

## Review of research on haploid production in cucumber and other cucurbits

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### ABSTRACT

This review provides a summary of haploid induction methods and factors affecting the efficacy of specific methodologies as applied to cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), winter squash (*Cucurbita maxima* Duch. ex Lam.), summer squash (*Cucurbita pepo* L.) and other cucurbits. This report is focused on studies that were carried out during the last 20 years.

The main objective of the research on the production of haploid cucurbit plants is to accelerate breeding programs through the use of homozygous double haploid lines (DHL) and to facilitate the selection of desired (e.g. disease-resistant) genotypes for breeding. Unfortunately, currently used protocols result in a low number of double haploids (DH). The most common and best-known method of obtaining haploid cucurbit plants is via pollination with irradiated pollen, which induces parthenogenetic development of haploid embryos *in planta*. The embryos are extracted from immature seeds and cultured *in vitro* to facilitate the maturation and development of plants. The studies described below were primarily aimed at the determination of an appropriate dose of radiation and the evaluation of the impact of the genotype and the time of year on the number of haploid embryos and plants obtained.

A less popular method of haploid production – ovule and ovary culture – is based on *in vitro* gynogenesis. The studies related to this method concentrated on optimising the composition of the medium and pre-treatment conditions (primarily temperature) to which the flower buds were subjected.

Recently, increasing attention has been paid to anther and microspore culture. As in the case of *in vitro* ovule and ovary culture, the medium composition and flower bud pre-treatment conditions were optimised. The most recent studies suggest that anther culture is comparable in effectiveness to the irradiated pollen technique.

Key words: androgenesis, Cucurbitaceae, double haploid, gynogenesis, microspore culture, *in vitro*, irradiated pollen

### INTRODUCTION

Haploid plants predominantly originate from meiotically reduced tissue that undergoes embryogenesis without fertilisation. Therefore, one can define haploids as plants containing a gametic

number of chromosomes in a sporophyte. Interest in haploids began with observations by Dorothy Bergner in 1921 of *Datura stamonium* L. haploid plants, described by Blakeslee et al. (1922).

The most important achievements and milestones, such as the development of protocols to

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produce haploid plants in the laboratory, the release of the first DH crop plant and the use of DHs in genetic analysis (e.g. establishing chromosomes maps), in haploid research and history were described by Forster et al. (2007). More recently Dunwell (2010) described in his comprehensive review a range of techniques that are available for the isolation or induction of haploids. Although several general reviews on haploid production have been published to date, only a few publications summarise the research in the field of cucurbit haploids. A short review by Przyborowski (1996) on haploids in cucumber (*Cucumis sativus* L.) was published in a series entitled *In Vitro Haploid Production in Higher Plants*. More recently, a brief report on haploid induction in Cucurbitaceae species was released by Gémes Juhász and Jakše (2005) in the *Biotechnology in Agriculture and Forestry* series. Still, no comprehensive review in this area has been published so far. The review presented here describes the most popular methods of obtaining haploid cucurbits, i.e. pollination with irradiated pollen, ovule/ovary culture and anther/microspore culture. It also analyses the factors that influence the success rate of each method. The most important results are summarized in Table 1. The listed publications do not refer to chromosome doubling of haploids.

#### ***The importance of double haploid lines in breeding***

The main objective of the haploid research is to obtain homozygous double haploid lines (DHLs) suitable for breeding programmes. The list of cultivars obtained through the application of DHLs (representing various genera and species) comprises nearly three hundred citations (<http://www.scri.ac.uk/assoc/COST851/DHTable2005.xls>). Half of the list (148 entries) is comprised of barley cultivars. In addition, the list includes rape (52), wheat (36), rice (11), melon (9), pepper (8), asparagus (7), tobacco (6), eggplant (5), triticale (3), Indian mustard (2) and turnip (1) cultivars.

The main emphasis in the literature describing cucumber haploids and double haploids (DHs) is on the efficiency of the methodology, i.e. the number of obtained haploids and DHs. Information about the number of DHs that were further advanced and used in the production of homozygous lines, which would be useful in breeding, is frequently missing.

In the case of cucumber, the development of homozygous parental lines using the traditional self-pollination method takes from six to eight years

(Gémes Juhász et al. 2002). According to Claveria et al. (2005), Dolcet-Sanjuan et al. (2006) and our experience with the Polish cucumber cultivars, the irradiated pollen method can be routinely applied to accelerate breeding programs and to achieve complete homozygosity in one generation, although the number of DHLs obtained is usually low.

