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Review

History and Current Status of Haploidization in Carrot (*Daucus carota* L.)

Agnieszka Kiełkowska [1,*] and Waldemar Kiszczak [2]

[1] Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, Al. Mickiewicza 21, 31-120 Krakow, Poland
[2] Department of Applied Biology, The National Institute of Horticultural Research, Konstytucji 3 Maja 1/3 Str., 96-100 Skierniewice, Poland
* Correspondence: a.kielkowska@urk.edu.pl

Abstract: The induction of haploid cell development into normal plants enables the production of doubled haploid lines, which are homozygous and can be used in breeding programs as an alternative to conventionally derived inbred lines. In this paper, we present the historical background and current status of the attempts of haploid induction in carrot (*Daucus carota* L.). Economically, carrot is one of the most important vegetables. It is an outcrossing diploid (2n = 2x = 18) species. Nowadays, the seeds of hybrid cultivars constitute the majority of the carrot seeds sold in the world. Hybrid cultivars of carrot are produced using inbred populations. Inbreeding in this species is difficult due to an inbreeding depression and is also time-consuming, as it is a biennial crop. Therefore, the implementation of the haploidization technology into the breeding programs of carrot is of high interest. Androgenesis, gynogenesis and induced parthenogenesis are the methods that have been used for haploid induction, and their potential in haploidization of carrot is discussed. The centromere-specific histone 3 variant (CENH3) and its manipulation in carrot is also acknowledged.

Keywords: androgenesis; anther culture; carrot; doubled haploids; haploids; homozygosity; induced parthenogenesis; isolated microspore culture

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1. Introduction

Umbellifers are important contributors to food consumption due to their diverse and unique flavoring and textural characteristics that complement many diets. *Apiaceae* includes vegetables such as carrot (*Daucus carota* L.), celery (*Apium graveolens* L.), parsnip (*Pastinaca sativa* L.) and parsley (*Petroselinum crispum* Mill.), as well as herbaceous plants, e.g., dill (*Anethum graveolens* L.), anise (*Pimpinella anisum* L.) and fennel (*Foeniculum vulgare* Mill.). Some umbellifers have edible leaves, petioles, stems (e.g., celery and fennel) and fruits, e.g., anise and cumin (*Cuminum cyminum* L.); however, vegetable umbellifers are grown for their edible storage root (e.g., parsnip, parsley), carrot being the most important. *Daucus carota* L. isadiploid (2n = 2x = 18), outcrossing, insect-pollinated biennial species. It is encountered amongst the top ten most consumed vegetables globally [1]. It is widely produced throughout Asia and the United States, and has the highest cultivation area and world production amongst all umbellifers. Cultivated carrot is rich in bioactive compounds, i.e., carotenoids, anthocyanins, chlorogenic acid, essential oils and dietary fibers [1,2]. Moreover, carrot is important crop for world nutritional security, as it is one of the main dietary sources of provitamin A carotenoids [3]. Carrot produces inflorescences defined as the compound umbels, which consists of pedicels (flower stalks) radiating from the common center at the end of the inflorescence stalk. Each pedicel is, again, umbellately branched, and each branch ends with a flower. A single carrot flower is a few (2–4) millimeters in diameter and consists of five sepals, five petals, five stamens and an inferior ovary. Carrot is protoandric and the stamens release pollen before the stigma of the same flower is receptive [2]. The breeding of F1 hybrids of carrot is based on genetic-cytoplasmic male

sterility (CMS). Hybrid varieties account for the majority of the carrot seeds sold in the world today and are produced using inbred populations obtained after several generations of self- or sib-pollination (usually 6 to 7) that also result in strong inbreeding depression [3].

Nowadays, new cultivars of many important edible plant species are mainly produced in high yield and good quality as hybrids derived from crosses between homozygous parents. Two approaches are utilized for the production of parental lines. A conventional method requires selfings of the selected plants, which is a time- and resource-consuming process due to many generation cycles, demand for plant isolation, and often observed effect of inbreeding depression. Alternatively, homozygous plants can be achieved by the induction of haploidization. In their sporophytes, haploid plants have a gametic chromosome number and by doubling their genome, doubled haploids (DH) are produced [4,5]. Doubled haploids are of great importance for plant breeding programs of edible crops for the following reasons: (1) true homozygous plants are generated, which is often not possible in conventional breeding; (2) the time required to obtain homozygous plants (usually one generation) is considerably shorter compared to inbreeding (several generations); (3) selection in DH population is more accurate and efficient, especially for traits caused by recessive genes in cross-pollinated crops; (4) relatively small populations are needed to breed the cultivar through the DH system, as the frequency ofsuperior gametes is always higher than the frequency of corresponding plants in F2 generation; (5) the obtained DHs are homozygous at all loci and can represent a new variety in self-pollinated crops or parental line for the production of hybrid cultivars in cross-pollinated crops [6–8].

Spontaneous haploids are observed in nature and can develop in the following ways: (1) semigamy (an abnormal type of fertilization whereby either reduced or unreduced male and female gametes participate in embryo formation, but fertilization does not occur); (2) polyembryony (the production of two or more embryos in one seed, owing to the existence and fertilization of more than one embryonic sac in one ovule); (3) androgenesis (embryo formation after failure of the egg nucleus to participate in fertilization); or (4) gynogenesis (embryo formation after failure of the sperm cell to fuse with the egg nucleus). However, the occurrence of spontaneous haploidization is rare [9,10]. On the other hand, haploids can be induced by various in vitro methods using female and male gametophytes (Figure 1).

