Yüzüncü Yıl Üniversitesi
Tarım Bilimleri Dergisi
(YYU Journal of Agricultural Science)

http://dergipark.gov.tr/yyutbd

Derleme Makalesi (Review Article)

An Overview of Doubled Haploid Plant Production in *Cucurbita* Species

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Article Info

Received: 21.05.2020
Accepted: 07.08.2020
Online Published 30.09.2020
DOI: 10.29133/yyutbd.741087

Keywords

Androgenesis,
Cucurbita spp.,
Double haploid,
Gynogenesis,
Parthenogenesis.

Abstract: F1 cultivars are currently used in vegetable production because of their many superior agronomic advantages (high yield and quality, resistance/tolerance to biotic and/or abiotic stress factors, wide adaptability, etc.). It is not possible to produce hybrid vegetables without homozygous pure parental lines with defined traits and high combining ability. However, the production of homozygous pure parents needs a long time, great efforts, and high production cost. Moreover obtaining an F1 hybrid variety takes 8-10 years with traditional breeding methods, especially in a highly open-pollinated species such as *Cucurbita* spp. Doubled haploid (DH) technologies (parthenogenesis, androgenesis, and gynogenesis), called biotechnological breeding methods, have become an alternative to classical breeding methods and provide to obtain pure lines within 1-2 years by adapting to these technologies in F1 hybrid breeding programs. Although the DH technologies considered a meaningful approach to enhance the production of valuable double haploid plants, the implementation of these techniques needs labor-intensive efforts, high experiences, and advanced technologies. The main objective of this review to provide a summary of DH techniques applied in the *Cucurbita* species in the current progress.

Cucurbita Türlerinde Dihaploid Bitki Üretimine Genel Bir Bakış

Makale Bilgileri

Geliş: 21.05.2020
Kabul: 07.08.2020
Online Yayınlanma 30.09.2020
DOI: 10.29133/yyutbd.741087

Anahtar kelimeler

Androgenesis,
Cucurbita spp.,
Dihaploid,
Gynogenesis,
Partenogenesis.

Öz: F1 çeşitler sahip oldukları birçok üstün zirai avantajlar sebebiyle (yüksek verim ve kalite, biyotik ve/veya abiyotik stress faktörlerine tolerans/dayanım, geniş adaptasyon yetenekleri v.b.) sebze üretiminde yoğun bir şekilde kullanılmaktadır. Özellikleri belirlenmiş ve üstün kombinasyon yeteneği sahip homozigot saf ebeveyn hatlar olmadan hibrit çeşitlerin üretilmesi mümkün değildir. Ancak, homozigot saf ebeveynlerin üretilmesi uzun bir zamana, yoğun emeğe ve yüksek yatırıma ihtiyaç duyar. Dahası, F1 hibrit çeşitlerin üretimi özellikle *Cucurbita* türleri gibi yüksek oranda yabancı tozlanan türlerde klasik ıslah yöntemleriyle 8-10 yıl sürer. Biyoteknolojik ıslah metodları olarak adlandırılan ve klasik ıslah yöntemlerine alternatif olarak düşünülen dihaplod (DH) teknolojilerinin (partenogenesis, androgenesis ve gynogenesis) F1 ıslah programlarına adapte edilmeleri sayesinde 1-2 yıl içerisinde saf hatların elde edilmesi mümkündür. DH teknolojileri ıslah açısından oldukça değerli olan dihaploid bitkilerin elde edilmesini sağlayan anlamlı bir yaklaşım olsa da, bu tekniklerin hayata geçirilmesi için yoğun işgücüne, yüksek tecrübeye ve ileri teknolojilere ihtiyaç vardır. Bu derlemenin temel amacı güncel çalışmaların ışığında *Cucurbita* türlerinde uygulanan DH teknikleri hakkında bir özet sunmaktır.

1. Introduction

Cucurbita pepo L. (summer squash), *Cucurbita maxima* Duch. (winter squash), and *Cucurbita moschata* Duch. (pumpkin) are widely cultivated species of *Cucurbita* genus. They are members of *Cucurbitaceae* family and originated from Central and South America. These species have superior adaptability and therefore they have spread over a wide area in the world (Wu et al., 2007). China (8 million tonnes) and India (5.1 million tonnes) are the biggest producers in the world (FAO, 2017). Summer squashes are cultivated for their immature fruit, but mature fruits of winter squash and pumpkin are consumed. The seeds of these species are the source of vegetable oil and used as an appetizer (Seymen et al., 2019). The hybrids of *C. maxima* x *C. moschata* are also used as a rootstock for melons, watermelons, and cucumbers (Karaağaç and Balkaya, 2013).

Today, F1 cultivars are extensively used in the production of *Cucurbita* species due to their superior traits (Kurtar et al., 2016; Yeşil, 2019). However, the production of invaluable pure lines, which are the starting material of F1 hybrid breeding, needs a long breeding cycle (recurrent selfing or sib-crossing), intense labor, experience and technology. Besides, they are not possible to reach 100% purity by classical methods. On the other hand, completely homozygous lines are successfully gained in a short time by adapting to DH techniques in breeding programs. DH breeding offers great advantages to the breeders in terms of labor, cost, time and effectiveness and thus accelerates breeding efforts on a large scale (Kurtar et al., 2009). This technique is used effectively in the *Cucurbita* spp. and studies have focused on parthenogenesis (*in situ* irradiated pollen technique), androgenesis (*in vitro* anther culture) and gynogenesis (*in vitro* ovule/ovary culture). Regardless of which technique is used; the most important and crucial point of a DH study is the healthy cultivation of donor plants as much as their genotypes. For this reason, the cultural practices (planting, fertilizing, irrigation, ensuring suitable climatic conditions, weed control, tillage, disease, and pest management, pruning, etc.) should be applied on time and proper manner during the growing season.