DHs can facilitate the selection of desirable genotypes for further breeding. Lofti et al. (2003) used hybrids resulting from crosses between lines with various disease resistance characteristics as donor plants for haploid and DH production to facilitate the recovery of melon (*Cucumis melo* L.) cultivars resistant to several viruses. Research on melon carried out by Kuzuya et al. (2003) indicated that the selection for resistance to powdery mildew caused by *Sphaerotheca fuliginea* could be applied to the haploid plants. More than half of the haploid plants displayed the same level of resistance as the donor plants. In addition, haploid and double haploid plants are also used in basic research. For example, Gonzalo et al. (2005) used DHL populations to construct a genetic map of melon.

#### ***The beginnings of haploid induction in the Cucurbitaceae family***

The first haploid plants in the Cucurbitaceae family were obtained in the 1950s. In 1958 Aalders isolated haploid embryos from normal seeds of cucumber, while Swaminathan and Singh (1958) observed a haploid shoot of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) on a diploid plant grown from an X-ray treated seed. Hayase (1954) obtained a haploid plant of winter squash (*Cucurbita maxima* Duch. ex Lam.) by pollination with *Cucurbita moschata* Duch. ex Poir. A similar method (pollination with *Cucumis ficifolius* A. Rich.) was used by Dumas de Vaulx (1979), who obtained the first haploids of *C. melo*.

The first haploid plants of *Cucurbita pepo* L. (summer squash) were obtained by Dumas de Valux and Chambonnet in 1986 using a non-fertilised ovule culture. Furthermore, Zagorcheva et al. (1987) found an apomictic haploid plant among the progeny of *C. ficifolius* obtained after isolation of a non-fertilised female flower. In the 1980s, attempts were made to obtain haploid plants from anthers of *C. maxima*, *C. moschata*, *C. pepo*, *Luffa cylindrica* Mill. (smooth loofah) and *Trichosanthes dioica* Roxb. (pointed gourd) (Kumar 1984, Shail and Robinson 1987). However, the data on the results is limited.

**Table 1.** Methods and results of selected protocols for haploid induction in cucurbits

Species	Method	Result	References
<i>C. lanatus</i> watermelon	A	p	Li et al. 2008
		pH	Xue et al. 1983
	Pirr	pH	Gürsöz et al. 1991; Sari et al. 1994
<i>C. ficifolius</i> cucumis	ap	pH	Zagorcheva et al. 1987 (apomixis in isolated flower)
<i>C. melo</i> melon	G	p	Lofti et al. 2003
		pD, pH, pM	Ficcadenti et al. 1999
	Pirr	pH	Sauton and Dumas de Vaulx 1987, 1988; Sauton 1988; Cuny et al. 1993; Ficcadenti et al. 1995; Yanmaz et al. 1999; Lim and Earle 2008; Godbole and Murthy 2012; Sari et al. 2010
		pD, pH, pM	Lofti et al. 2003; Ari et al. 2010
	px	pH	Dumas de Vaulx 1979 (pollination with <i>C. ficifolius</i> )
	<i>C. sativus</i> cucumber	A	co
p			Lazarte and Sasser 1982,
pD, pH,			Ashok Kumar et al. 2003, 2004; Ashok Kumar and Murthy 2004
pD, pDH, pH, pM, pT			Song et al. 2007
G		e	Gémes Juhász et al. 1997
		p	Suprunova and Shmykova 2008
		pDH, pH	Dirks 1996 (patent)
		pDH, pH, pM	Gémes Juhász et al. 2002
		pD, pDH, pH, pT	Diao et al. 2009
		co	Suprunova and Shmykova 2008
Pirr	M	p	Zhan et al. 2009
	Pirr	pDH, pH,	Chen et al. 2008 (patent)
		pH	Truong-Andre 1988; Sauton 1989; Przyborowski and Niemirowicz-Szczytt 1992; Niemirowicz-Szczytt et al. 1995; Çağlar and Abak 1996a,b, 1999a,b; Faris et al. 1999; Lofti et al. 1999; Niemirowicz-Szczytt et al. 2001; Claveria et al. 2005; Dolcet-Sanjuan et al. 2006; Sztangret-Wiśniewska et al. 2006; Lofti and Salehi 2008
		pA, pH	Przyborowski and Niemirowicz-Szczytt 1994
		pA, pD, pH	Niemirowicz-Szczytt and Dumas de Vaulx 1989
		pD, pH	Le Deunff and Sauton 1994
		sp	pH
<i>C. maxima</i> winter squash	A	co	Shail and Robinson 1987
	Pirr	pH	Kurtar and Balkaya 2010
	px	pH	Hayase 1954 (pollination with <i>C. moschata</i> )
<i>C. moschata</i> squash	A	co	Shail and Robinson 1987
	G	e	Kwack and Fujieda 1988
	Pirr	pH	Kurtar et al. 2009
<i>C. pepo</i> summer squash	A	p	Shail and Robinson 1987
		pA, pD, pH	Mohamed and Refaei 2004
		pD, pH	Metwally et al. 1998b
	G	co	Shail and Robinson 1987
		p	Xie et al. 2006
		pH	Dumas de Vaulx and Chambonnet 1986; Gémes Juhász et al. 1997;
		pD, pH	Metwally et al. 1998a; Shalaby 2007
		pA, pD, pM, pP	Chambonnet and Dumas de Vaulx 1985
Pirr	pH	Kurtar et al. 2002	