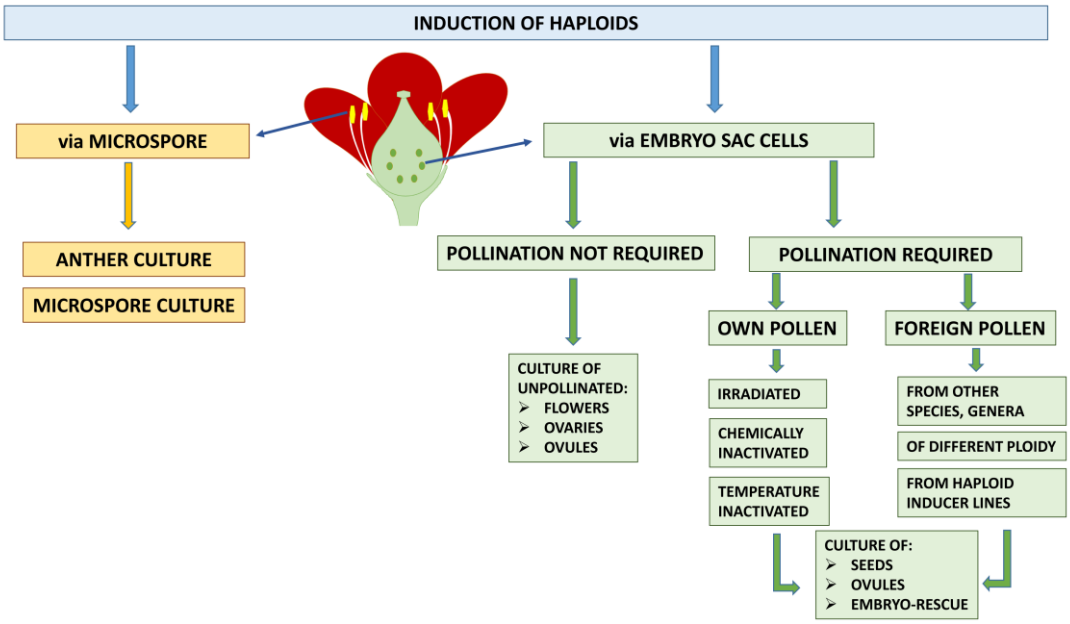

Figure 1. Methods and techniques of haploid plant production in vitro via male (yellow path) and female (green path) gametophytes.

Androgenesis in vitro is the method in which the embryo develops from microspores (immature pollen grain) and contains only paternal chromosomes. Gynogenesis in vitro refers to female parthenogenesis in which the embryo contains only maternal chromosomes. Interspecific crossing might result in the development of a haploid embryo by fertilizing an ovule with pollen of another species and the subsequent elimination of the chromosomes of the pollen. In other methods, the haploid embryo develops as a result of the pollination of flowers with their own, but inactivated (irradiation, chemical, temperature), pollen that, nevertheless, is capable of introducing cellular divisions in the embryo sac cells leading to the development of the embryo containing only maternal chromosomes.

In general, the *Apiaceae* family has been considered recalcitrant to haploidization [11]. Confirmed haploid or doubled haploid plants were regenerated in celery, carrot, caraway (*Carum carvi* L.) and *Bupleurum falcatum* L. [12–16]. Vurtz et al. [17] presented an analysis of economic efficiency of the production of carrot lines using classical breeding and biotechnology-supported methods. The analysis involved, among others, energy costs, labor, chemicals (plant protection and fertilization, media components), water, storage, as well as materials and equipment for in vitro culturing. It was shown that the economic costs of obtaining carrot breeding lines using doubled haploid technology was reduced almost five-fold when compared to the classical approach involving self-pollination and the maintenance of male-sterile plants and selection. It has been shown that the implementation of doubled haploid technology has improved and accelerated breeding programs in many important species, such as corn, wheat, maize, barley, onions, peppers or rapeseed [7,8]. Therefore, the application of this technology into carrot plant breeding is of interest for carrot breeders all over the world. In carrot, the potential of androgenesis, gynogenesis and induced parthenogenesis has been reported for haploid induction. Moreover, centromere-specific histone 3 variant (CENH3) and its manipulation in carrot is discussed in the present review.

2. Haploid Induction from Male Gametophyte (Androgenesis)

2.1. Male Gametophyte Development

Pollen development consists of two major phases: microsporogenesis (I in Figure 2) and microgametogenesis (II in Figure 2). The primary sporogenous layer within the anther gives rise to diploid microsporocytes (pollen mother cells, meiocytes) (Figure 2a). Meiotic division first produces dyads (Figure 2b) and then tetrads (Figure 2c) of four haploid microspores enclosed within a callosic (β-1,3-glucan) cell wall. Callose is degraded by the activity of an enzyme complex (callase) secreted by the tapetum leading to the separation of tetrads into individual microspores (Figure 2d). Microspore growth and development proceed through a progressive cycle of vacuole biogenesis (events of its fusion and fission) [18]. Vacuole expansion is associated with the strong polarization of the microspore nucleus against the microspore wall (Figure 2e). Polarization of the microspore nucleus may provide a signal for entry into the highly asymmetric cell division at pollen mitosis I. Pollen mitosis I gives rise to two daughter cells with different structures and cell fates (Figure 2f). The large vegetative cell has dispersed nuclear chromatin and constitutes the majority of the pollen cytoplasm. In contrast, the smaller generative cell has condensed nuclear chromatin and contains relatively few organelles. The vegetative cell exits the cell cycle at the G1 phase, whereas the generative cell undergoes mitosis II to form the two sperm cells (Figure 2g) [19].

2.2. Androgenesis In Vitro

The induction of androgenic haploids can be obtained by culturing anthers or by isolation microspores from the anthers. The unicellular microspores or pollen at the early bicellular stage (Figure 2) are able to switch the developmental pathway to form multicellular structures, then embryos, and finally mature plants with a haploid or dihaploid number of chromosomes [20]. The phenomenon of pollen embryogenesis was first demonstrated by Guha and Maheshwari [21] in *Datura innoxia* Mill. Then, the application of anther and isolated microspore cultures was documented in many species, including carrot (Table 1) [22].

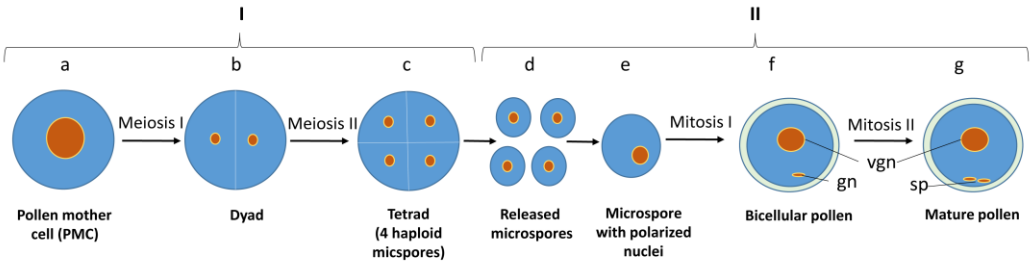

Figure 2. Schematic diagram illustrating pollen development in plants. Pollen development occurs in anthers located within a flower. Phase I is microsporogenesis and phase II is microgametogenesis. During microsporogenesis, the PMC (**a**) undergoes meiosis (**b**) and four haploid spores are produced (**c**). Microgametogenesis begins with releasing the microspores (**d**), followed by polarization of nuclei in the microspores (**e**). After the first mitosis, bicellular pollen develops (**f**) and contains vegetative (vgn) and generative nuclei (gn). Next, mitosis of generative nuclei leads to the development of mature pollen (**g**) with vegetative nuclei and two sperm cells (sp).

Table 1. Reports on the induction of development in vitro from male gametophyte in *Daucus carota* L. Androgenesis was induced both in anther culture and isolated microspore cultures. Legend: 1n—haploids; 2n—diploids; ppl—polyploids (3x, 4x, 6x); mixo—mixoploids (1x + 2x, 2x + 4x); an—aneuploids (2n = 10, 2n = 11).

