Gynogenesis in Agricultural Crops

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Gynogenesis is a viable methodology with promising results in recalcitrant species for the generation of doubled haploids, which uses unpollinated female gametophytes. This technique has been successful in loquat (*Eriobotrya japonica* (Thumb) Lindl.), citrus (*Citrus grandis* (L.) Osbeck), spinach (*Spinacia oleracea* L.), cucurbits, red beet (*Beta vulgaris* L.) and *Gentiana* ssp. crops, where it is feasible to apply this technique in breeding.

Keywords: Gynogenesis ; Agricultural Crops ; Irradiated pollen ; Wide Hybridization ; In vivo haploid inducers

1. Gynogenesis in Agricultural Crops

Haploid regeneration by means of unpollinated female gametophytes is one of the most commonly used alternatives in species where androgenesis has not been effective; this method is called haploid gynogenesis or haploid parthenogenesis. The term gynogenic haploid regeneration is used for all haploid induction methods in which a female gametophyte is used as the origin of the haploid cells, regardless of whether it is a pseudofertilization process or not; therefore, there are four variants: (a) in vitro culture of unfertilized ovaries or ovules [1], (b) pollination with pollen irradiated with cobalt-60 (^{60}Co) [2][3], (c) wide hybridization [2] and (d) in vivo haploid inducers [3][4].

1.1. In Vitro Culture of Ovaries or Ovules

In the case of self-pollinated species, in vitro culture of unfertilized female gametes is achieved by culturing flower buds prior to anthesis, while in male-sterile or self-incompatible plants it is performed at any stage of ovule development, since they show a favorable response to gynogenic induction [5]. This technique is successfully employed in species of the genus *Allium*, where it is the main technique to derive DHs [6]. For example, Panahandeh et al. [7] achieved a gynogenic induction range of 5 to 12% by culturing unpollinated flower buds of *Allium hirtifolium* Boiss., which allowed callus formation with a success rate of 20%, of which the efficiency of obtaining haploid plants was 70 to 77%. This technique is also viable in both wild and improved species of the genus *Gentiana* L. spp. [8][9][10]. Although the results obtained were promising in both species mentioned, the authors agree that it is necessary to continue with the establishment of efficient protocols because the average response in obtaining haploid plants does not exceed 5% (**Table 1**).

Table 1. Examples of protocols used for successful haploid induction mediated in vitro culture of unfertilized ovaries or ovules.

Species	Common Name	Pathway	Ploidy Level Determination	Haploid Induction Rate	Reference
***Beta vulgaris* L.**	**Red beet**	**Unfertilized ovule culture**	**Flow cytometry and chromosome counting**	**25%**	**Zayachkovskaya et al. [11]**
***Gentiana* spp.**	**Gentians**	**Unfertilized ovule culture**	**Flow cytometry and molecular marker analysis**	**32.5%**	**Takamura et al. [8]**
***Allium hirtifolium* Boiss**	**Persian shallot**	**Unfertilized ovary**	**Squash root**	**0–77%**	**Panahandeh et al. [7]**
Gentiana triflora	**Gentians**	**Unfertilized ovules**	**Flow cytometry and Feulgen staining**	**23.5–56%**	**Doi et al. [10]**
***Solanum lycopersicum* L.**	**Tomato**	**Non-fertilized ovary culture**	**-**	**0%**	**Bal et al. [12]**

1.2. Irradiated Pollen

Irradiated pollen allows the development of haploid embryos by fertilizing an ovule with mature pollen whose genetic material is inactive, i.e., it is capable of inducing cell divisions in the ovule and the normal development of the embryo [2].

There are many favorable examples involving the use of irradiated pollen in different vegetable and fruit species in which androgenesis was not an option (**Table 2**).

Table 2. Examples of successful haploid induction methods by induced parthenogenesis by irradiated pollen in recalcitrant species.