**Table 1.** (continued)

Species	Method	Result	References
<i>L. cylindrica</i> smooth loofah	A	eD, eH	Kumar 1984
<i>M. charantia</i> bitter melon	A	co	Tang et al. 2012
<i>T. dioica</i> pointed gourd	A	eD	Kumar 1984

Methods: A – anther culture, ap – apomixis, G – ovule and/or ovary culture, M – microspore culture, Pirr – pollination with irradiated pollen, sp – spontaneous parthenogenesis, px – interspecific pollination induced parthenogenesis;  
Results: e – embryos only (no plants), co – callus only, p – plants, A – aneuploid, D – diploid, DH – double haploid, H – haploid, M – mixoploid, P – other polyploids, T – tetraploid.

## METHODS OF CUCURBIT HAPLOID INDUCTION

There are several processes leading to obtaining haploid and DH cucurbits. These are primarily haploid parthenogenesis (induced primarily by pollination with irradiated pollen), *in vitro* gynogenesis (during *in vitro* culture of ovules and ovaries) and *in vitro* androgenesis (during *in vitro* culture of microspores and anthers).

### *Pollination with irradiated pollen*

The induction of parthenogenetic development of the egg cell by pollination with irradiated pollen followed by haploid embryo rescue was first successfully used to obtain haploid embryos of melon and cucumber over 20 years ago (Sauton and Dumas de Vaulx 1987, 1988, Truong-Andre 1988, Niemirowicz-Szczytt and Dumas de Vaulx 1989). During the subsequent years, research was focused on the effect of the genotype, time of the year and radiation dose on the number of obtained haploids (Cuny et al. 1993, Przyborowski and Niemirowicz-Szczytt 1994, Çağlar and Abak 1996a, 1999a, b, Faris et al. 1999, Lofti et al. 1999). Haploid plants of watermelon and summer squash were also obtained using irradiated pollen (Gürsöz et al. 1991, Sari et al. 1994, Kurtar et al. 2002).

Experiments on cucumber haploid induction with irradiated pollen have been conducted for about 20 years at the Department of Plant Genetics, Breeding and Biotechnology of the Warsaw University of Life Sciences in Poland. Pollination is routinely carried out on 40 plants per cultivar twice a year – in the spring and in late summer. The pollen is irradiated with a dose of 300 Gy (Sztangret-Wiśniewska et al. 2006).

Recently, the application of this method resulted in haploid induction in *C. maxima* and *C. moschata* (Kurtar et al. 2009, Kurtar and Balkaya 2010). Further attempts have also been made to optimise

the method for cucumber and melon (Lofti et al. 2003, Claveria et al. 2005, Lofti and Salehi 2008, Ari et al. 2010, Sari et al. 2010, Godbole and Murthy 2012). So far pollination with irradiated pollen has been the leading method of haploid induction in these two species.