Technique	Response	Plant Regeneration	Ploidy of Regenerants (%)	Verification of Homozygosity of Regenerants	Reference
Anther culture	embryos/calli	yes	1n—16.8% 2n—70.0% ppl—8.9% mixo—4.3%	no	[12]
	embryos/calli	yes	1n—88.8% an—11.2%	no	[13]
	embryos/calli	yes	1n—8.3% 2n—91.7%	no	[23]
	embryos	yes	1n—1.0% 2n—89.0% mixo—1.0%	no	[24]
	embryos/calli	yes	1n—1.0% 2n—87.0% ppl—5.0% mixo—7.0%	yes PGI locus homozygous—1.0%	[25]
	embryos	yes	no	no	[26]
	embryos	yes	2n—90.0%, remaining 10.0% not specified	yes PGI and AAT loci homozygous—74.0%	[27]
	embryos/calli	yes	2n—94.0% 6% not specified	no	[28]
	embryos	yes	1n—1.8% 2n—89.1%, 4x—9.1%	yes PGI and AAT loci homozygous—53.7%	[29]
	embryos	yes	no	no	[30]
	embryos	yes	2n—100.0%	yes PGI and AAT loci homozygous—76.4%	[31]

Table 1. *Cont.*

Technique	Response	Plant Regeneration	Ploidy of Regenerants (%)	Verification of Homozygosity of Regenerants	Reference
Isolated microspore culture	calli	no	no	no	[23]
	embryos	yes	1n/2n—frequency not specified	no	[16]
	embryos	yes	2n—100.0%	no	[32]
	embryos	yes	1n/2n—frequency not specified	no	[11]
	embryos/calli	yes	1n—68.6% 2n—29.9%, 3x—1.5%	no	[33]
	embryos	yes	1n—18.7% 2n—71.3%, 3x—10.0%	no	[34]
	embryos	no	2n—100.0%	no	[35]
	embryos	no	no	no	[36]

2.2.1. Anther Culture

This technique of androgenesis utilizes whole anthers as the explants. The first report on anther cultures in carrot was published by Andersen et al. [12]. Anthers selected for culture initiation contained microspores at the mid-uninucleate stage of microgametogenesis. Anthers were cultured on B5 media [37] with the addition of 1-naphthaleneacetic acid (NAA) and dichlorophenoxyacetic acid (2,4-D). The pre-culture of carrot umbels at a low temperature (1–2 days at 7 °C) enhanced the efficiency of embryo and callus development in anther culture. A high sucrose concentration (10%) during the initial phase and a lower sucrose level (2%) in the post-induction stage resulted in the best anther response. As a result, 89 plants were obtained, among which 15 were haploid, 62 were diploid and 11 were tetra- or mixoploids. From the population of haploid and diploid plants, eight agronomically well-performing DH lines were produced, and crossed with a cytoplasmatically male-sterile female line to obtain first hybrids. Four of the eight hybrid combinations investigated demonstrated uniform, vigorous and well-shaped hybrids. However, results concerning the production of carrot hybrids using induced haploids were not reported. Three years later, Hu et al. [13] reported an experiment in MS [38] solid medium with different combinations and concentrations of 2,4-D and 6-furfurylaminopurine (kinetin). The average frequency of calli development was 4.8%, while that of embryos was 0.6%. However, the highest rate of embryo development (15%) was obtained on the medium containing 2,4D without kinetin. Embryos were regenerated into plants, from which 16 were haploid and 2 was aneuploid (2n = 10 and 2n = 11). Matsubara et al. [23] cultured anthers of carrot, and two other members of the *Apiaceae* family: fennel and mitsuba (*Cryptotaenia japonica* Haask). Anthers were cultured on media based on MS and B5 supplemented with 2,4-D and 6-benzylaminopurine (BAP). The anthers selected for culture contained tetrads (stage 1), a mixture of microspores with central and polarized nuclei (stage 2) and anthers prevalent on microspores with polarized nuclei (stage 3). The majority of the embryos were obtained from anthers in stage 1 and 2, but in stage 3, anthers mostly developed calli. The effectiveness of embryo development from anthers was 1.8–4.3%. Embryos were regenerated into plantlets. Observation of the root tip cells in carrot regenerants showed that 92% were diploids, and the remaining plants were haploids. Fennel and mitsuba anthers formed calli exclusively.

To induce androgenesis in carrot, Tyukavin and co-workers [24] pretreated the floral buds. After collection, the flower buds from donor plants were cultured in the dark on MS

medium with 2,4D for 2–4 weeks. Following this treatment, anthers were isolated onto the same medium used bud pretreatment, but cultures were kept under a photoperiod of 16 h. The authors reported that 5–95% of cultured anthers produced embryos. Embryos were regenerated into plants, among which 89% were diploids.

In another study, 21 different genotypes of carrot were examined as anther donors. Anthers for culturing contained a mixture of uninucleate microspores with central and polarized nuclei. Around 26,000 anthers were plated on five different B5- and MS-based media supplemented with plant growth regulators (PGRs) (2,4D, 1-napthaleneacetic acid (NAA)) and amino acids (glutamine, glycine, serine). Calli and embryos were formed with a frequency of 0–20.8% and0–1.2%, respectively. Embryos developed mainly on MS medium containing 2,4-D and 2% sucrose. Plants were regenerated from both calli and embryos. The obtained population of regenerants comprised 3% of haploids, 86% of diploids and 11% of polyploids [25]. Górecka et al. [26] examined five carrot cultivars with respect to androgenesis in anther culture. They used anthers with microspores at the late uninucleate and early binucleate stage. Anthers were placed onto B5 medium supplemented with 2,4-D and NAA, glutamine, serine, and 10% sucrose. The authors obtained from 0 up to 46.5% of embryos. However, there was no information about the ploidy of the obtained plants. In another study, Górecka et al. [27] evaluated the response of 20 carrot cultivars. Only anthers containing uninucleate microspores were used. Anthers were cultured on the same medium as in a previous study [26]. The embryo development varied depending on the genotype and ranged from 0 to 11.5%. Plants were regenerated from the embryos. Flow cytometry analyses showed that 90% of the plants were diploids. Zhuang et al. [28] evaluated 39 carrot accessions including open-pollinated cultivars, inbred lines, F1 and F2 generations for their response in anther culture. Thirty accessions produced calli exclusively, with the highest frequency reaching 37%. Embryos developed only in six accessions (inbreed lines mostly), with the highest frequency reaching 4%. Embryos were mostly produced on B5 medium supplemented with 2,4-D and NAA. The addition of $AgNO_3$ to this medium increased the frequency of embryo development in one accession up to 10%. They also tested the effect of temperature shock on the anthers. The authors applied a cold (4 °C) treatment for 1–3 days. Cold treatment for 1 day was effective for embryo development only for one accession; a longer treatment increased callus development. The experiments resulted in 121 plantlets. Cytogenetic analysis showed that 94% of the regenerants were diploids. No information on the ploidy level of the remaining plants was provided.