Recently, Galazka and Niemirowicz-Szczytt (2013) published a review on DH in cucurbits, especially on irradiated pollen technique in cucumber. Dong et al. (2016) comprehensively discussed the effects of genotype, environment, pre-treatments, culture medium and media composition on androgenesis, gynogenesis, and parthenogenesis in *Cucurbitaceae* family. Nevertheless, it is considered that there is a need for a review that will include studies on DH technology in recent years for *Cucurbita* species. In this review, methodologies and protocols of irradiated pollen technique, anther culture, and ovule/ovarium culture are discussed in *C. pepo*, *C. maxima* and *C. moschata* in the highlight of current reports.

2. DH Techniques in *Cucurbita* Species

2.1. Parthenogenesis (*in situ* pollination with irradiated pollen)

2.1.1. Irradiation, pollination, isolation and harvest

In this technique, parthenogenesis stimulated by gamma rays (Co^{60}) or X-rays (Table 1). In the pollination process, the most suitable physiological period of plants was determined as the beginning or middle of the female flowering. This period is changing by growing conditions and seasons. In *C. pepo*, the best pollination time May or September in greenhouse and June in open-field conditions (Kurtar et al., 2002). June is the best pollination time in *C. maxima and C. moschata* species which have to be raised at open field conditions (Kurtar and Balkaya 2010). Fruit-set rate (FSR) is an important point in the pollination process and it ranged from 20% to 60% in *C. pepo* (Kosmrlj et al., 2013). FSR was found 26.3% in *C. moschata* and 75% in *C. maxima* pollination with 0th-day (d) old pollens (Kurtar, 2009). 4 weeks (w) old immature fruits are harvested for extraction. Otherwise, lots of necrotic embryos were extracted from later harvests (Kurtar and Balkaya, 2010).

2.1.2. Embryo rescue, culture conditions, and regeneration

Embryos obtained from the seeds are cultured in the growth medium as soon as possible, and solid E20A and CP medium produced better results. In general, the embryos are kept at 23 - 28 ºC,

16/8 h photoperiod and 3000 lux light intensity in the climate room until they turn into a complete plant. Medium refreshment encourages the transformation of small embryos into the plants at 7-15 d intervals. Considering that the genotype is the most important factor, it should be said that irradiation doses and seasons are effective on the number of embryos and the number of haploid embryos per fruit. The doses with the highest number of embryos ranged from 50 Gray to 300 Gy, while it was 50 Gy and 100 Gy for both pumpkin and winter squash (Table 1). The number of embryos per fruit is between 0.8 and 39.0 in *C. pepo* (Kurtar et al., 2017), 1.5 and 29.0 in *C. moschata* (Kurtar et al., 2009) and 1.0 and 34.7 *C. maxima* (Kurtar et al., 2010). Frequency of haploid embryos was 1.2, 10.4 and 0.7 in 100 seeds, 100 embryos, and fruit in *C. pepo* (Kurtar et al., 2002) and it was 0.24, 0.94 and 0.33 in *C. moschata* (Kurtar et al., 2009) and 0.11, 1.17 and 0.28 in *C. maxima* (Kurtar and Balkaya, 2010), respectively.

The growing season of donors is one of the important issues that affect success in the irradiated pollen technique. In *C. pepo*, the best season for the obtention of the highest number of haploid embryos per fruit was between April – June and September – November in greenhouse and May - July in open field conditions (Berber, 2009; Kurtar et al., 2002; Bektemur et al., 2014; Kurtar et al., 2017). Between May-August has a beneficial effect on haploid embryo frequency in *C. moschata* and *C. maxima* (Kurtar et al., 2009; Kurtar and Balkaya, 2010).

In general, smaller embryos produced more haploid plants than larger ones. Globular to torpedo type embryos produced 100 % haploid plants, on the contrary, cotyledon type embryos gave only diploid plants. Otherwise, torpedo and heart-type embryos produced both haploid (53.8 % and 23.1 %, respectively) and diploid plants in summer squash (Kurtar et al., 2002; Ebrahimzadeh et al., 2013). Similar findings were also reported in pumpkin (Kurtar et al., 2009) and winter squash (Kurtar et al., 2010).

Table 1. DH protocols and results of irradiated pollen technique in *Cucurbita* species

Species	Irradiation Dose and Source (IR), Plantlets Initiation (P), Regeneration (R)	Fecundity (F), Ploidy (P)	References
Summer squash (*C. pepo*)	IR: 25-50 Gy (γ) P/I: E20A + 28ºC +16/8 h-3000 lux	F: 16.8 p/100 em P: 43.7% H, 56.3% D	Kurtar et al., 2002
	IR: 100-150 Gy (γ) P: E20A + 25ºC + 16/8 h R: MS + 25ºC + 16/8 h	P:42.6% H, 57.3% D	Berber, 2009
	IR: 50-75 Gy (γ) M: E20A I: 26ºC + 16/8 h-3000 lux	F: 34.6 p/100 em P: 38.6% H, 61.4% D	Ebrahimzadeh et al., 2013
	IR: 200-300 Gy (X-Ray) P/I: E20A + 23ºC + 16/8 h	F: 1.1 – 4.4 H p/100 em P: 1.01% H, 97.4% D, 0.3% Tr, 1.6% T	Kosmrlj et al., 2013
	IR: 150 Gy (γ) P/I: CP + 30 g l^{-1} S + 0.08 mg l^{-1} B12 + 0.02 mg l^{-1} IAA + 25ºC + 16/8 h	F: 1.89 – 20.7 H em/100 seeds P: 11.0% H, 89.0% D	Baktemur et al., 2014
	IR: 150 Gy (γ) P/I: MS + 0.1 mg l^{-1} IAA + 26ºC + 16/8 h-3000 lux	F: 1.29-2.92 H p/100 em P: 2.56% H, 97.44% D	Kurtar et al., 2017
Winter squash (*C. maxima*)	IR: 50-100 Gy (γ) P/I: E20A + 28ºC +16/8 h-3000 lux	F: 1.17 H p/100 em P: 10.96% H, 89.04 D	Kurtar and Balkaya, 2010
Pumpkin (*C. moschata*)	IR: 50-100 Gy (γ) P/I: E20A + 28ºC +16/8 h-3000 lux	F: 16 p/100 em P: 0.94% H, 99.06% D	Kurtar et al., 2009

S: sucrose; h: hour; p:plant; em: embryo; H: Haploid; D: Diploid; T: Triploid; T: Tetraploid

2.2. Androgenesis (*in vitro* anther culture)

The best androgenic response obtained from the pollens at the middle to late uninucleate microspore stages. Flower buds at a suitable stage are collected from healthy, well-formed and younger donor plants at the full-flowering time, in the morning.