### *Efficiency of haploid recovery*

In cucumber, Sauton (1989) obtained an average of 0.9 haploid embryos per fruit (e/f). Przyborowski and Niemirowicz-Szczytt (1994) were able to obtain 4 e/f, while Çağlar and Abak (1996b) were able to achieve 16 e/f. In summer squash, Kurtar et al. (2002) obtained 7.5 e/f, while Sari et al. (2010) had 0.7 embryos per one melon fruit. Recently, an average efficiency of 13.7 embryos per fruit was obtained in winter squash (Kurtar and Balkaya 2010). The efficacy of embryo induction in cucumber and melon is determined by several factors, such as radiation dose, the genotype of the mother plants and the time of the year during which the pollination took place (Sauton 1988, 1989).

### *Irradiation and pollination*

A low dose of gamma radiation, typically about 300 Gy, was shown to be effective in experiments carried out on melon (Sauton 1988), and was also optimal in cucumber haploid production (Niemirowicz-Szczytt et al. 1995, Çağlar and Abak 1996a, 1999a). Faris et al. (1999) examined in detail the effect of the radiation dose on haploid embryo development using five lines and three hybrids of cucumber. The authors applied doses ranging from 50 to 300 Gy and found that 100 Gy was the optimal dose to induce haploid embryo development. Claveria et al. (2005) obtained higher induction levels with a higher dose of 500 Gy. Cuny et al. (1993) used doses of gamma radiation ranging from 150 to 2,500 Gy, and did not observe a significant effect of the dose on the induction of melon haploids. These results suggest

that the preferred radiation dose is specific to each particular experiment.

Cobalt ( $^{60}\text{Co}$ ) is most commonly used as the source of  $\gamma$ -rays. Gamma radiation is easy to apply and, at the same time, it has good tissue penetration, induces a high mutation rate, and has low lethality (Kurtar and Balkaya 2010). However, other sources of radiation can be used as well, for example Antos et al. (2001) obtained cucumber haploids using pollen irradiated at 300 Gy using X-rays.

Usually, radiation is applied to flowers (frequently after the removal of sepals and petals) or to isolated anthers. For instance, Kurtar et al. (2009) used anthers of *C. moschata*, while Sari et al. (1994) used flower buds with partly removed sepals and petals in watermelon. Information about the pollination method is limited. It is known, however, that a large amount of irradiated pollen applied to stigmas can stimulate a parthenogenetic response by increasing the number of pollen tubes reaching the egg cell. This phenomenon was observed in melon (Sauton and Dumas de Vaulx 1987, Cuny et al. 1993).

#### Donor plant genotype and vigour

A significant effect of the genotype of the donor plants on haploid production was observed in cucumber, melon and summer squash (Sauton 1988, 1989, Cuny et al. 1993, Przyborowski and Niemirowicz-Szczytt 1994, Niemirowicz-Szczytt et al. 1995, Kurtar et al. 2002). Furthermore, vigour and physiological state of parental plants was important for the formation of melon haploids (Sauton 1988, Cuny et al. 1993).

Niemirowicz-Szczytt et al. (1995) used cucumber inbred lines and their hybrids as mother plants. The total number of haploid embryos obtained was higher when using hybrids as compared with inbred lines. Sestili and Ficcadenti (1996) observed significant differences among hybrids generated through diallel crosses of various lines as shown by the numbers of melon haploids obtained via pollination with irradiated pollen. Therefore, plant vigour seems to play an important role in the induction of haploid embryos.

#### Time of the year

The time of the year and the month during which pollination with irradiated pollen takes place also affects the outcome. Sauton (1988) reported the highest yield of melon haploids between June and September. In the case of cucumber, the number of embryos developing into haploid plants was higher

in the summer than in the spring (Przyborowski and Niemirowicz-Szczytt 1994, Sztangret-Wiśniewska et al. 2006). Similarly, the highest number of haploid cucumber plants was obtained by Çağlar and Abak (1999a, b) in June and July. In the case of *C. pepo*, after pollination carried out from May to September, the largest number of embryos per fruit was obtained in September and June (Kurtar et al. 2002). In *C. lanatus*, *C. moschata* and *C. maxima*, a positive influence of the spring season on the yield of haploid embryos was observed (Gürsöz et al. 1991, Kurtar et al. 2009, Kurtar and Balkaya 2010).