Species	Common Name	Pathway	Ploidy Level Determination	Haploid Induction Rate	Reference
***Eriobotrya japonica* (Thunb.) Lindl.**	**Loquat**	**γ–irradiated pollen**	**Flow cytometry**	**0.007–0.008%**	**Blasco et al. [13]**
***Citrus grandis* (L.) Osbeck**	**Pummelo**	**γ–irradiated pollen**	**Flow cytometry**	**1%s**	**Wang et al. [14]**
***Spinacia oleracea* L.**	**Spinach**	**γ-irradiated pollen**	**Flow cytometry**	**-**	**Keleş et al. [15]**
***Cucumis melo* L.**	**Melon**	**γ-irradiated pollen**	**Flow cytometry**	**14–33%**	**Lotfi et al. [16]**
***Cucumis melo* L.**	**Melon**	**γ-irradiated pollen**	**Chromosome counting**	**23.65%**	**Nasertorabi et al. [17]**
Citrus reticulata	**Mandarin**	**γ-irradiated pollen**	**Flow cytometry**	**2.58–8.33%**	**Jedidi et al. [18]**

Thus, Hooghvorst et al. [3] and Kurtar et al. [19] reported that in cucurbits, a family containing crops of high economic value such as pumpkin, melon and cucumber, pollination with γ-ray-irradiated pollen is the most efficient method to induce haploidy because it has not been possible to take advantage of androgenesis in these crops. In *Cucumis melo* L., pollen irradiated with 250 Gys of ^{137}Cs was more effective compared to in vitro culture of unpollinated ovules [16]. Likewise, Nasertorabi et al. [17] obtained 48 Cucumis melo L. plants induced from embryos obtained with pollen irradiated with 550 Gys of ^{60}Co, of which 94% were haploid.

In citrus, this technique has proven to be very useful to obtain haploid plants with high value for breeding. For example, Wang et al. [14] were able to induce haploid plants in *Citrus grandis* L. *Osbeck* by irradiating pollen with γ-rays with doses lower than 500 Gys and in vitro culture of immature embryos. Likewise, Jedidi et al. [18], by irradiating pollen at 250 Gys with γ-rays, obtained seven seedlings that were used to generate homozygous lines in *Citrus reticulata* Blanco.

1.3. Wide Hybridization

The third variant of gynogenesis consists of interspecific crosses, through which it is possible to induce the formation of haploid embryos due to the fertilization of an ovule with pollen from a distant species, allowing double fertilization. However, cell divisions in the zygote eliminate the chromosomes of the male parent [2][20]. Thus, Santra et al. [21] published an efficient protocol to obtain completely homozygous lines in only two years by wide hybridization to obtain DHs from wheat pollinated with maize pollen.

Although wide hybridization is most commonly used in cereals, in recent years its application in leafy vegetables has been shown to have acceptable results in the induction of haploid plants (**Table 4**). For example, Piosik et al. [22] carried out distant hybridization of *Lactuca sativa* L. with *Helianthus annus* L. and *Helianthus tuberosus* L., with which they established an effective methodology to induce haploidy in lettuce. In addition, Wei et al. [23] obtained haploid offspring by embryo rescue and subsequent duplication of chromosomal material with colchicine using a commercial variety of *Brassica oleracea* var. *alboglabra* as the male parent and a variety of *Brassica rapa* var. *parachinensis* as the female parent. Similarly, haploid plants were obtained by crossing *Brassica rapa* × *Brassica oleracea* and in vitro culture of immature embryos [24].

Table 3. Summary of haploid induction methodologies by wide hybridization.

Species	Common Name	Pathway	Ploidy Level Determination	Haploid Induction Rate	Reference
***Triticum aestivum* L.**	**Wheat**	**Wheat × maize crossing**	**-**	**-**	**Wiśniewska et al. [25]**

Species	Common Name	Pathway	Ploidy Level Determination	Haploid Induction Rate	Reference
Lactuca sativa L.	Lettuce	Cross-pollination with *Helianthus annus* L.	Flow cytometry and chromosome counting	15%	Piosik et al. [22]
Lactuca sativa L.	Lettuce	Cross-pollination with *Helianthus tuberosus* L.	Flow cytometry and chromosome counting	16%	Piosik et al. [22]
Solanum lycopersicum L.	Tomato	Cross-pollination with *S. sisymbriifolium* Lam.	Chromosome counting	0%	Bal et al. [26]
Solanum lycopersicum L.	Tomato	Cross-pollination with *S. sisymbriifolium* Lam.	Flow cytometry and chromosome counting	~10% cells haploids	Chambonnet [27]