2.2.1. Pre-treatments, the culture of anthers and incubation

For induction of embryogenesis, microspore development has to be switched from the gametophytic to sporophytic pathways via specific pre-treatment conditions. Low-temperature applications have also been reported to be essential for maintaining pollen viability (Xie et al., 2005). In summer squash and *Cucurbita* interspecific hybrids, the cold pre-treatments at 4 ºC for 4 d produced the best androgenic response, otherwise, the best embryogenic response obtained from buds that have not been pre-treated in winter squash and pumpkin (Kurtar et al., 2016) (Table 2). In many experiments, MS medium was used with the addition of different levels of sucrose (S) and 2.4-D (2.4-Dichloro phenoxy acetic acid).

In addition to cold treatments, in initial cultures, high-temperature shock and dark conditions are essential to enhance androgenetic response. In *C. pepo*, male flower buds incubated at 35 ºC for 1 w; subsequently at 25 ºC for 4 w at dark. The buds were also kept 32 ºC for 1 w (Shalaby, 2006), 25 ºC for 4 w (Habiba, 2016) and 32 ºC for 4 w (Araghi et al., 2017) at dark. Recently, Kurtar et al. (2016) pointed out that the efficiency of microspore embryogenesis in *C. moschata* and *C. maxima* were higher at 32 ºC for 1 w at dark conditions. In *Cucurbita* interspecific hybrids, incubation of buds at 35 ºC for 1 w and then at 25 ºC for 9 w at dark conditions was found to be optimal for embryogenic callus induction (Table 2).

2.2.2. Callogenesis and embryogenesis,

In androgenesis, the indirect pathway is preferred and the direction of callogenesis and microspore embryogenesis can be converted by changing the amounts of some additive substances. Different treatments resulted in different callogenesis efficiencies and callus induction is strongly influenced by both S, 2.4-D and BAP (Benzyl Amino Purine) concentrations. The combination of 9% S and 1 mg l^{-1} 2.4-D was optimal for callus induction and plantlet regeneration in *Cucurbita* interspecific hybrids**.** Besides, 12% S+2 mg l^{-1} 2.4-D enhanced the callogenesis; subsequently 12% S+2 mg l^{-1} 2.4-D+0.5 mg l^{-1} BAP has a positive effect on callus maturation and embryogenesis in *C. moschata* and *C. maxima***.** On the other hand, the combination of high S (15%) and 2.4-D (5 mg l^{-1}) was to be optimal for callogenesis in *C. pepo*, while this combination inhibited the callogenesis in *Cucurbita* interspecific hybrids (Table 2). It is reflected that a combination of relatively higher S and 2.4-D is superior for the induction of microspore embryogenesis in *Cucurbita* species.

Induction medium consists of different amounts of KIN (Kinetin) (0.05-0.5 mg l^{-1}), NAA (Naphthalene Acetic Acid) (0.05-0.5 mg l^{-1}) and BAP (1-4 mg l^{-1}). The calli are incubated in a growth chamber at different temperatures (22-26 ºC), photoperiods (12/12-16/8 h), light intensities (1000-3000 lux) and durations (4-6 w). Embryoids can be seen (particularly globular type embryos) in callus surfaces within 4 - 6 w, and the plantlet initiation observed from some embryos within 7 or 8 w of culture (Kurtar et al., 2016). To maintain the viability and maturation of calli and induction of embryogenesis, media refreshment is a crucial point, thus calli should be subcultured at 7-10 d intervals.

2.2.3. Recovery of embryos and regeneration

When the embryogenic plantlets reach visible size are gently transferred to the regeneration medium as soon as possible. MS medium supplemented with 0.01 mg l^{-1} IAA (Indole Acetic Acid) (Kurtar et al., 2016) and 1 mg l^{-1} IBA (Indole Butyric Acid) (Mohamed and Refaei, 2004) or without growth regulators is used for root and shoot elongation. Considering that each plant obtained comes from different pollens and have a different genetic structure, it is very important to keep all plants alive. Rooted and elongated plants were individually transferred onto fresh MS medium.

Donors play a predominant role and genotypes and species show a wide range of androgenetic responses. Some genotypes produce a good response, while others were extremely poor or non-responsive. The reason for these very variable results may be due to genetic structure and genotype-environment interactions. Concerning species and genotypes, *C. pepo* shows a higher androgenic response than *C. moschata* and *C. maxima*. The highest rate of embryogenic calluses (38.7 %) and the number of plantlets per callus (8.9) were obtained from *C. pepo* cv. Yellow Bik F1, while some

genotypes non-respond (Shalaby, 2006). Likewise, *Cucurbita* interspecific hybrid gave the optimal results (7.5 plants/callus) than the cultivars, and these findings have revealed that hybridization process presented more successful results (Rakha et al., 2012). On the other hand, *C. maxima* (var. 57Sİ21) showed higher haploid plants/callus results (2.48) than *C. moschata* (var. G9) (1.26) (Kurtar et al., 2016). Besides, Mohamed and Refaei (2004) proved that the season has also great importance in embryogenesis, and November (260 plants) was better than March (118 plants) in *C. pepo.*

Table 2. DH protocols and results of anther culture technique in *Cucurbita* species.