#### Embryo detection, excision and culture

In addition to the factors mentioned above, the method of embryo detection and isolation as well as the manner of handling the seeds can affect the number of recovered haploids. The endosperm does not develop after pollination with irradiated pollen (Sauton and Dumas de Vaulx 1987, Sauton 1989, Faris and Niemirowicz-Szczytt 1999). The embryo grows initially even without the presence of endosperm, however its development is arrested at an early stage which necessitates embryo rescue and *in vitro* culture. Otherwise the embryo dies. The medium most commonly used for the rescue is the E20A medium, which was developed by Sauton and Dumas de Vaulx (1987) for melon haploid production.

One of the most common methods used for the detection and isolation of embryos consists of the physical opening of all seeds (Faris et al. 1999, Kurtar et al. 2002, Sari et al. 2010, Godbole and Murthy 2012). At the Department of Plant Genetics, Breeding and Biotechnology cucumber seeds are inspected over a light source and the transparent (empty) seeds are discarded. Another efficient method of embryo detection is the application of X-ray radiography of seeds (Savin et al. 1988, Le Deunff and Sauton 1994, Claveria et al. 2005, Dolcet-Sanjuan et al. 2006), which also enables the detection of seeds containing developing embryos.

Lofti et al. (2003) and Lofti and Salehi (2008) show that about 10 days of culture of seeds in liquid E20A medium, prior to embryo isolation, increases the number of haploid plants, likely due to the increased visibility of the embryos. However, Ari et al. (2010) pointed out the higher labour costs of the seed culture technique as compared to the inspection of seeds placed over a light source immediately after harvesting. Moreover,

a liquid medium culture increases the risk of plant material losses due to microbial infection (Lofti et al. 2003).

### ***Ovule and ovary culture***

As compared to parthenogenesis induced by pollination with irradiated pollen, the information on successful ovule and/or ovary culture (*in vitro* gynogenesis induction) is rather sparse. This technique was used successfully to obtain haploids of summer squash, cucumber and melon (Dumas de Vault and Chambonnet 1986, Gémes Juhász et al. 1997, Metwally et al. 1998a, Ficcadenti et al. 1999, Gémes Juhász et al. 2002, Shalaby 2007, Suprunova and Shmykova 2008, Diao et al. 2009). Dirks (1996), Metwally et al. (1998a), Shalaby (2007) and Diao (2009) also obtained DHs in cucumber and summer squash by inducing *in vitro* gynogenesis.

Nunhems Zaden BV, a Dutch seed company, patented the method of obtaining haploids and DHs of cucumber with ovary culture (Dirks 1996); however, no data on the efficacy of this method and its subsequent application was released. The highest reported efficiency of regeneration was 7.1 and 9 regenerated plants per 100 cucumber ovaries cultured (Gémes Juhász et al. 2002 and Diao et al. 2009, respectively), and 11 regenerated plants per 100 *C. pepo* ovaries (Metwally et al. 1998a). Thus, as compared to pollination with irradiated pollen, ovary culture is a low efficiency method.

Optimisation experiments involved evaluations of the developmental stage of the embryo sac, medium composition and temperature pre-treatment (Chambonnet and Dumas de Vault 1985, Metwally et al. 1998a, Gémes Juhász et al. 2002). Histological studies of cucumber female gametophyte showed that a late stage of development (characterised by the presence of cells similar in shape and structure) was the most suitable for embryo induction. It was also possible to obtain haploid embryos from a mature embryo sac; however, it was impossible to obtain them from megaspores and during early stages of embryo sac development (Gémes Juhász et al. 2002). In cucumber, most embryos were obtained after harvesting ovaries six days before flowering (Gémes Juhász et al. 2002). *C. pepo* ovaries harvested one day before flowering yielded the highest number of embryos in the experiments of Chambonnet and Dumas de Vault (1985) and Metwally et al. (1998a). Various types of media have been used for *in vitro* gynogenesis induction. Metwally et al. (1998a)

applied Murashige and Skoog (MS) medium with an addition of 1-5 mg dm<sup>-3</sup> 2,4-D and 30 g dm<sup>-3</sup> of sucrose. Xie et al. (2006) used N6 medium with the addition of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene-1-acetic acid and benzyladenine (BA). Gémes Juhász et al. (2002) carried out induction on their own CBM medium, enriched with 0.02 mg dm<sup>-3</sup> of thidiazuron and 40 g dm<sup>-3</sup> of sucrose.