Polyamines are biogenic amines found in animals, plants, as well as fungi and bacteria. Polyamines are involved in many physiological processes, such as flowering, fruit development, senescence and stress response [39]. Hoverer, exogenous polyamines can regulate growth and development, including embryogenesis and organogenesis in vitro [40]. Górecka and co-workers [30] examined the effect of the exogenous polyamines, such as putrescine and spermidine supplemented into B5 media, on the efficiency of androgenesis in anther cultures of two carrot cultivars. The authors obtained over 250% more embryos on the medium with the addition of putrescine, and over 40% more on the medium supplemented with spermidine, in comparison to the control. The study of Kiszczak et al. [31] showed a positive effect of low temperature (4 °C) treatment applied on the donor plants. Plants were treated with a low temperature for 9, 12 and 21 d, and after treatment, carrot anthers were isolated from the floral buds and cultured on the B5 medium [26]. The most effective treatment was at 4 °C for 12 d. Under these conditions, the authors obtained over six-fold more embryos compared to the control (donor plants were not subjected to cold treatment).

2.2.2. Isolated Microspore Cultures

In this technique, explants are the microspores released form the anthers. Isolated microspore culture has some advantages over the anther culture: (1) the sporophytic tissues development is avoided by removing the anther walls; (2) a homogenous population of microspores can be induced to convert into embryos directly; (3) the media components

and culture treatments have direct access to the microspores; (4) microspores can be used to study embryogenesis, cell development and cell biology [7]. This technique has been successfully applied in many species, including *Nicotiana tabacum* L., *Brassica oleracea* L., *Brassica napus* L. and triticale (x *Triticosecale* Wittm.) [8,22].

For carrot, there are relatively few studies in this field (Table 1). The first report on microspore cultures in carrot was given by Matsubara et al. [23]. They cultured microspores of carrot, fennel and mitsuba on different media based on MS and B5 supplemented with 2,4-D and BAP, but they did not observe any tissue development in all tested species. Ferrie and co-workers [16] reported trials on the isolation of microspores of 15 members of the *Apiaceae* family, including anise, caraway (*Carum carvi* L.), dill, fennel, lovage (*Levisticum officinale* Koch.), parsnip and carrot. However, neither detailed methodological information nor the efficiency of androgenesis for carrot were presented.

The first detailed and successful report on microspore cultures in carrot was given by Górecka et al. [32]. The keysteps in the procedure of isolated microspore culture in carrot are summarized in Figure 3. The uninucleate microspores isolated from donor plants were cultured on B5 media [37] with modifications [41] supplemented with 2,4-D, NAA, glutamine, serine and 10% sucrose (Figure 3a–e). The cultures were incubated in the dark at 27 °C for four weeks (Figure 3f). The culture resulted in the development of embryo-like structures. These structures were visible to the naked eye after approximately 4 weeks of culture; however, the appearance of the embryonic structures was observed even up to 6 months of culture (Figure 3g). The obtained embryogenic structures were exposed to continuous light at 27 °C and were regenerated into plants (Figure 3h). Out of 90 plants planted in a growth chamber, 47% adapted to ex vitro conditions. Analysis of the ploidy of these plants, performed using flow cytometry, showed that they were all diploids.

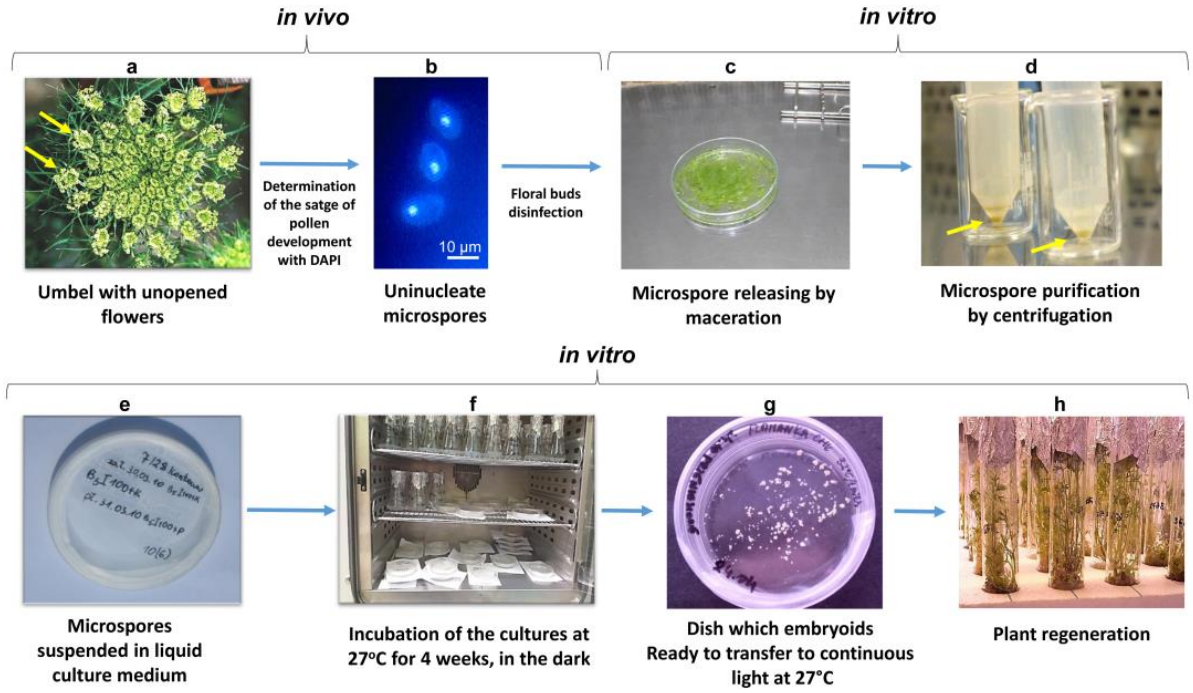

Figure 3. Steps in obtaining androgenetic carrot plants in isolated microspore cultures. Blue arrows indicate transition between the steps. (**a**) Umbel of the donor plant, with arrows marking the umbels containing optimal buds to androgenesis; (**b**) uninucleate microspores of carrot after 4,6-diamino-2-phenylindole (DAPI) staining; (**c**,**d**) microspore release and purification, where the arrows in picture 'd' show microspores collected in the pellet; (**e**,**f**) culture of carrot microspores incubated in the dark at 27 °C; (**g**) young embryoids visible with the naked eye after approximately 4 weeks of culture; (**h**) microspore-derived carrot plantlets at the regeneration stage.