Species	**Pre-treatments (P) / Culture (C) / Incubation - Callogenesis (I) / Embryogenesis (E) / Regeneration (R)**	**Fecundity (F), Ploidy(P)**	**References**
Summer squash (*C. pepo*	P: 4ºC for 4 d; C: MS+15% S+5 mg l^{-1} 2.4-D I: 35ºC for 1 w+25ºC for 4 w, subculture 25ºC for 4 w at dark; E: MS+0.057 mg l^{-1} KIN+0.05 mg l^{-1} NAA+25◦C+16/8 h-1000 lux z+4 w ; R: MS+25◦C+16/8 h-3000 lux+4 w	F: 1.93 p/anther P: 50% H, 50% D	Metwally et al., 1998a
	P: 4ºC for 4 d; C: MS+12% S+5 mg l^{-1} 2.4-D I: 35ºC for 1 w+25ºC for 4 w at dark E: MS+0.23 mg l^{-1} KIN+0.27 mg l^{-1} NAA R: MS+25◦C+16/8 h-3000 lux+4 w	P: D	Kurtar et al., 1999
	P: 4ºC for 4 d; C: MS+10% S+6 mg l^{-1} 2.4-D I: 35ºC for 1 w+25ºC for 4 w, subculture 25ºC for 4 w at dark; E: MS+0.05 mg l^{-1} KIN+0.05 mg l^{-1} NAA, 25◦C+16/8 h-3000 lux+4 w; R: MS+4 w, then MS+1 mg l^{-1} IBA+25◦C+16/8 h-3000 lux	F: 0.13 – 2.6 p/anther P: 60% H, 13% D, 17% A	Mohamed and Refaei, 2004
	P: 4ºC for 4 d; C: MS+10% S+5 mg l^{-1} 2.4-D I: 32ºC for 1 w+25ºC for 4 w at dark; E: MS+0.05 mg l^{-1} KIN+0.05 mg l^{-1} NAA, 25 ◦C+16/8 h-3000 lux+4 w ; R: MS+25◦C+16/8 h-3000 lux+4 w	F: 0.0 – 8.90 p/callus P: 48.3% H, 51.7% D	Shalaby, 2006
	P: 4ºC for 4-7 d; C: MS+9% S+2 mg l^{-1} 2.4-D I: 25ºC for 4 w at dark; E/R: MS+0.5 mg l^{-1} KIN+0.5 mg l^{-1} NAA+ 22˚C+16/8 h for 6 w	F: Calli and shoots	Habiba, 2016
	P: 4ºC for 4 d; C: E20A+9% S+2.5 mg l^{-1} 2.4-D+1 mg l^{-1} BAP; I: 32ºC for 4 w at dark,	F: Calli	Araghi et al., 2017
Winter squash (*C. maxima)*	I: 32ºC for 1 w at dark C: MS+12% S+2 mg l^{-1} 2.4-D for 1 w, then MS+12% S+2 mg l^{-1} 2.4-D+0.5 mg l^{-1} BAP for 3-5 w at 1500 lux; E: MS+4 mg l^{-1} BAP+0.05 mg l^{-1} NAA, 26 °C+12/12 h-1500 lux+1 w, then 26°C+16/8 h-3000 lux+3-5 w; R: MS+0.01 mg l^{-1} IAA+26°C+16/8 h-3000 lux	F: 0.0 – 2.48 H p /callus P: 50% H, 50% D	Kurtar et al., 2016
Pumpkin (*C. moschata)*	I: 32ºC for 1 w at dark; C: MS+12% S+2 mg l^{-1} 2.4-D for 1 w, then MS+12% S+2 mg l^{-1} 2.4-D+0.5 mg l^{-1} BAP for 3-5 w at 1500 lux; E: MS+4 mg l^{-1} BAP+0.05 mg l^{-1} NAA, 26 °C+12/12 h-1500 lux+1 w, then 26°C+16/8 h-3000 lux+3-5 w ; R: MS+0.01 mg l^{-1} IAA+ 26°C+16/8 h-3000 lux	F: 0.0 – 1.26 H p/callus P: 37.5% H, 63.5% D	Kurtar et al., 2016
Others (*Cucurbita* interspecific hybrids)	P: 4ºC for 4 d ; C: MS+9% S+1 mg l^{-1} 2.4-D I: 35ºC for 1 w then 25ºC for 9 w at dark E: MS+0.05 mg l^{-1} KIN+0.05 mg l^{-1} NAA+25◦C+ 16/8 h-3000 lux+4 w; R: MS+25◦C+16/8 h-3000 lux+4 w	F: 0.0 – 7.5 p /callus P: 50% H, 50% D	Rakha et al., 2012

S: sucrose; h: hour; d: day; w: week; p:plant; H: Haploid; D: Diploid; A: Aneuploid

2.3. Gynogenesis (*in vitro* ovule/ovarium cultures)

Gynogenesis has been one of the most studied and popular DH technique and presented remarkably results in recent years. In gynogenesis, the maturation phase of female flowers is strongly effective in the embryogenic division. In the case of the appropriate flower stage, previous reports have expressed different arguments in *Cucurbita* species (Table 3). As a consequence, nearly mature or mature ovaries produced favorable results in *Cucurbita* species.

2.3.1. Pre-treatments, culture of ovules or ovarium slices and incubation

In gynogenesis, ovary slices or ovules were used for embryogenesis in squash, winter squash and pumpkin. Ovules and ovary slices are cultured with or without pre-treatments. Shalaby (2007) and Domblides et al. (2016) recommended cold pretreatment at 4 ºC for 4 d in summer squash. However, the embryogenesis was significantly reduced at the cold treatments, and untreated controls produced a higher embryogenic response in summer squash**,** pumpkin and six interspecific *Cucurbita* hybrids. Besides, pre-treated ovaries at 5 °C for 2 d produced the optimal embryogenesis. In light of these findings, it is clear that untreated ovaries provide better results. MS medium has been reported as the most suitable initial medium. CBM and N6 media were also used for initiation. MS medium was used with the addition of different levels of S, 2.4-D, NAA, BAP, KIN, TDZ (Thidiazuron), EBL (*Epibrassinolide*) and polyamines (Spermidine and Putrescine) (Table 3).