The impact of temperature pre-treatment (low or high temperature) on *in vitro* gynogenesis was also tested. Metwally et al. (1998a) found that haploid induction in summer squash was more efficient without a low temperature stress pre-treatment. However, a high temperature pre-treatment of ovaries (28°C or 35°C) in the presence of thidiazuron increased the number of embryos and plants obtained in cucumber (Gémes Juhász et al. 2002). The optimisation of the methodology was continued by Shalaby (2007), Suprunova and Shmykova (2008) as well as Diao et al. (2009), who also studied the role of the genotype, flower position on shoots, temperature pre-treatment and medium composition on cucumber haploid production. The results of the presented studies indicate that *in vitro* ovule and ovary culture is a low efficiency method of cucurbit haploid induction, requiring further optimisation.

### ***Anther and microspore culture***

The results of the first studies on the induction of *in vitro* androgenesis in the Cucurbitaceae family, carried out on a limited scale on cucumber, watermelon, melon and summer squash, were limited to the production of mostly callus and only a small number of haploid plants (Lazarte and Sasser 1982, Dryanovska and Ilieva 1983, Xue et al. 1983, Shail and Robinson 1987). *In vitro* culture was also applied to the anthers of smooth loofah and pointed gourd by Kumar (1984), who obtained haploid and diploid embryos. In anther culture of balsam pear (*Momordica charantia* L.). Tang et al. (2012) observed only callus formation. In 1998 Metwally et al. (1998b) exposed *C. pepo* male flower buds to cold pre-treatment for four days at 4°C. This treatment was applied to male flower buds with microspores at the mid or late uninucleate stage. When the anthers were placed on MS medium with 5 mg dm<sup>-3</sup> 2,4-D and 150 g dm<sup>-3</sup> of sucrose, the regeneration level reached 1.9 plants per anther (p/a). Similar experiments carried out by Mohamed and Refaei (2004) gave slightly better results (2.6 p/a).

The *in vitro* culture of cucumber anthers resulted in callus development, followed by embryo development and organogenesis. However, haploid plants were not recovered and it was not reported from which tissues callus was formed (Lazarte and Sasser 1982).

Additional reports of cucumber anther culture have been published in the last 10 years. The anthers used contained microspores at the mid to late uninucleate stage. Ashok Kumar et al. (2003) examined in detail the effect of medium composition and flower bud cold pre-treatment on the regeneration of haploid and diploid plants. The highest rate of anthers producing embryogenic callus (54.4%) was observed with a two-day cold pre-treatment. Similar results were obtained by Xie et al. (2005). Ashok Kumar et al. (2004) and Ashok Kumar and Murthy (2004) improved the method by enriching the induction medium with various kinds of sugars (sucrose, maltose, glucose and fructose), amino acids (glutamine, glycine, arginine, asparagine and cysteine) and polyamines (putrescine and spermidine). Their last experiments produced 1.6 embryos and 1.35 plantlets per anther placed on Gamborg B5 medium supplemented with 0.44 mg dm<sup>-3</sup> 2,4-D, 0.22 mg dm<sup>-3</sup> BA, 85 g dm<sup>-3</sup> sucrose and 29 mg dm<sup>-3</sup> spermidine (Ashok Kumar et al. 2004). In 2007 Song et al. released the data of six experiments aimed at a comprehensive analysis of the impact of such factors as cold pre-treatment, physical conditions during *in vitro* culture, composition of induction medium and genotype on regeneration. As a result, the authors developed a protocol for anther culture that gave the highest efficiency (three embryos per anther and 0.9 DH plants per anther). The best medium for embryonic callus induction was MS medium supplemented with 1 g dm<sup>-3</sup> BA, 0.5 g dm<sup>-3</sup> 2,4-D, 1 g dm<sup>-3</sup> kinetin and 30 g dm<sup>-3</sup> sucrose while suitable temperature stress depended on the place of origin of each cultivar. The application of their protocol resulted in the production of double haploids, haploids, diploids and plants of other ploidy.

The formation of haploids and DHs from microspores as well as the formation of diploid plants from somatic tissue was observed in anther culture of cucumber (Ashok Kumar et al. 2003, Ashok Kumar and Murthy 2004, Song et al. 2007). Song et al. (2007) noted embryo regeneration only from callus (indirect embryogenesis), while Ashok Kumar and Murthy (2004) reported both direct and indirect embryogenesis. In the case of regenerated diploid plants one needs to determine their

origin, whether it is gametophytic or sporophytic, e.g. with the use of molecular markers. This additional analysis could be avoided with the application of microspore culture instead of anther culture.