A few years later, Li et al. [33] reported the results of culturing the microspores of 47 carrot accessions (including cultivars, inbred lines, F1–F4 and BC generations). Carrot buds containing late uninucleate microspores (with polarized nuclei) and early binucleate pollens were used for the study. The microspores were cultured on the NLN [42] medium containing 2,4-D, NAA and 13% sucrose. In their study, only slightly more than half (28 out of 47 accessions) of accessions responded to the culture. Responsive accessions produced either calli or calli and embryos, and none of these accessions produced embryos exclusively. The embryos or calli developed in the culture between 2 and 6 months. Embryos/calli development was observed in 0 up to even 100% of Petri dishes, depending on the accession. The dishes containing isolated microspores were exposed to temperature shocks (33 °C for two days and later 4 °C for 2 days, or 33 °C for 2 days). Cold and heat pretreatment generally had a negative impact on the induction of microspore embryogenesis, but a short pretreatmentat elevated temperature showed a positive influence on some accessions. The plants were regenerated from the experiment and the results of flow cytometry analysis revealed that 69% were haploids, 30% were diploids, and single triploids were detected among 137 plantlets tested. In both of the above-cited experiments, the authors expressed the microspore culture response as the 'Petri dishes with embryos' [32] or 'ratio of Petri dishes with embryos' [33]. It seems to be not precise and does not give information about the frequency of development in the culture, as even one responding microspore per Petri dish was considered as a success, which cannot be quantitatively compared to a Petri dish with many responding microspores. This, of course, does not make this study less important, but it must be considered during interpretation.

Shmykova et al. [34] applied a half-strength NLN medium supplemented with 2,4-D, kinetin and 13% sucrose to induce embryogenesis from microspores in eight carrot accessions. Microspores were subjected to a low-temperature shock treatment for three days at 5–6 °C, followed by incubation at 25 °C in the dark. The first divisions of microspores were observed on the third day of culture, and continued until the globular embryos were visible with the naked eye, which took place at about the fourth week of culture. The development in the culture was genotype-dependent, as some responsive (five accessions) and recalcitrant (three accessions) genotypes were observed. In this study, for the first time, the authors present reliable results on the efficiency of the development in the isolated microspore culture in carrot, as they present their results as the number of embryos per single Petri dish. The highest yield of embryos was 53 embryoids per Petri dish. In low-responding accessions, the yield was roughly 3–5 embryos per dish. The obtained embryos were subjected to regeneration. Ploidy analyses of regenerants showed that 19% were haploids and 71% were diploids. What was interesting, and has never been reported for carrot previously, is that they observed the albino plants among regenerants from microspore cultures. In 'Nantskaya 4′cultivar, from 256 embryoids obtained, 162 regenerated into albino plantlets, whereas in 'Chantenay' cultivar, 97 embryoids regenerated 45 albino plantlets. In another study, the authors [35] established culture, from microspores at the tetrad and early uninuclear stage, in two F1 carrot cultivars. The isolation of microspores was performed in B5 medium supplemented with 13% sucrose and 5% mannitol, and, later, microspores were cultured in NLN medium supplemented with 2,4-D, NAA, casein hydrolysate and 13% sucrose. For the induction of microspore division, high-temperature treatment (33 °C) was applied for 5 days. The efficiency of embryo development was, on average, 0–5 embryoids per Petri dish. All obtained regenerants were identified as diploids.

Romanova et al. [36] applied different combinations of cold (5 °C for 1–3 days) and heat shock (32 °C for 1–2 days) stress on carrot microspores in the culture.The results showed that the response for a single shock treatment (either cold or hot) had no significant effect on the number of embryos, as they were similar to the control (15 per Petri dish). In a separate experiment, they combined a cold pre-treatment of explants (5 °C for 1 and 2 days) with heat treatment of isolated microspores (32 °C for 2 days). This joint stress combination increased embryogenic induction (28.9–58.2 embryoids per Petri dish) for some accessions

to the level exceeding the controls (0.5–30 embryoids per Petri dish). The authors did not present the data on the ploidy status of regenerants.

3. Haploid Induction from the Female Gametophyte (Gynogenesis and Induced Parthenogenesis)

3.1. Female Gametophyte Development

Development of the embryo sac (female gametophyte) takes place in tissues embedded within the ovule located in the ovary. In the ovule, the differentiation of a nucellus parenchyma cell into a megaspore mother cell (megasporocyte) occurs. The developing embryo sac is situated within the body of nucellus parenchyma cells, which are covered by the integuments. In the most popular pattern of embryo sac development (monosporic; for a review, see [43]), a single megasporocyte differentiates within an ovule and becomes a megaspore mother cell (Figure 4a). This cell then passes through meiosis to yield four haploid megaspores (Figure 4b,c). Typically, three of the megaspores degenerate (Figure 4d), while one survives and develops into an embryo sac (Figure 4e,f). The remaining megaspore undergoes three rounds of nuclear division, followed by cellularization. A mature embryo sac consisting of eight nuclei in seven cells (egg cell, synergids and antipodals and polar nuclei (Figure 5)). All these cells are haploid including non-fused polar nuclei [44]. *Daucus carota* L. embryo sac development corresponds to the monosporic development and is classified as the *Polygonum*-type of the female gametophyte [45].

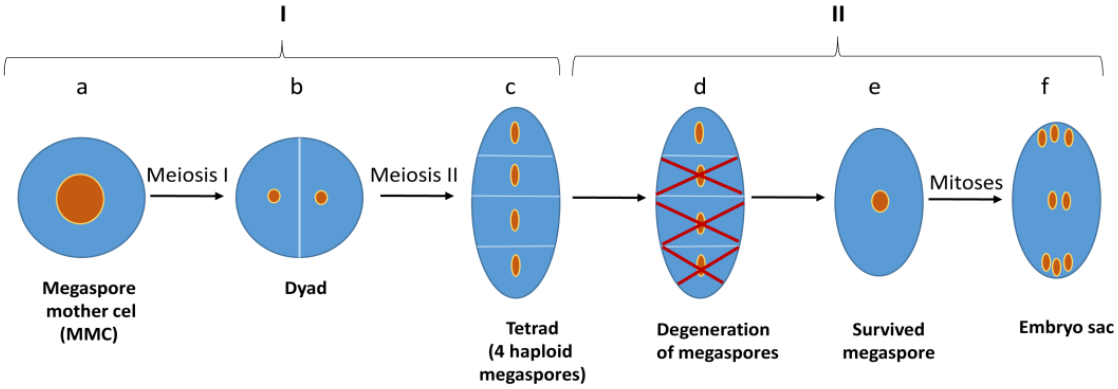

Figure 4. Schematic diagram illustrating monosporic path of the embryo sac development. Embryo sac development occur in ovaries located within a flower. Phase I is macrosporogenesis, phase II is macrogametogenesis. During microsporogenesis, the MMC (**a**) undergoes meiosis (**b**) and four haploid spores are produced (**c**). During macrogametogenesis, three spores degenerate (**d**), and the survived megaspore (**e**) undergoes mitosis, leading to the development of an eight-nucleus embryo sac (**f**).

