53.33(46.92)	68.88bc(59.74)	
			35	80(68.07)		
			4	73.33(64.22)		
2	I2		25	73.33(59.21)	71.11b(57.80)	54.81cd(47.98)

		12 h B/A	35	66.66(54.99)		
			4	73.33(59.21)		
		A T/A	25	53.33(46.92)	48.88efg(44.35)	
			35	40(39.23)		
			4	53.33(46.92)		
		12 h A/A	25	46.66(43.07)	44.44fgh(41.79)	
			35	46.66(43.07)		
			4	40(39.23)		
3	I3	12 h B/A	25	53.33(46.92)	46.66fgh(43.07)	48.8de(44.44)
			35	46.66(43.07)		
			4	40(39.23)		
		A T/A	25	46.66(43.07)	44.44fgh(41.79)	
			35	40(39.23)		
			4	46.66(43.07)		
		12 h A/A	25	60(51.14)	55.55efg(48.45)	
			35	60(51.14)		
			4	46.66(43.07)		
4	I4	12 h B/A	25	53.33(46.92)	53.33efg(46.92)	42.96e(40.73)
			35	53.33(46.92)		
			4	53.33(46.92)		
		A T/A	25	46.66(43.07)	33.33h(34.88)	
			35	26.66(30.79)		
			4	26.66(30.79)		
		12 h A/A	25	46.66(43.07)	42.22gh(40.38)	
			35	46.66(43.07)		
			4	33.33(35.01)		
5	I5	12 h B/A	25	53.33(47.30)	60cde(52.81)	73.3b(62.51)
			35	66.66(60)		
			4	60(51.14)		
		A T/A	25	93.33(81.14)	88.88a(75.24)	
			35	86.66(72.29)		
			4	86.66(72.29)		
		12 h A/A	25	73.33(59.21)	71.11b(59.47)	
			35	60(51.14)		
			4	80(68.07)		

6	I6	12 h B/A	25	80(68.07)	88.88a(76.78)	82.22a(71.10)
			35	93.33(81.14)		
			4	93.33(81.14)		
		A T/A	25	86.66(76.92)	86.66a(75.38)	
			35	86.66(76.92)		
			4	86.66(72.29)		
		12 h A/A	25	73.33(63.84)	71.11b(61.14)	
			35	53.33(47.30)		
			4	86.66(72.29)		

LSD_{stage} :N.S., LSD_{PGRs} :6.86, $LSD_{temperature}$:N.S., $LSD_{stage*\ PGRs}$:12.27, $LSD_{stage*temperature}$:N.S., $LSD_{PGRs\ *temperature}$:N.S., $LSD_{stage*\ PGRs\ temperature}$: N.S.

In Fig.3.5, graph is describing the results for callus formation and flower stage as parameter. Highest value is seen 63% for 12 hours before anthesis, 60 % is for at the time of anthesis and last is 58% for 12 hours after anthesis. LSD test value is not significant which mean only flower stage is not sufficient in providing virtuous results.

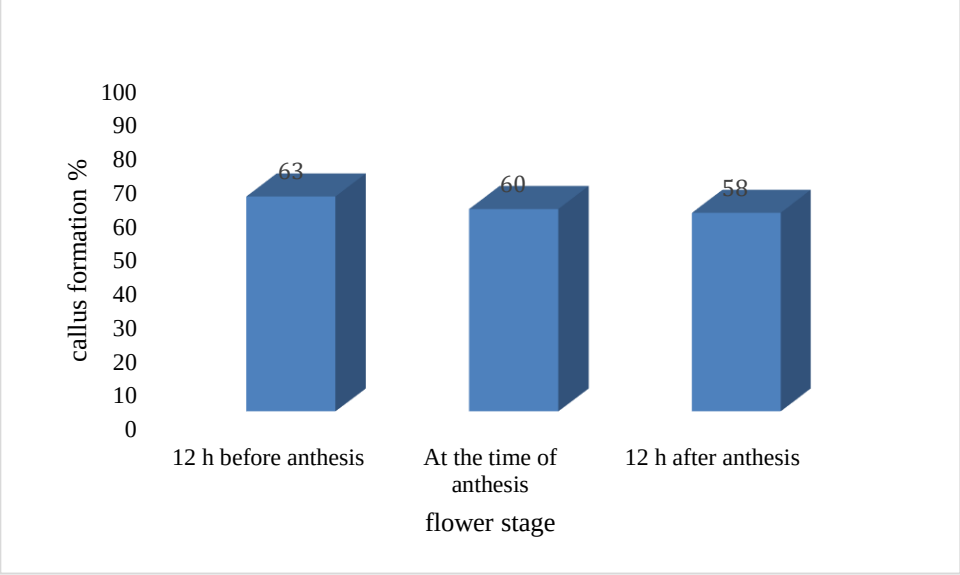

Fig. 3.5. Results Of Callus Formation By Flower Stage In 'Silah'