In addition to cold treatments, in the initial cultures, high thermal shock in dark conditions (incubation) is essential to enhance gynogenetic response. In *C. pepo*, Metwally et al. (1998b) and Xie et al. (2006) incubated female flower buds at 25 ºC for 4 w in dark. The ovaries were also exposed to 35 ºC for 1 w (Yılmaz, 2005), 32 ºC for 4 d (Domblides et al., 2016) and 32 ºC for 4 d (Shalaby, 2007) at dark. Recently, Zou et al. (2020) proved that the gynogenetic response remarkably enhanced at 35 ºC for 5 d at dark; similarly, Kurtar et al. (2018) obtained favorable results at 35 ºC for 3 d at dark conditions in *C. moschata* and *C. maxima*. Thermal shock induces the greenish calli form, embryogenesis and plantlet initiation in pumpkin. On the other hand, in *Cucurbita* interspecific hybrids, incubation of ovaries at 25 ºC for 1 w in dark was found to be optimal for embryogenesis (Table 3).

2.3.2. Callogenesis and embryogenesis,

In gynogenesis, the indirect pathway preferred in *Cucurbita* species. Different treatments resulted in different callogenesis efficiencies and callus induction is strongly influenced by both S and PGR's concentrations. In callus induction media, 2.4-D is essential for callogenesis and used at 1 - 5 mg l^{-1} concentrations. To induce the embryogenic greenish calli form, 0.5 mg l^{-1} BAP should be added initially of the culture (Kurtar et al., 2018). In *Cucurbita* species, 3% S concentration produced the highest percentage of embryogenesis. Besides, Shalaby (2007) found that higher S concentrations (over 6%) do not produce embryos. Conversely, Kurtar et al. (2018) proved that higher S concentration (12%) induced the embryogenic calli in *C. moschata* and *C. maxima.*

In callogenesis, 12% S+2 mg l^{-1} 2.4-D+35 ºC for 3 d at dark enhanced the callogenesis; subsequently, calli were subcultured on callus maturation media (12% S+2 mg l^{-1} 2.4-D+0.5 mg l^{-1} BAP+26 ºC +12/12 h-1500 lux) for 1 w, then 16/8 h-1500 lux for 3-5 w has a positive effect on embryogenesis in *C. moschata* and *C. maxima* (Kurtar et al., 2018). 3% S+1 mg l^{-1} 2.4-D+25 ºC for 8 w was optimal for callus induction in *Cucurbita* interspecific hybrids (Rakha et al., 2012). These results proved that the maturation of calli has an indicative role in embryogenesis and 2.4-D can be used in combination with other PGR's to increase gynogenetic response. The media should be refreshed 7-10 d intervals.

Table 3. DH protocols and results of ovule/ovarium cultures in *Cucurbita* species.

Species	Collecting Time (CT), Pre-treatments (P), Culture (C), Incubation - Callogenesis (I), Embryogenesis (E), Regeneration (R)	Fecundity (F), Ploidi (P)	References
Summer squash *(C. pepo*	CT: 1 d BA (O); P: 4ºC for 0 d ; C: MS+5 mg l^{-1} 2.4-D; I: 25ºC for 4 w at dark; E: MS+25ºC+ 16/8 h+4 w; R: MS	F: 8.8 - 9.1 p/100 O P: 25% H, 75% D	Metwally et al., 1998b
	CT: A (OS); I: 35ºC for 1 w at dark; E: CBM+3% S+0.1 mg l^{-1} TDZ+25ºC+16/8 h-1600 lüx+ 4 w; R: CBM+0.5 mg l^{-1} BAP+0.05 mg l^{-1} NAA	F: Calli	Yılmaz, 2005
	CT: 1 d BA (O); P: 4ºC for 0 d ; I: 25ºC for 4 w at dark; E: MS+3% S+4 mg l^{-1} 2.4-D+0.25 mg l^{-1} NAA+1 mg l^{-1} BAP; R: N6+2% S	P: H, D, M	Xie et al., 2006
	CT: 1 d BA (O); P: 4ºC for 4 d at dark C/I: MS+1 mg l^{-1} 2.4-D+25 ºC+16/8 h+4 w E: MS+3% S+1 mg l^{-1} KIN+1 mg l^{-1} 2.4-D+25ºC +16/8 h-3000 lüx+4 w; R: MS	F: 3 – 14 p/25 O P: 65% H, 35% D	Shalaby, 2007
	CT: 1 d BA (O); P: 4ºC for 4 d ; C: MS+3% S+ 2 mg l^{-1} 2.4-D; I: 32ºC for 4 d at dark; E: MS+0.2 mg l^{-1} TDZ+0.0001 µM EBL; R: MS	F: 0.23 – 8.5 p/100 O P: H, M	Domblides et al., 2016
Winter squash *(C. maxima)*	CT: A (O); C: MS+12% S+2 mg l^{-1} 2.4-D; I: 35ºC for 3 d at dark, then MS+12% S+2 mg l^{-1} 2.4-D+0.5 mg l^{-1} BAP+26ºC +12/12 h-1500 lux for 1 w, then 16/8 h-1500 lux for 3-5 w at light ; E: MS+4 mg l^{-1} BAP+0.05 mg l^{-1} NAA+ 0.1 mg l^{-1} TDZ+26ºC +16/8 h-3000 lux ; R: MS+0.01 mg l^{-1} IAA+1 mg l^{-1} BAP	F: 0.0 – 3.72 p/per callus P: 57.4% H, 37.2% D, 5.3% M	Kurtar et al., 2018
	CT: 1 d BA (OS); P: 4ºC for 0 d ; I: 35ºC for 5 d at dark; E: MS+3% S+1 mg l^{-1} BAP+8 w; R: MS	F: 0.0-5.67 p/100 OS P: 18.64% H, 3.40% M, 32.20% D, 45.76% T	Zou et al., 2020
Pumpkin *(C. moschata)*	CT: 1 d BA (OS) ; C: MS+3% S+4 mg l^{-1} 2.4-D+0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP; I: 35ºC for 6 d at dark; E/R: MS+3% S+0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP	F: Embryogenic structures	Sun et al., 2009
	CT: A (O); I: 35ºC for 5 d at dark ; E: MS+3% S+0.04 mg l^{-1} TDZ ; R: MS	P: H, D	Min et al., 2016
	CT: A (O); C: MS+12% S+2 mg l^{-1} 2.4-D I: 35ºC for 3 d at dark, then MS+12% S+2 mg l^{-1} 2.4-D+0.5 mg l^{-1} BAP+26ºC+12/12 h (1500 lux) for 1 w, then 16/8 h-1500 lux for 3-5 w at light ; E: MS+4 mg l^{-1} BAP+0.05 mg l^{-1} NAA+0.1 mg l^{-1} TDZ ; R: MS+0.01 mg l^{-1} IAA+1 mg l^{-1} BAP+26 ºC+16/8 h-3000 lux	F: 0.0 – 2.12 p/per callus P:57.1% H, 39.3% D, 3.6% M	Kurtar et al., 2018
	CT: 1 d BA(O) ; C: MS+5 mg l^{-1} 2.4-D+40 µM Spermidine+40 µM Putrescine	F: Calli	Kara and Sarı, 2019
	CT: 1 d BA (OS); P: 4ºC for 0 d ; I: 35ºC for 5 d at dark; E: MS+3% S+1 mg l^{-1} BAP+8 w; R: MS	F: 0.0-10.18 p/100 OS P: 18.64% H, 3.40% M, 32.20% D, 45.76% T	Zou et al., 2020
Others (*Cucurbita* hybrids)	CT: 1 d BA (O); P: 4ºC for 0 d ; I: 25ºC for 1 w at dark; C/E: MS+3% S+1 mg l^{-1} 2.4-D+25ºC+16/8 h for 8 w; R: MS	F: 0.0 – 13.33 p/100 O P: 60% H, 40% D	Rakha et al., 2012