A formation of haploid plants from cells of the female gametophyte is a potentially attractive alternative, where androgenesis is either not applicable or is fraught with some difficulties. This approach seems to be the only way to produce haploids of male-sterile lines and female clones of dioecious species [46]. There are several techniques for stimulating the female gametophyte for haploid development. In some species, haploids are induced without the necessity of transferring pollen onto stigma, and in others, this is required.

Haploids from female gametophytes can be produced in plants after pollination by pollen of distantly related species via the development of a hybrid zygote. After successful fertilization and subsequent cell division in the zygote, a unique phenomenon occurs—the elimination of paternal chromosomes—resulting in a haploid embryo formation containing the chromosome set of the female parent. The endosperm usually aborts early in the seed, and in consequence, the haploid embryo must be rescued by in vitro culture. This very unique system (called the 'bulbosum method' or the 'chromosome elimination method') works well in some cereals, i.e., barley (*Hordeum vulgare* L.), triticale (x *Triticosecale* Wittm.), rye (*Secale cereale* L.) and oats (*Avena sativa* L.) [47], but not in other species.

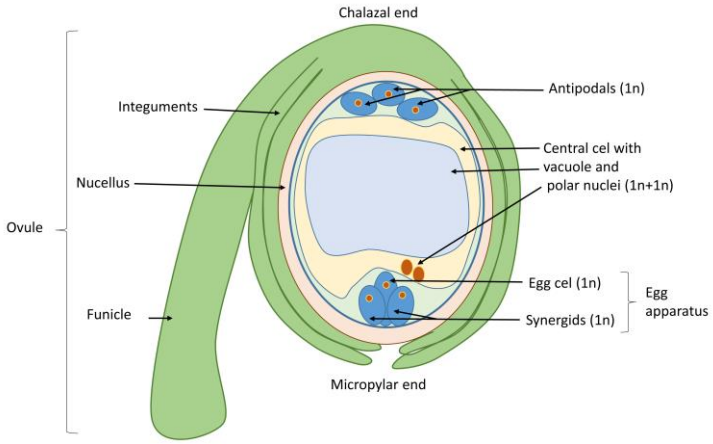

Figure 5. Schematic drawing of the ovule of higher plants, containing female gametophyte (embryo sac). Development of the embryo sac takes place in tissues embedded within the ovule attached to the maternal tissues by funicle. The embryo sac is situated within the body of the nucellus, which is covered by integuments. The mature embryo sac consists of four types of cells: the egg cell and the two synergids (egg apparatus) at the micropylar end, the antipodals at the chalazal end and the central cell containing the two polar nuclei and a large vacuole. 1n—haploid nucleus.

Haploid regeneration using non-pollinated female gametophytes is usually described as gynogenesis or haploid parthenogenesis, although there are some inconsistencies in nomenclature. In the animal kingdom, the term "gynogenesis" has been reserved for haploid embryogenesis induced by the presence of a male sperm cell, which does not contribute to embryo formation, resulting in the development of female-type offspring [48]. For plants, some studies [49–51] have used the term "gynogenesis" for all methods in which a female gametophyte is the origin of haploid cells, regardless of whether a pseudo-fertilization process is involved. Some studies [52–54] have reserved this term only for haploid regeneration through unpollinated female gametophytes (unpollinated egg); however, when inactivated/incompatible/distant pollen is used as a stimulator for haploid induction, then the method is called induced parthenogenesis. In this article, the second approach is supported.

3.2. Origin of Haploids from the Female Gametophyte

The haploids developed from female gametophyte in vitro can originate via parthenogenesis (the production of an embryo from an egg cell, without the participation of the male gamete) or apogamy (the production of an embryo from a gametophytic haploid nucleus other than the egg (see Figure 5) [6,7]. Most studies tracking the origin of haploid embryo have determined the egg cell as the predominant source. Such evidence has been shown for tobacco (*Nicotiana tabacum* L.) [55], *Melandrium album* Mill. [56], table beet (*Beta vulgaris* L.) [57], onion (*Allium cepa* L.) [58], *Citrus* sp. [59] and lettuce (*Lactuca sativa* L.) [60]. An antipodal or synergid origin of embryos has been observed in barley (*Hordeum vulgare* L.) [61] and rice (*Oryza sativa* L.) [62]. There is no evidence in the literature on the development of haploids from polar nuclei, as in many species they tend to fuse at different stages of embryo-sac development, forming diploid nuclei [44].

3.3. Methods Based on In Vitro Gynogenesis

Gynogenesis would exploit the ability of haploid nuclei of the embryo sac (Figure 5) to develop a haploid zygote without fertilization—this would, therefore, be a form of female haploid parthenogenesis. Methodologically, this technique is implemented by in vitro culture of unopened flower buds (unpollinated, but containing mature embryo sacs), isolated ovaries or ovules. This method was found to be the most successful for sugar beet (*Beta vulgaris* L.) [63], onion (*Allium cepa* L.) [64] and leek (*Allium ampeloprasum* L.) [65]. In sugar beet, inflorescences are subjected to a cold treatment of 8 °C for one week. To improve the response of isolated ovaries or ovules, during the induction phase, high temperatures

(30 °C) are applied [66]. In alliums, flower buds or ovaries are placed on to artificial media and no particular stress pre-treatment is applied [67]. An induction medium contains PGRs (BAP and 2,4-D) and elevated sucrose (9–10%); however, the regeneration medium has reduced PGRs or is PGR-free. Regenerated plants are usually haploids and require chromosome doubling treatments. Doubled haploids have been used in the breeding of sugar beets [68] and onion [8], including the production of F1 hybrids using DH parents.