In Fig. 3.6, graph is illustrating the relation between temperature as a parameter in callus formation and callus %. It is clearly seen that values are not much different from each other. The highest value is 62% for 25°C and the lowest is 58% for 35°C. LSD test value is not significant which means temperature is not worthy enough.

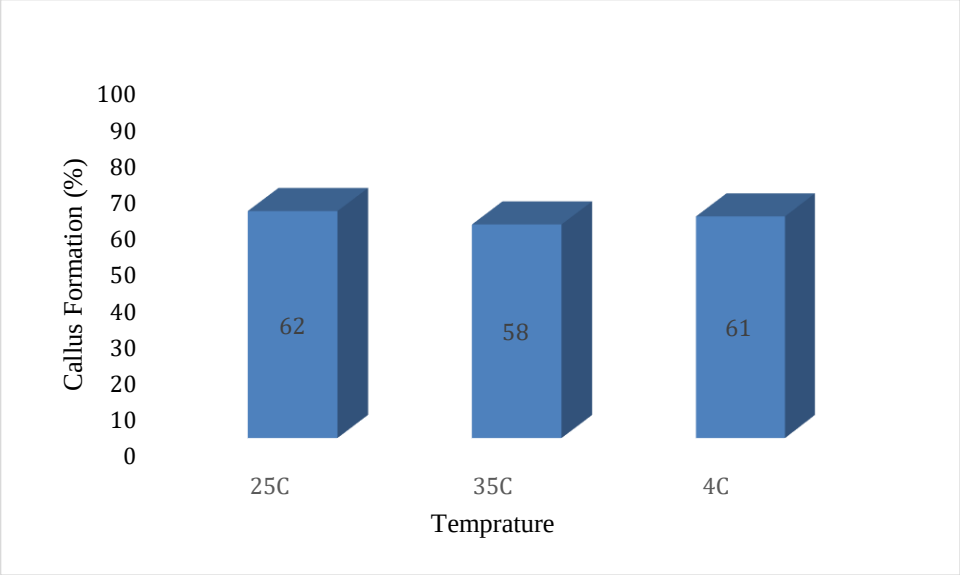

Fig. 3.6. Results Of Callus Formation By Temperature In 'Silah'

In Fig. 3.7, the relation between temperature*flower stage and callus formation % is shown in the form of bar graph. The highest value of 64% is observed for the combination 1) 12 hours before anthesis+4°C, 2) 12 hours before anthesis+ 25°C and 3) at the time of anthesis+ 25°C. the lowest value is for the combination at the time of anthesis+35°C which is 57%. LSD test value is not significant that means this parameter did not seem to be crucial in callus formation.