1.4. In Vivo Haploid Induction

In the past decade, methodologies applied to induce in vivo haploidy to accelerate the production of double haploid lines have been developed for several target crops [3][28]. These methodologies take advantage of the specific gene expressions that regulate the formation of maternal haploids (**Table 4**). In maize, the generation of in vivo haploid inducer lines of maternal haploidy via the expression of the genes *MATL* [29], *NLD* [30] and *ZmPLA1* has been possible [31]. In wheat, the genetic edition of the gen *MTL* permitted to observe that the alleles *mtl-AD*, *mtl-BD* and *mtl-ABD* were effective to generate inducer lines from self-pollinated and cross-pollinated progenies; its rate of success ranged between 7.8% and 15.6% [32]. However, these genes do not work in dicot species [33]. On the other hand, the haploid induction from aneuploidy is possible via CRISPR/Cas9 mutation of the *CENH3* gene in both monocot and dicot crops [3][28]. These two methodologies are very promising and are used in cereals because they have been more efficient than the in vitro methods.

Table 4. Summary of haploid induction reports via in vivo haploid inducers.

Species	Common Name	Pathway	Ploidy Level Determination	Haploid Induction Rate	Reference
Zea mays L.	Maize	Inducer inbred lines	Morphological markers	2.5–15.7%	Qu et al. [34]
Zea mays L.	Maize	BHI Bulk	Embryo coloration (R1-nj)	11.2–16.8%	Trampe et al. [35]
Zea mays L.	Maize	Frame-shift mutation in *MATRILINEAL* (*MTL*)	Flow cytometry	6.7%	Kelliher et al. [29]
Zea mays L.	Maize	Eliminate native CENH3- gene	Flow cytometry	0.05–0.31%	Kelliher et al. [4]
Zea mays L.	Maize	Inducer lines (NOT LIKE DAD)	Morphological markers	0–3.59%	Gilles et al. [30]
Triticum aestivum L.	Wheat	Edited the MTL alleles using CRISPR/Cas9	Chromosome counting	0–15.6%	Tang et al. [32]
Arabidopsis thaliana	Arabidopsis	Edited the DMP genes using CRISPR/Cas9	Flow cytometry	0–4.41%	Zhong et al. [33]
Brassica napus L.	Oilseed rape	Knocked out of BnaDMP using CRISPR/Cas9	Flow cytometry	1.5 +-0.63%	Li et al. [36]
Brassica napus L.	Oilseed rape	DMP CRISPR/Cas9 mutagenesis	Flow cytometry	0–4.44%	Zhong et al. [37]
Nicotiana tabacum	Tobacco	DMP CRISPR/Cas9 mutagenesis	Flow cytometry	0–1.63%	Zhong et al. [37]
Nicotiana tabacum	Tobacco	DMP CRISPR/Cas9 mutagenesis	Flow cytometry and cytological observation	1.52–1.75%	Zhang et al. [38]
Medicago truncatula Gaertn	Barrel medic	DMP CRISPR/Cas9 mutagenesis	Flow cytometry	0.29–0.82%	Wang et al. [39]

Species	Common Name	Pathway	Ploidy Level Determination	Haploid Induction Rate	Reference
***Solanum lycopersicum* L.**	**Tomato**	**DMP CRISPR/Cas9 mutagenesis**	**Flow cytometry**	**0.5–3.7%**	**Zhong et al. [40]**
***Solanum lycopersicum* L.**	**Tomato**	**Edition of the CENH3 gen with GFP-tailswap disruption**	**Flow cytometry**	**0.2–2.3%**	**Op Den Camp et al. [41]**

In contrast, the conservation of the *DMP* genes in dicot species opens up the possibility to apply this haploidy induction system [33]. From this starting point, protocols for some horticultural crops have been developed. In *Brassica napus* L., *bnaDMP* mutation could induce amphihaploidy [36][37]. In *Nicotiana tabacum* L., it was reported that the simultaneous *MtDMP1*, *MtDMP2* and *MtDMP3* mutations can trigger maternal haploidy at rates from 1.52% to 1.75% [38]. In contrast, the inactivation of the *MtDMP8* and *MtDMP9* alleles in *Medicago truncatula* Gaertn would facilitate in vivo maternal haploid induction at a rate from 0.29% to 0.82% in mutant progeny [39]. Despite these results, the use of *DMP* genes is not very frequent because there are not transformation systems (CRISPR/Cas9) or TILLING populations in major crops [3][37].

2. Gynogenesis in Tomato

Due to the few successful results obtained by androgenesis for haploidy induction and the formation of doubled haploids in tomato, some research groups have sought alternatives to achieve this goal. The options employed are variants of gynogenesis: wide hybridization, unfertilized ovule culture and irradiated pollen [42]; and haploid inducers/CRISPR/Cas9 [40]; however, it is not yet fully known what these could mean for the breeding of this crop.