O: ovule; OS: ovary segment; A: anthesis; BA: before anthesis; S: sucrose; h: hour; d: day; w: week; p:plant; H: Haploid; D: Diploid; M: Mixoploid; T: Tetraploid

2.3.3. Recovery of embryos and regeneration

Plantlets at the suitable size (4 – 6 mm) are transferred to the regeneration medium as soon as possible to prevent embryo losses. Standard MS medium is widely used for regeneration. On the other hand, plantlets are rooted and elongated on MS+0.01 mg l^{-1} IAA+1.0 mg l^{-1} BAP (Kurtar et al., 2018), MS+0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP (Sun et al., 2009) and N6+2% S (Xie et al., 2006). Well rooted and elongated plants were individually subcultured onto fresh MS medium for further development. The gynogenetic response is closely related to the genetic structure of the genotypes, and varied between species and cultivars. Some genotypes produce a good gynogenetic response, while others were poor or non-responsive.

3. Micropropagation, Acclimatization and Ploidy Determination

3-4 w old *in vitro* plants micro-propagated for 2-3 times. Thus, plant losses are prevented and the desired number of clones is reproduced from each plant for acclimatization, ploidy determination, and chromosome doubling processes (Kurtar et al., 2016). Then, micro-propagated clones acclimatized as described by Kurtar and Balkaya (2010).

The plantlets obtained from double haploidy processes have different ploidy levels such as haploid, diploid, tetraploid, aneuploid and mixoploid. Disturbances in the ploidy level associated with the diploid anther tissues (especially the anther wall lead to the development of diploids plants), spontaneous duplication, and irregular meiosis. Besides, spontaneous DH lines are valuable because they do not need chromosome doubling (Alan et al., 2003). The ploidy level of plantlets is determined by indirect (flow cytometry, morphological observations and cytological analysis) and direct (chromosome counting in root tips) methods in *Cucurbita spp*. Isozyme analysis could also help to determine the origin of spontaneous DH plants (Kosmrlj et al., 2013).

Flow cytometry (FCM) is used as a technique to determine the ploidy level according to the amount of DNA in the cell. Ploidy levels of DH plants were determined by FCM in *C. pepo* (Ebrahimzadeh et al., 2013; Kosmrlj et al., 2013) and *C. moschata* and *C. maxima* (Zou et al., 2020). Although FCM is a very reliable and exhibiting accurate result in ploidy determination, requires special techniques and equipment. Morphological observations such as leaf and flower shape and sizes, pollen production, internode length, and fruit-set are used for determining the putative haploid and diploid plants in *Cucurbita* spp. But, this method takes a long time because needs an appropriate growing period and conditions (Kurtar et al., 2016). In cytological analyses, the 4th or 5th leaves from shoot apex were used to measure stomata size, stomata density and chloroplast number. This technique is not time-consuming, simple and low cost (Kurtar et al., 2002). Chromosome counting in root tips of *in vitro* and *in vivo* plants was used in *C. pepo*, *C. moschata* and *C. maxima*.

As a consequence, all methods can be used safely, but chromosome counting is tedious, morphological observations take a long time and FCM is expensive, do not reach easily, and labor-intensive, thus stomatal observations, as a simple and more practical technique, seems to be recommendable for determine the putative haploid and diploid plants in *Cucurbita* spp.