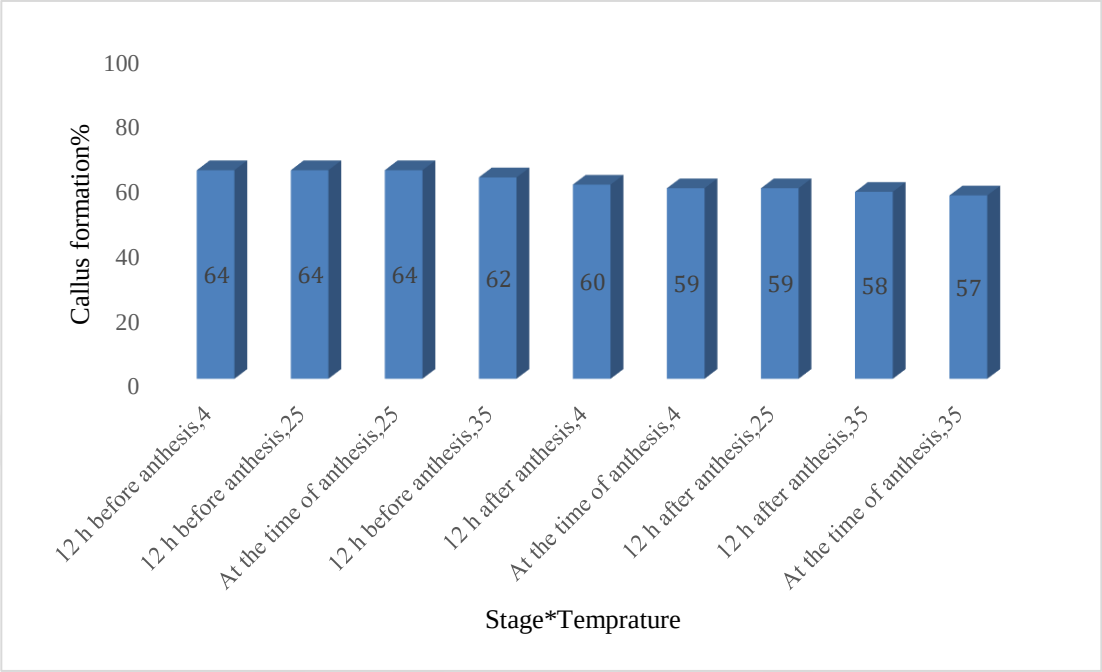

Fig. 3.7. Results Of Callus Formation By Temperature* Stage In 'Silah'

In Fig. 3.8, graph is demonstrating the relation between parameter temperature*hormone and callus formation. The highest value 89% is seen in combination 0.12 mg/L TDZ+4°C and the lowest value of 38% is observed in the case of 1.5mg/L 2,4D Kn. + 4°C. LSD test value for parameter temperature*hormone is not significant which means it is worthful to be used as a single factor in callus formation.

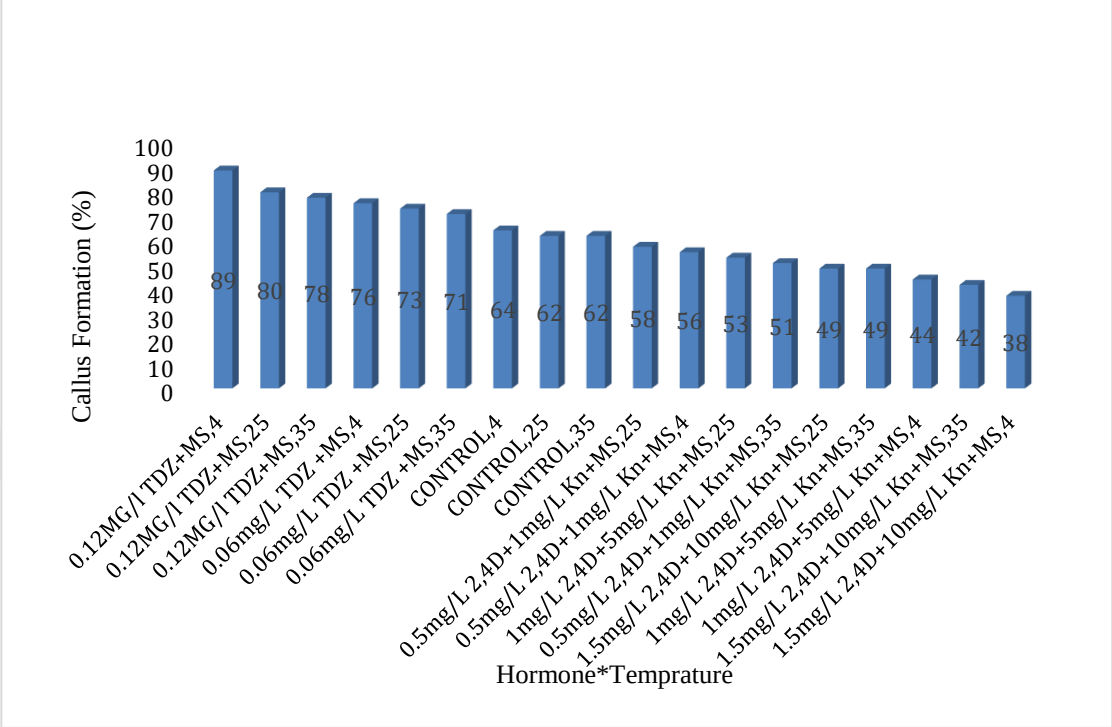

Fig.3.8. Results Of Callus Formation By Temperature*PGRs In 'Silah'

3.4. Results Of Embryo Formation

After 4-6 weeks of callus culture, ELS was observed in the petri plates. Data was recorded as per type of PGRs. In this case, the only PGRs was used as a parameter and no other parameters were selected. 4 different types of PGRs combination were selected as shown in table 2.1. Callus were cultured in those 4 hormones and kept at 25°C growth incubator. Unluckily embryo was not formed but we got ELS as shown in Fig. 3.9.