There are only few reports dealing with the induction of gynogenesis in carrot (Table 2). The first small study was undertaken by Tyukavin and Shmykova [69]. They intended to induce gynogenesis in carrot by culturing unpollinated ovules. Induction media composition was not specified. The formation of embryos was recorded. Embryos were regenerated into plants, and the presence of diploids and haploids was observed, but no detailed information on their frequency was provided. In another study, performed for a single breeding line of carrot, ovaries were isolated from unpollinated flowers, and the induction of calli was observed on the ovaries [70]. The obtained callus was transferred onto the MS medium with calcium chloride and elevated 9% sucrose. Embryo formation was observed on the calli after 30 days of culture. The average yield of embryoids was 0.1–7%. The regeneration of the plantlets from the obtained embryoids was conducted on the MS medium with kinetin. The embryoids were regenerated into plants; however, no information on ploidy and genetic status of the regenerants was provided. Later, a more detailed study was reported by Domblides [71]. The disinfected carrot buds of two carrot accessions were pre-cultured on the MS medium with the addition 2,4-Dfor 2–3 weeks at 25 °C in the dark. Then, the ovules were excised from the ovaries and placed on the MS medium of the same composition, but under light. After five weeks of culture, some ovules enlarged and produced calli and embryoids at their micropylar end. Out of 871 ovules plated, 36% produced calli and 0.6% produced embryos. A cytological study of the cultured ovules showed that the first divisions of the embryo sac cells located at the micropylar end occurred after 3 days of culture. In ovules cultured for 3–4 weeks, the cell clusters within the embryo sac were enlarged; however, no embryos were found during cytological examination. Embryoids and calli developed on the ovules cultured in vitro were further transferred to the MS medium with kinetin. Embryoids did not develop into plants. Plants were regenerated from callus tissue only on the MS medium without PGRs. Cytological analysis of the root cells showed that all plants had a diploid set of chromosomes.

Table 2. Reports on the induction of the development from female gametophyte in *Daucus carota* L. Legend: 1n—haploids; 2n—diploids; ppl—polyploids (3x, 4x, 6x); mixo—mixoploids (1x + 2x, 2x + 4x).

Method/Type of Culture	Response	Plant Regeneration	Ploidy (%)	Verification of Homozygosity of Regenerants	Reference
Gynogenesis					
unpollinated ovaries	embryos/calli	yes	no	no	[70]
unpollinated ovules	embryos	yes	1n/2n—frequency not specified	no	[69]
	embryos/calli	yes	2n—100.0%	no	[71]
Induced parthenogenesis					
irradiated pollen	embryos—2 pcs	yes	1n—100.0%	no	[72]
	embryos	no	no	no	[73]
foreign pollen	embryos/calli	yes	1n—0.1% 2n—97.9% ppl—2.0%	yes PGI locus homozygous—49.0%	[74] [75]
	embryos/calli	yes	1n—21.1% 2n—75.4% ppl—2.6% mixo—0.9%	yes PGI, chs2 and ipi3 loci homozygous—72.6%	[76]

3.4. Methods Based on Induced Parthenogenesis

Parthenogenesis (from the Greek: *parthénos*—virgin, *genesis*—creation) is a form of reproduction in which an egg can develop into an embryo without being fertilized by a sperm. This phenomenon was observed both in animals and plants. Parthenogenesis as a reproduction means was observed in some invertebrates (scorpions, nematodes aphids, bees, wasps and ants) and certain vertebrates (some fish, reptiles and a few birds). In plants, it is a common type of reproduction and it is a part of the process called apomixes. It occurs in mosses, about 10% of ferns, and about 1% of higher flowering plants [77].

Naturally occurring haploid parthenogenesis is an insufficient method for haploidization, as it is a rare event occurring only in some species (i.e., apple, pear, plum and mango) [78,79]; therefore, to use this approach for a broader scale, an additional factor must be applied to trigger unfertilized egg cell divisions leading to the development of haploid embryos. This process is called induced parthenogenesis or in situ parthenogenesis [80]. Parthenogenesis can be induced by pollination with pollen without fertilization capability [81,82], pollen of distantly related species [51,74], pollen with an altered ploidy level [49] or by applying thermal or chemical [54] treatment on pollen or during pollen meiosis. This review will focus on the approaches tested for carrot only (Table 2).

3.5. In Situ Parthenogenesis Induced by Irradiated Pollen

A dose-dependent treatment of pollen grains by ionizing radiation (X-ray, γ-ray) may cause mutations, breakage of the chromosomes and disorganization of the nucleus [83]. The irradiated pollens maintain their ability for germination, but are incapable of successful fertilization. Several studies have shown that irradiated pollen can germinate on the stigma, grow within the style and reach the embryo sac, but cannot fertilize the egg cell and the polar nuclei [52,73,84–88]. However, it can stimulate the division of the egg cell, inducing parthenogenic embryos or the development of parthenocarpic fruits. Parthenogenetic embryos usually die if they are left in the ovary, due to the absence of endosperm or abnormalities in its development. Embryos must be rescued by transfer on to artificial media. The selection of an efficient radiation dose, the optimization of the pollination method, the embryo rescue timing, the developmental stage and theculture media and conditions are important factors affecting the success of this method [84]. Parthenogenesis achieved by in situ pollination with irradiated pollen has been reported for fruit trees, such as apples (*Malus domestica* Mill.) [85], pears (*Pyrus communis* L.) [52], kiwi (*Actinidia deliciosa* Chev.) [86], oranges (*Citrus* spp.) [87], cocoa (*Theobroma cacao* L.) [88] and some Cucurbitaceae species [54,84].

The first report on the use of irradiated pollen for haploid induction in carrot was described by Rode and Dumas de Vaulx [72]. Whole umbels of the male-fertile parent were irradiated with gamma rays (irradiation dose 300–500 Gy) and used for pollination of two male-sterile accessions (female parent). Fifteen days after pollination, the in vitro culture of immature seeds was established. The seeds were cultured on half-strength B5 medium with 2% sucrose. First embryos emerged from the immature seed 30–34 days after plating. The effectiveness of the process of embryo germination was very low: from 992 immature seeds plated, only two haploid embryos were obtained. Secondary embryogenesis was observed on these embryos, which allowed clonal propagation. In total, the authors obtained 110 plants from one embryo, and 63 from the other. Amongst these plants, some were haploids and some were diploids, as confirmed by chromosome counts in the root tips; however, specific data were not provided. Eight years later, Doré et al. [73] repeated an experiment with irradiated pollen for haploid induction in carrot. They applied the same dose of irradiation as reported by Rode and Dumas de Vaulx [72] on the male fertile parent, and treated pollen on the stigma of male-sterile female parent. They applied the embryo-rescue technique; however, culture conditions were not specified. The culture resulted in the obtainment of embryos, but most of them showed abnormalities in shape, size and development. Researchers concluded that the method did not appear to be feasible for

the haploidization of carrot, as it could not be easily repeated and the number of embryos obtained was very low.