4. Chromosome Doubling (Dihaploidization)

The haploid plants produced via irradiated pollen technique, androgenesis and gynogenesis are sterile due to they have only one (n) set of chromosomes. Therefore, the chromosome numbers must be multiplied to restore the ploidy, fertility and maintain the dihaploid lines in a DH effort. In general, chromosome doubling realized by *in vitro* and *in vivo* techniques and colchicine (CL) is the mainly used anti-mitotic agent. Besides, trifluralin, amiprophos methyl, and oryzalin have been reported as alternative antimitotic agents for colchicine due to less phytotoxicity, high efficiency at low concentration, and insignificantly morphological abnormalities (Alan et al., 2007). In *Cucurbita* spp, the first comprehensive methodology was reported by Kurtar (2018) in winter squash and pumpkin and the highest fertile DH efficiency (93.3%) was obtained from *in vivo* multiple treatments of 1% colchicine for an hour to shoot tips of haploid plants.

5. Conclusion and perspective

It is quite obvious that DH efficiency of irradiated pollen, ovule/ovary culture, and anther culture has not expected level in *Cucurbita* yet. However, the most important factor preventing the widespread use of these techniques is that they are genotype-dependent and the haploid frequency shows a great variation among the genotypes. However, when an evaluation is made among these three methods, it is possible to say that the irradiated pollen technique is more advantageous than the others for *Cucurbita* F1 hybrid breeding. Besides, to improve these techniques, further studies should focus on fecund genotypes, profitable PGRs and their combinations, pretreatments, culture procedure (stress treatments), and culture conditions (especially light intensity). In low-frequency genotypes, the frequency should be increased by hybridizing with high-frequency genotypes. On the other hand, as an alternative technique, isolated pollen culture should be adapted to the DH program quickly.

In line with the agronomic targets in the cultivar breeding of vegetable species, DH technology offers great advantages to today's breeders and scientists dealing with biotechnology. With the development and optimization of DH techniques in the future, the breeding period will be shortened and the breeding efficiency will increase in many vegetable species. This should be considered as a great advantage, especially in countries that are still dependent on the outside in terms of vegetable seed production. Vegetable seed breeding organizations that stand out in seed production have established tissue culture laboratories and included DH technology in their breeding programs. When DH technology is combined with biotechnology and some other breeding methods (mutation breeding, backcrossing, F1 hybrid breeding) success in breeding will further increase and will play a decisive role in future vegetable breeding studies.

References

Alan, A.R., Mutschler, M.A., Brant, A., Cobb, E., & Earle, E.D. (2003). Production of gynogenic plants from hybrids of *Allium cepa* L. and *A. roylei* Stearn. *Plant Science, 165,* 1201–1211.

Alan, A.R., Lim, W., Mutschler, M.A., & Earle, E.D. (2007). Complementary strategies for ploidy manipulations in gynogenic onion (*Allium cepa* L.). *Plant Science, 173,* 25–31.

Araghi, Z.M., Abdollahi, M.R., & Asl, A.M. (2017). The study on the effect of anther orientation, type and composition of culture medium and ovary co-culture on callus induction from cultured anthers of pumpkin (*Cucurbita pepo* var. styriaca). *Agr Biotec, 8(1),* 19-32.

Bektemur, G., Yücel, N.K., Taşkın, H., Çömlekçioğlu, S., & Büyükalaca, S. (2014). Effects of different genotypes and gamma-ray doses on haploidization using irradiated pollen technique in squash. *Turk J Biol, 38,* 318–327.

Berber, M. (2009). *Production of haploids in naked seed pumpkins (Cucurbita pepo L. var. styriaca) by pollination with irradiated pollen.* (MSc), Cukurova University, Institute of Natural and Applied Science, Adana, Turkey.

Domblides E.A., Shmykova N.A., Zayachkovskaya T.V., Khimich G.A., Korotseva I.B., Kan L.Y., & Domblides A.S. (2016, October). *Doubled haploid plant production from in vitro culture of unpollinated ovules of summer squash (Cucurbita pepo L.).* Proc of the VII Int Sci and Prac Congress, Yalta, Crimea, Russia.

Dong, Y., Zhao, W.X., Li, X.H., Liu, XC, Gao, N.N., Huang, J.H., Wang, W.Y., Xu, X.L., & Tang, Z.H.(2016). Androgenesis, gynogenesis, and parthenogenesis haploids in cucurbit species. *Plant Cell Rep, 35*, 1991–2019.

Ebrahimzadeh, H., Lotfi, M., Azizinia, S.H., & Ghanavati, F. (2013). Production of haploids in *Cucurbita pepo* L. through parthenogenesis induced by gamma-irradiated pollen. *Crop Biotech, 4,* 99.

FAO. (2017). FAOSTAT Statistical Databases. www.fao.org. Erişim tarihi: 11.04.2020.

Galazka, J., & Niemirowicz-Szczytt, K. (2013). Review of research on haploid productionin cucumber and other cucurbits. *Folia Hort, 25(1),* 67–78.

Habiba, R.M.M. (2016). Effect of genotypes, sucrose concentrations and their interaction on anther culture response on summer squash. *J Agr Chem and Biotec, Mansoura Univ, 7 (4),* 113–120.

Kara, B., & Sarı, N. (2019). Effects of spermidine and putrescine applications on haploid plant obtention via ovule culture in Sakız and Dark Green squashes (*Cucurbita pepo* L.). *Turk J Agr Res,6(2),* 206-213.

Karaağaç, O., & Balkaya, A. (2013). Interspecific hybridization and hybrid seed yield of winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) lines for rootstock breeding. *Sci Hort, 149,* 9-12.

Kosmrlj, K., Murovec, J., & Bohanec, B. (2013). Haploid induction in hull-less seed pumpkin through parthenogenesis induced by X-ray irradiated pollen. *J Am Soc Hort Sci, 138(4),* 310–316.

Kurtar, E.S. (1999). Haploid plant propagation by anther culture of squash (*Cucurbita pepo* L.). *J Agr. Fac. O.M.U., 14(2),* 33-45.

Kurtar, E.S., Sari, N., & Abak, K. (2002). Obtention of haploid embryos and plants through irradiated pollen technique in squash (*Cucurbita pepo* L.). *Euphytica, 127 (3),* 335-344.