Statistical analysis was done separately for both genotypes focusing on a single parameter "hormone". In both genotypes highest value of ELS formation is seen for the same PGRs combination.

In table 3.3, ELS % is given for the genotype 'Kros'. According to the table, a higher % of ELS is 15 for the hormonal combination of 1mg/L NAA 3 mg/L BAP. LSD test value is not significant. It might be due to the fewer number of ELS formation that PGRs combination seems to be not that much important.

In table 3.4, ELS % is given for the genotype ‘Silah’ with respect to the hormonal combination. Highest value is 25% for hormonal combination of 1mg/L NAA 3mg/L BAP. LSD test value is not significant in this case.

NAA and BAP played important role in callus maturation and ELS formation in both genotypes. More number of ELS was obtained for genotype ‘Silah’ as compared to genotype ‘Kros’, however difference is not much notable.

Table 3.3. Percentage Of ELS Formation In ‘Kros’ Cultivar

Variety	Percentage Of ELS Formation In ‘Kros’	
‘Kros’	PGRs	ELS formation
	M1	0(-0.00)
	M2	15(16.44)
	M3	10(9.80)
	M4	0 (-0.00)

LSD_{PGRs}: N.S.

Table 3.4. Percentage Of ELS Formation In ‘Silah’ Cultivar

Variety	Percentage Of ELS Formation In ‘Silah’	
‘Silah’	PGRs	ELS formation
	M1	0(0.00)
	M2	25(29.73)
	M3	10(9.80)
	M4	5 (6.64)

LSD_{PGRs}: N.S.

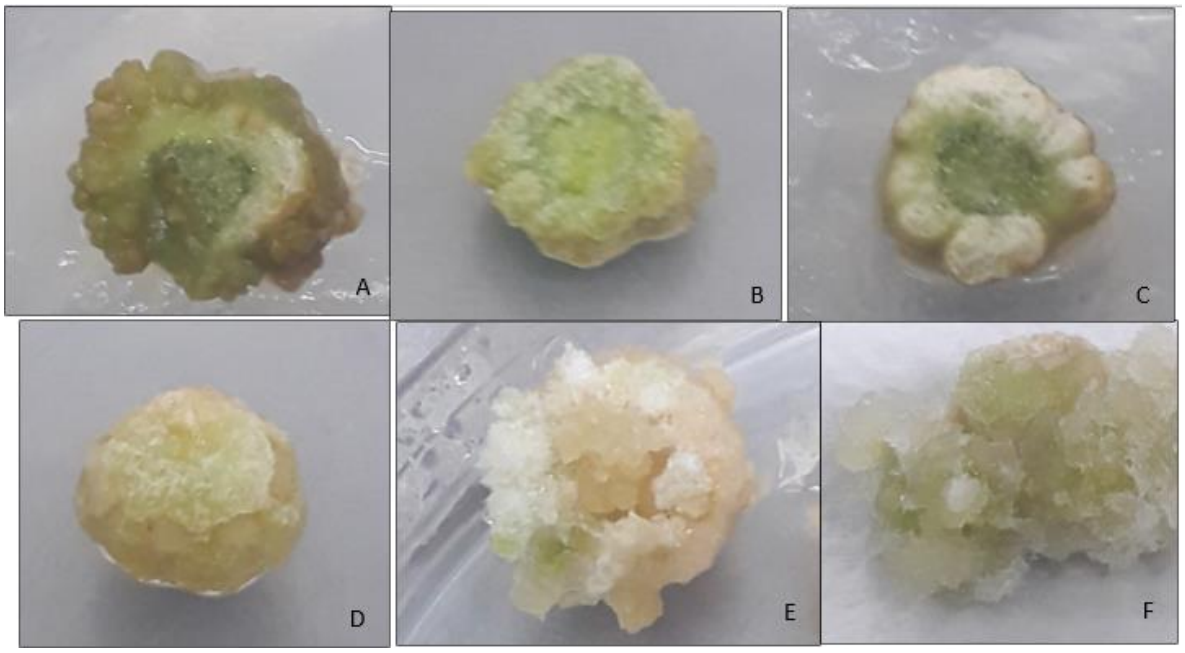

Fig.3.9. Callus and ELS formation; A, B) Callus formation in ‘Kros’, C) Callus formation in ‘Silah’, D, E) ELS formation in ‘Kros’, F) ELS formation in ‘Silah’