2.1. Wide Hybridization

Wild species phylogenetically related to tomato are commonly used for crop improvement to incorporate alleles of interest into crop breeding programs, most notably *S. pimpinellifolium* [43], *S. arcanum* Peralta [44], *S. sitiens* I. M. Johnst [45], *S. pinnelli* L. [46], *S. chilense* (Dunal) Reiche [47], *S. neorickii* D. M. Spooner, G. J. Anderson & R. K. Jansen [48], *S. habrochaites* S. Knapp & D. M. Spooner [49] and *S. sisymbriifolium* Lam. [50].

The general use of wide crosses in this species is not only performed to induce haploidy, as some studies have attempted to apply them to generate DHs (**Table 4**). For example, *S. sisybriifolium* pollen was used unsuccessfully to induce haploids [26]. In contrast, *S. sisybriifolium* pollen allowed obtaining haploid and di-haploid genotypes of maternal origin.

Even though only ~10% of embryos were rescued and only two plants were generated, the results suggest that it may be a viable alternative; however, the author suggests that the procedure needs to be modified to improve results [27].

2.2. Unfertilized Ovule Culture

Few attempts have been made to obtain haploid tomato plants by in vitro culture of unfertilized ovules (**Table 2**). In tomato, this objective was not possible despite the fact that ovules have a variable response to different culture media [12]. Moreover, Zhao et al. [51] designed a very efficient in vitro protocol with which they isolated, from a single ovary in tomato, between 100 and 150 ovules with which they were able to induce gynogenic callus; despite this, they were unsuccessful in regenerating haploid plants.

2.3. Irradiated Pollen

Regarding the use of irradiated pollen in tomato, the work carried out is limited, although the results are promising (**Table 3**). Thus, Nishiyama et al. [52] reported that *S. pimpinellifolium* pollen maintains its germination capacity and that it is possible to generate fruits with some seeds with doses of 2000 to 7000 Gys of X-rays. In addition, Nishiyama et al. [53], when applying between 100 and 1100 Gys in increments of 100 Grays with X and γ radiation to *S. pimpinellifolium* pollen, found that it has the same effect on germination and fruit set, with a pollen germination capacity of less than 50% with doses higher than 300 Gys. These studies suggest the possibility of obtaining tomato fruits and seeds from irradiated pollen, although the doses used did not allow inactivating the genetic material of the microspore and inducing haploid parthenogenesis. However, the success of this technique obtained in other crops allows people to assume that it is essential to determine the median lethal dose (LD_{50}), which could vary according to the genotype and species [1][54].

For this methodology to be used in tomato breeding programs for haploidy induction, the optimum dose for the inactivation of genetic material in pollen must be determined. In recent years, Akbudak et al. [55] irradiated pollen from different tomato hybrids with doses of 100, 200, 300 and 400 Gys of γ-rays without obtaining fruit in any treatment although radiation doses higher than 200 Gys correspond to LD50. Likewise, Bal et al. [42] mentioned their own unpublished work on haploidy induction in this crop using irradiated pollen, where 1000 Gys caused the loss of viability and germination capacity of the pollen; however, with 800 Gys, fruits were generated, which were aborted in the early stages of development.

2.4. In Vivo Haploid Inducers

In tomato, the use of CRISPR/Cas9 has been applied to achieve objectives such as introgression breeding [56], plant architecture, fruit development and ripening [57], herbicide-resistance [58], leaf development [59] and ToBRFV-resistant tomato [60]. This suggests that it is possible to generate protocols to use the *DMP* and *CENH3* genes that regulate the gynogenesis to facilitate the generation of maternal haploid inducer males, as reported in maize [29][30][31] and wheat [32]. Thus, Zhong et al. [40] obtained *sldmp* tomato mutants using CRISPR/Cas9, with a rate of 1.9% for haploidy induction. Likewise, KEYGENE N. V. (Wageningen, Netherlands) has a patent for a methodology to generate haploids via GFP-tailswap disruption that by editing the *CENH3* gene produces 0.5–2.3% of haploids [41]. These achievements produced by genetic edition show the potential of the in vivo haploid inducers to obtain DH lines in tomato and other recalcitrant crops.

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