Kurtar, E.S. (2009). Influence of gamma irradiation on pollen viability, germinability and fruit and seed-set of pumpkin and winter squash. *Afr J Bio, 8,* 6918–6926.

Kurtar, E.S., Balkaya. A., Ozbakır, M., & Ofluoglu T. (2009). Induction of haploid embryo and plant regeneration via irradiated pollen technique in pumpkin (*Cucurbita moschata* Duchesne ex. Poir). *Afr J Bio, 8,* 5944–5951.

Kurtar, E.S., & Balkaya, A. (2010). Production of *in vitro* haploid plants from *in situ* induced haploid embryos in winter squash (*Cucurbita maxima* Duchesne ex Lam.) via irradiated pollen. *Plant Cell Tis and Org Cul, 102,* 267-277.

Kurtar, E.S., Balkaya, A., & Kandemir, D. (2016). Evaluation of haploidization efficiency in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) through anther culture. *Plant Cell Tis and Org Cul, 127,* 497–511.

Kurtar, E.S., Balkaya, A., & Kandemir, D. (2016). Screening for salinity tolerance in developed winter squash (*Cucurbita maxima*) and pumpkin (*Cucurbita moschata*) lines. *YYU J Agr Scı, 26(2), 183-195.*

Kurtar, E.S., Balkaya, A., Göçmen, M., & Karaağaç, O. (2017) Hıyara (*Cucumis sativus* L.) anaç olabilecek ümitvar kabak (*Cucurbita spp.*) genotiplerinde ışınlanmış polen tekniği ile dihaploidizasyon. *Selcuk J Agr Food Sci, 31(1),* 34-41.

Kurtar, E.S., Balkaya, A., & Ozbakir Ozer, M. (2018). Production of callus mediated gynogenic haploids in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.). *Check J of Gen and Plant Bre, 54(1),* 9–16.

Kurtar, E.S. (2018). The effects of anti-mitotic agents on dihaploidization and fertility in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) androgenic haploids. *Acta Sci. Pol. Hort Cul, 17(5),* 3–14.

Metwally, E.I., Moustafa, S.A., EI-Sawy, B.I., & Shalaby, T.A. (1998a). Haploid plantlets derived by anther culture of *Cucurbita pepo*. *Plant Cell Tiss Org Cult, 52(3),* 171–176.

Metwally, E.I., Moustafa, S.A., El-Sawy, B.I., Haroun, S.A., & Shalaby, T.A. (1998b). Production of haploid plants from *in vitro* culture of unpollinated ovules of *Cucurbita pepo* L. *Plant Cell Tiss Org Cult, 52(3),* 117–121.

Min, Z.Y., Li, H., Zou, T., Tong, L., Cheng, J., & Sun, X.W. (2016). Studies of *in vitro* culture and plant regeneration of unfertilized ovary of pumpkin. *Chin Bull Bot, 51(1),* 74–80.

Mohamed, M.F., & Refaei, E.F.S. (2004). Enhanced haploids regeneration in anther culture of summer squash (*Cucurbita pepo* L.). *Cucurbit genetics. Coop Rep, 27,* 57–60.

Rakha, M.T., Metwally, E.I., Moustafa, S.A., Etman, A.A., & Dewir, Y.H. (2012). Evaluation of regenerated strains from six *Cucurbita* interspecific hybrids obtained through anther and ovule *in vitro* cultures. *Aust J Crop Sci, 6(1),* 23–30.

Seymen, M., Kurtar, E.S., Dursun, A., & Türkmen, Ö. (2019). *In Sustainable Agriculture: Assessment of plant growth promoting rhizobacteria in cucurbitaceous vegetable crops.* In: Maheshwari D., Dheeman S. (eds) Field Crops: Sustainable Management by PGPR. Sustainable Development and Biodiversity, vol 23. Springer, Cham.

Shalaby, T.A. (2006). Embryogenesis and plantlets regeneration from anther culture of squash plants (*Cucurbita pepo* L.) as affected by different genotypes. *J Agr Res Tanta Univ, 32(1),* 173–183.

Shalaby, T.A. (2007). Factors affecting haploid induction through *in vitro* gynogenesis in summer squash (*Cucurbita pepo* L.). *Sci Hortic, 115(1),* 1–6.

Sun, S.R., Zhai, Q.H., Hu, J.B., Chen, J.F., & Zhang, P. (2009). Effects of several physiological factors on embryo formation in unpollinated ovary culture of pumpkin (*Cucurbita moschata* Duch. ex Poiret). *Plant Phy Com, 45(10),* 977–980.

Wu, T., Zhou, J., Zhang, Y., & Cao, J. (2007). Characterization and inheritance of a bush-type in tropical pumpkin (*Cucurbita moschata* Duchesne). *Scientia Horticulturae*, 1-4.

Xie, M., Qin, L.Y., Pan, J.S., He, H.L., Wu, A.Z., & Cai, R. (2005). Flower morphogenesis and microspore development versus anther culture of cucumber. *Acta Bot Bor-Occ Sin, 25(6),* 1096–1100.

Xie, B., Wang, X.F., & Fan, Z.C. (2006). Improved conditions of *in vitro* culture of unpollinated ovules and production of embryonary sac plants in summer squash (*Cucurbita pepo* L.). *Sci Agric Sin, 39(1),* 132–138.

Yeşil, S. (2019). Some virus diseases of edible seed squash (*Cucurbita pepo* L.) in Aksaray province, Turkey. *YYU J Agr Sci, 29, 63-71.*

Yılmaz, Ö. (2005). *Obtention of haploid plants via ovary culture in summer squash (Cucurbita pepo L.).* (MSc), Sutcu Imam Univ Ziraat Fak, Kahramanmaras, Turkey.

Zou, T., Song, H., Chu, X., Tonga, L., Lianga, S., Gonga, S., Yanga, H., & Suna, X. (2020). Efficient induction of gynogenesis through unfertilized ovary culture with winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.). *Sci Hort, 264,* 109152.