CHAPTER 4

DISCUSSION, CONCUSION AND SUGGESTION

4.1. Discussion

Cucumber is a species with significant production figures in the world and in Türkiye. In Türkiye, cucumber is grown both in the greenhouse and in open fields intensively. After tomato and watermelon, it is the most cultivated vegetable in Türkiye.

Genotypes of donor plants significantly affected ELS and callus formation abilities among five Thai cucumber cultivars (Tantasawat et al., 2015). In our study, we also get different results in both varieties. we got a higher number of ELS in 'Silah' as compared to 'Kros'..

Temperature shock (high or low temperature) is preferred to improve gynogenesis by diverting normal gametophytic development into a sporophytic mode of production, resulting in callus and ELS formation (Shalaby, 2007; Chen et al., 2011). Recently, it has been shown that thermal shock pre-treatment of ovary slices at 35°C for 2–4 days significantly induced the ELS formation of six Chinese cucumber cultivars (Diao et al., 2009). Gemes-Juhasz et al., (2002) also reported that 35°C thermal shock pre-treatment was effective in the cucumber ovary culture of five parthenocarpic breeding lines and a hybrid variety. Using six cucumber hybrids, it has been reported that the highest proportion of ELS was obtained with 3 days of 35°C pre-treatment (Moqbeli et al., 2013). Similarly, pre-treatment of summer squash ovaries at 4 or 32°C for 4 days also produced a better ELS response than untreated control (Shalaby, 2007). Likewise, in both varieties, we got different results for heat and cold shock treatments. In ''Kros', callus formation was observed highest at 35C while in 'Silah' best results were seen at 25C. Insensitivity to thermal and cold pre-treatment on several plants has also been reported (Metwally et al., 1998; Gugsa et al., 2006). It was seen that squash ovules without cold pre-treatment at 4°C produced a better ELS response than the ones treated at 4°C for 2, 4, or 8 days (Metwally et al., 1998). Similarly, pre-treatment at 4°C for up to 9 days or at 32°C for 1 day did not improve gynogenic

development of tef pistil culture (Gugsa et al., 2006). Our research also shows that temperature is not much significant parameter for callus formation.

ELS and callus formation potentials varied significantly when using different plant growth regulators. Overall M2 was the best maturation medium for ELS formation, while I5 and I6 were the best induction medium for callus formation in both varieties. Although some researchers were successful in obtaining haploid/doubled haploid plants from ovule-derived calli (Wei et al., 2006; Diao et al., 2009; Pathirana et al., 2011) while there were other who did not succeed in obtaining haploids like Lazarte and Sasser, (1982), used cucumber to produce haploid plants through *in vitro* culture, reported that ELSs developed following callus development, but haploid plants could not be obtained. Similarly we were also unable to regenerate any plant from calli or ELS. Therefore, M2, which induced the highest ELS formation, was considered the best maturation media for efficient haploid/doubled haploid production too. It is interesting to note that in both varieties, the highest ELS formation was induced by M2 that contains NAA and BAP and for callus induction, I5 and I6 performed best in both varieties containing TDZ either 0.06mg/L or 0.12mg/L with MS. TDZ has been frequently reported as the most efficient growth regulator for gynogenesis and calli induction in cucurbit crops (Gemes-Juhasz et al., 2002; Suprunova and Shmykova, 2008; Diao et al., 2009; Malik et al., 2011; Li et al., 2013).

The % of ELS formation obtained in 'Silah' by M2, which contained 0.06 mg/L TDZ (38.4 to 64.6%) was comparable to TDZ is used by many researchers like it was reported by Diao et al., (2009) using 0.02 mg/L TDZ with six Chinese cucumber cultivars (20 to 65.7%), confirming the TDZ efficiency of the ovary culture of cucumber. However, at higher TDZ concentration (0.04 mg/L), they found up to 72.7% ELS formation. With two different Chinese cucumber inbred lines, the highest ELS induction frequencies (11.1–12.1%) were achieved using 0.07 mg/L TDZ (Li et al., 2013). In melon, 0.04 mg/L TDZ could also induce ELS formation of a Chinese cultivar up to 76.6% when combined with 4°C pre-treatment for 4 days (Malik et al., 2011). When using I5 and I6, which contained a high TDZ concentration

(0.06mg/L and 0.12mg/L respectively) together with MS, we were able to induce callus formation efficiency (up to 80% and 79% in ‘Kros’ while 72% and 83% in ‘Silah’).
These results suggested a synergistic effect of TDZ, which was consistent with those reported by Saini and Chopra (2012). This new induction medium will be very useful for haploid/doubled haploid production in the future.