Only a few researchers attempted to obtain cucumber haploids via the use of microspore culture and the results were unsatisfactory in most cases. In the experiments carried out by Suprunova and Shmykova (2008), microspore culture resulted in callus formation from vegetative cells. In China Chen et al. (2008) patented a method for isolated microspore culture of cucumber. The patent describes a procedure from flower bud collection to haploid or DH plant regeneration. However, the data presented is general in nature and does not include the method's efficacy. The following year the same researchers released a publication on cucumber microspore culture (Zhan et al. 2009). Fourteen plants were obtained from two out of four genotypes used. The embryo recovery was 1.5-33.4 embryos per 2 cm<sup>3</sup> of a liquid medium containing 2×10<sup>5</sup> microspores. The authors reported that a two-day cold pre-treatment of buds containing microspores at a late uninucleate stage and the addition of 0.5 mg dm<sup>-3</sup> 2,4-D and 0.2 mg dm<sup>-3</sup> BA to NLN or B5 medium stimulated haploid embryogenesis. Recently, an attempt to regenerate plants from cucumber microspores has been made by Kielkowska and Havey (2011). Although the authors experimented with three genotypes of cucumber, applying eight liquid media, a diversified microspore suspension density and low and high temperature pre-treatment, no microspore divisions were observed.

## CHROMOSOME DOUBLING IN HAPLOIDS

In the case of cucurbits, haploid plants are often obtained first, and are subsequently subjected to chromosome duplication. The plants derived from parthenogenetically developed embryos are predominantly haploid and genetically stable, whereas *in vitro* culture of ovaries and anthers leads to the formation of both haploids and DHs of cucumber (see Table 1).

The procedures for chromosome doubling of cucurbit haploids are mainly based on colchicine treatment (Çağlar and Abak 1997, Nikolova and Niemirowicz-Szczytt 1996, Sari and Abak 1996, Yetisir and Sari 2003), a method that is constantly being revised (Lim and Earle 2008, 2009). Çağlar and Abak (1997) analysed the influence of

colchicine concentration and treatment duration on chromosome doubling of cucumber haploids *in vitro* and noted that the highest efficiency of chromosome doubling (60% on average) was obtained with the immersion of plantlets in 0.5% colchicine solution for four hours. Earlier experiments by Nikolova and Niemirowicz-Szczytt (1996) showed that more favourable results could be obtained by multiple applications (3-5 times) of 0.1% colchicine solution on apical meristems of plants grown *in vitro*.

Another chromosome doubling method designed for cucumber employs the direct regeneration of plants from haploid leaf explants (Niemirowicz-Szczytt et al. 1995), which showed to be more effective than colchicine treatment (Sztangret-Wiśniewska et al. 2006). In their previous research Faris et al. (2000) evaluated the usefulness of direct and indirect regeneration for cucumber haploid doubling. The doubling proved to be more effective (showing higher rate of DH plants among regenerated plants) when indirect regeneration was applied; however, the results are difficult to compare as the haploids submitted to direct and indirect regeneration were derived from different donor plants. Faris et al. (2000) used explants from the first and second haploid leaf in order to directly regenerate DHs of cucumber. Plants were regenerated from both types of leaves (388 and 210, respectively), however, all plants derived from the first leaf were haploids, whereas the second leaf yielded 70.5% haploids, 28.2% diploids and 1.3% mixoploids. This shows that the developmental age of the tissue being regenerated may be of major significance for the effectiveness of chromosomal doubling.

Whether with the application of colchicine treatment or regeneration from haploid leaves, it is possible to obtain DHs, haploids and plants of different ploidy (i.a. Faris et al. 2000, Claveria et al. 2005, Sztangret-Wiśniewska et al. 2006, Lim and Earle 2008, 2009). Cytological observations have to be performed in order to evaluate the ploidy level of the obtained plants. Typically, the cells of the root apical meristem are used for chromosome count or leaf samples are used in flow cytometry. In cucumber one can distinguish between haploids, diploids and tetraploids on the basis of leaf and flower shape (Sauton 1989, Nikolova and Niemirowicz-Szczytt 1996). However, in the study of Nikolova and Niemirowicz-Szczytt (1996), the ultimate ploidy indicator was the number of chromosomes in the maternal cells of anther.

## CONCLUSIONS

The first haploid plants in the Cucurbitaceae family were obtained in the 1950s. To date, haploid and DH plants were obtained in eight species. Most of these species, especially cucumber, squash, melon and watermelon, are widely grown and are of economic importance. Information on the use of DHLs in breeding programs of particular species is rather sparse. Nevertheless, the main objective of haploid research is to facilitate and accelerate breeding programs, although the number of DHLs produced remains low.

Researchers in various research centres all over the world were successful in inducing haploids in many cucurbit plants through pollination with irradiated pollen. This most popular method of haploid induction is being continuously optimised and applied to an increasing number of cucurbit species. It is also being employed in breeding programmes.

The number of publications on the successful application of *in vitro* gynogenesis in cucurbit plants is rather small. The results of *in vitro* ovule and ovary culture indicate a low efficacy of the method and the need for its further optimisation. Recently, interest has been focused on *in vitro* anther culture and, though to a lower extent, microspore culture as well. A direct comparison of the irradiated pollen technique and anther culture is difficult. In cucumber it is possible to obtain up to 5-10 haploid embryos (which require doubling) per fruit using irradiated pollen. *In vitro* culture of anthers allows DHs to be obtained directly, with regeneration of up to three embryos per anther. The newest reports suggest that anther culture can provide an equivalent or alternative method of cucumber haploid induction to that of pollination with irradiated pollen. However, our experiments on anther and microspore culture do not confirm the high efficiency of these two methods (unpublished).

Plants obtained from parthenogenetic embryos are predominantly haploids, while the use of ovule or ovary culture and anther or microspore culture allows the induction of both haploids and DHs. Chromosome doubling in haploids is obtained primarily by colchicine application. In cucumber chromosome doubling by plant regeneration from haploid leaf explants has also proved to be effective.

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#### PRZEGLĄD BADAŃ NAD HAPLOIDAMI OGÓRKA I INNYCH ROŚLIN DYNIOWATYCH

Streszczenie: Niniejsze opracowanie obejmuje historyczny i metodyczny opis metod otrzymywania haploidów ogórka (*Cucumis sativus* L.), melona (*Cucumis melo* L.), arbuza (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), dyni olbrzymiej (*Cucurbita maxima* Duch. ex Lam.), dyni zwyczajnej (*Cucurbita pepo* L.) i innych roślin dyniowatych oraz czynniki wpływające na wydajność poszczególnych metod. W szczególności opracowanie dotyczy badań prowadzonych w ciągu ostatnich dwudziestu lat.

Głównym celem badań nad haploidami jest otrzymanie linii podwojonych haploidów, które są homozygotyczne, co może przyspieszyć proces hodowli, chociaż liczba otrzymywanych linii jest zwykle mała. Zastosowanie podwojonych haploidów w trakcie hodowli może również ułatwić wyselekcjonowanie określonych roślin, na przykład odpornych na choroby.

Najczęściej stosowaną i najlepiej poznaną metodą otrzymywania haploidów roślin dyniowatych jest zapylenie napromieniowanym pyłkiem, które indukuje partenogenetyczny rozwój haploidalnych zarodków na roślinie (*in planta*). Zarodki izoluje się i poddaje kulturze *in vitro* w celu regeneracji roślin. Prace nad zastosowaniem tej metody poświęcone były przede wszystkim ustaleniu odpowiedniej dawki promieniowania oraz wpływu genotypu i pory roku na liczbę otrzymywanych zarodków i roślin haploidalnych.

Mniej popularną metodą otrzymywania haploidów jest kultura załączków i załączni, wykorzystująca zjawisko gynogenezy *in vitro*. W przypadku tej metody badania skupiły się na ustaleniu składu pożywki i sposobu traktowania wstępnego pąków kwiatowych (wysoka lub niska temperatura).

W ostatnich latach wzrosło zainteresowanie kulturą *in vitro* pylników, oraz w mniejszym zakresie kulturą mikrospor (androgeniza *in vitro*). Podobnie jak w przypadku gynogenezy *in vitro*, optymalizacji podlegały przede wszystkim skład pożywki oraz traktowanie wstępne pąków kwiatowych. Wyniki najnowszych badań pozwalają przypuszczać, że kultura pylników może być równorzędną metodą otrzymywania haploidów ogórka w stosunku do zapylenia napromieniowanym pyłkiem.

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