Tantasawat et al., (2015) found that more ELSs developed after transferring to the differentiation medium having NAA and BAP. However, all differentiation media led to equivalent %s of ELS and callus formation. In our study, we also used NAA and BAP as ELS maturating medium and the highest ELS obtained from M2 (1mg/L NAA and 3m/L BAP).
The first study on "Ovule-Ovarian culture" in the Cucurbitaceae family was the study of Chambonnet and Dumas de Vaulx, (1985) on squash. In this study, the researchers reported that the ovules they isolated from the squash ovaries taken 1 or 2 days before the anthesis period gave the most successful results. In our research, we collected flowers at three different stages (12 h before anthesis, at the time of anthesis and 12 h after anthesis) however callus formation was higher only at 12 h before anthesis in ‘Silah’ while in ‘Kros’ it was seen at 12 h before and at the time of anthesis.

4.2. Conclusion And Suggestions

Breeding studies with commercially grown cucumber plants in the world are very important. Obtaining inbred pure lines is considered the basis of vegetable breeding. That can be used directly as a variety or a superior new pure line which are ultimately the source of cultivars and high-yielding hybrids. It is seemed to be the basic and first step in any breeding program (Veilluex, 1994). Due to the natural cross-pollination nature of vegetable species in the family Cucurbitaceae, hence, breeding takes a very long time (Lower and Edwards, 1986). Shortening of this period, facilitating selection, increasing breeding efficiency, etc. has been studied. In recent years, with the development of the seed industry, breeding studies has also gained momentum.

For haploid plant production, the basic need is to develop haploid embryos that would grow into haploid plants. For producing haploid embryos, different methods like ovary culture, anther culture, parthenogenesis, etc. can be utilized. In this experiment, I tried to achieve haploids through the ovary culture technique. I succeed in getting callus and then ELS. This study is of great significance as we used multiple parameters like genotype, PGRs combination, temperature and flower stage. We did a statistical analysis based on 7 parameters (PGRs, temperature, flower stage, PGRs*temperature, PGRs*flower stage, temperature*flower stage, and PGRs*flower stage*temperature) and discussed results in % as well as angle transformation.

Overall if I compare both genotypes, then we can see hormone TDZ perform best among other hormones in producing callus while 1 mg/L NAA+3mg/L BAP PGRs pair bring about superior results in ELS formation. If we look at the flower stage, then two flower stages which are at the time of anthesis and 12 hours before anthesis put on good results in both genotypes. More or less it is due to the fact that ovaries are still unpollinated. Moving towards temperature, it stands not significant in both genotypes but still highest values were observed at 25C in genotype ‘Silah’ and at 35C in genotype ‘Kros’.

Gynogenesis is considered one of the prime methods of obtaining haploid plants. Still, there are many complications and difficulties. We tried our best while working with multiple parameters at the same time and following all essential protocols and requirements. Working with more than one factor and analyzing different factors and their combinations allow to understand this process in more depth. This work will be a foremost source of knowledge and cognizance in cucumber breeding and haploid development.

Today, there is large number of contributors in the science dealing with breeding. In addition to the researcher, there are many private institutions dealing with breeding. New competition in developing varieties is increasing day by day. Biotechnological institutions that want to take a step forward by using methods of haploidization techniques are of great interest. Any work that will contribute to this topic and increase the success rate will certainly be important.

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RESUME

PERSONAL INFORMATION

Name Surname: Huma MAJEED

Nationality: Pakistani (PAK)

EDUCATION INFORMATION

Degree	**Instiute**	**Graduation year**
Masters	Erciyes University, Agricultural Biotechnology	2022
Bachleors	PMAS Arid Agriculture University, Plant Breeding and Genetics	2018
Intermediate	FSc. Pre-Medical, Attock	2013

FOREIGN LANGUAGE

English

Turkish