3.6. In Situ Parthenogenesis Induced by Foreign Pollination (Inter-Specific Crosses)

Distant pollination between different species or genera is a well-known path for obtaining haploids in plants. As it has been mentioned above, in some wide crossing (i.e., barley or wheat crossed with maize), a unique mechanism of paternal chromosome elimination in hybrid embryos occurs and, as a result, haploid embryos of female parent are produced [6]. Unfortunately, this approach works only for cereals, and has not been reported in any vegetable plant. However, in some cases, the mere presence of foreign pollen grains on the stigma or pollen tube in the style can stimulate female gametophyte to develop haploid embryos. In such cases, different mechanisms operate: pre-zygotic incongruity barriers, such as inhibition of pollen grain germination on the stigma and inhibited or erratic pollen tube growth in the style, might cause the absence of micropyllar penetration and the fusion of gametes [89]. Haploid embryos developed through this pathway are mostly of parthenogenic origin. Parthenogenesis induced by wide pollination resulting in haploid plant development was reported for *Brassica oleracea* L. crossed with *Eruca sativa* Mill. [90] or *Mimulus luteus* L. after pollination withpollen from *Torenia fournieri* Lind. [91]. Virk and Gupta [92] obtained haploid plants of *Pisum sativum* L. after pollination with *Lathyrus odoratus* L. Haploids were also obtained in *Cichoriumintybus* L. after pollination with *Cicrebita alpina* Walbr. [93]. *Hibiscus cannabinus* L. has been reported to cause in situ parthenogenesis in cotton hybrids (*Gossypium barbadense* × *G. hirsutum*) [94]. Pollination of *Lactuca sativa* L. with the pollen of *Helianthus annuus* L. or *H. tuberosus* L. resulted in lettuce haploid embryo development [60].

Trials of the induction of haploid plants using foreign pollen were also undertaken in carrot (Figure 6). Selected carrot flowers from thirteen cultivars were hand-pollinated with pollen from other species: three *Apiaceae*, i.e., parsley, parsnip and celery, and one *Brassicaceae*, i.e., cabbage [74,95]. Pollen tube growth of all pollinators in carrot pistil and control (carrot pollinated with carrot) was investigated with the aniline blue fluorescence method. The pollens of parsley, parsnip and celery germinated on the carrot stigma and entered the transmitting tissue of the carrot pistil. However, none of foreign pollen tubes reached the carrot embryo sac even 24 h after pollination. In combinations where cabbage was a pollen donor, pollen tube growth was inhibited mainly at the carrot stigma. The highest number (95%) of carrot pistils with numerous germinating pollen tubes was observed in the control combination (carrot × carrot). Among the remaining combinations, the highest number (68%) of carrot pistils containing pollen tubes was observed after pollination with parsley pollen. After pollination of carrot pistils with parsnip and celery pollen, the total number of pistils with pollen tubes was lower (30–40%) compared to parsley. Cabbage pollen grains placed onto carrot pistils germinated very poorly and very short and single pollen tubes were observed on only 9% of pistils [75,96]. Fifteen days after the pollination of carrot flowers with foreign pollens, the ovules were isolated from the ovaries and placed onto artificial media. After four weeks of culture, the development of embryos or calli was observed. Development in the culture depended on the genotype, induction medium composition and pollen source. The study showed that calli were formed with a frequency of 0 to 3.9% and embryos from 0 to 2.1%. All tested cultivars responded during the culture, but the efficiency of the development varied (from 0.2% in 'Regulska' to 2% in 'Karlena'). A medium based on $\frac{1}{2}$ MS and supplemented with IAA and 2% sucrose was the most efficient for embryo development (1.5%) while A12 medium, based on B5, supplemented with 2,4D and BA, was the most suitable for callus formation (3.4%). The experiment showed that parsley was the best pollen source for embryo development in the experiment [74,75]. The ploidy level of the obtained regenerants was estimated by flow cytometry, analysis showed that about 98% of the regenerants were diploids, and the remaining were haploids and polyploids (Table 2).

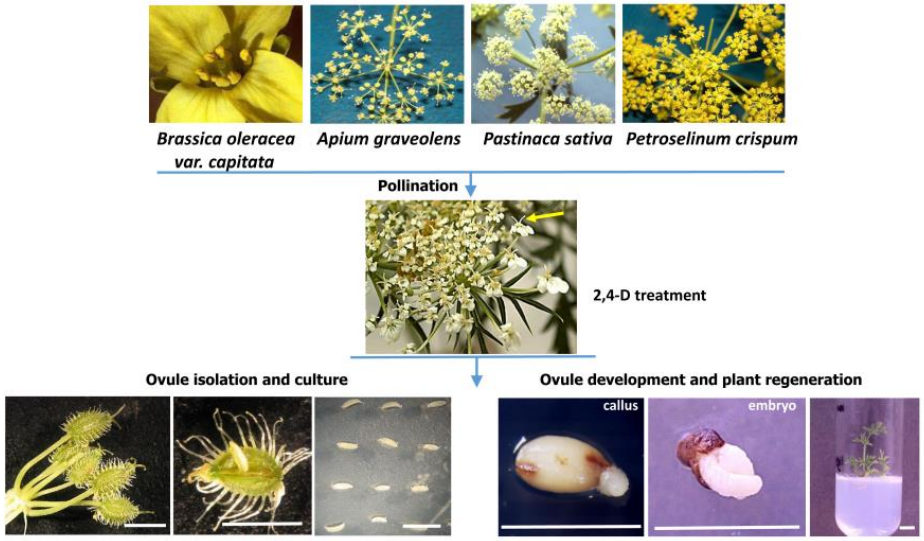

Figure 6. Flowchart of the experiment on the development of the carrot ovules isolated from the flowers pollinated with foreign pollen. Blue arrows indicate the transition between the steps. Description of figures from top to bottom: Flowers and inflorescences of pollen donors; Close-up image of the carrot inflorescence showing flowers in the receptive stage (the yellow arrow indicates that the styles have separated from each other, forming a V-shape); Ovule isolation and culture: enlarged carrot ovaries before ovule isolation, ovule excised from the ovary, ovules placed onto culture medium; Ovule development and plant regeneration: callus tissue developing at the micropylar region of the ovule, embryo emerging from the ovule, regenerated plantlet. Scale bar 0.5 cm.

To verify the above-mentioned results, another set of experiments was performed in which the effects of pollination with parsley pollen and 2,4D treatment on the stimulation of parthenogenesis was studied on 30 cultivars and breeding lines of carrots [76]. Ovules were isolated from flowers pollinated with parsley pollen. Ovules cultured in vitro developed embryos and calli. The application of 2,4D on pollinated flowers stimulated mainly callus development and was not useful for increasing the frequency of embryo development. The efficiency of embryo development was genotype dependent and varied from 0 to 24%. The additional experiments validating the optimal set of treatments and culture condition favoring embryo development showed that the highest frequency of embryo development was observed from ovules excised from ovaries 20–22 d after pollination with parsley pollen, omitting 2,4D treatment. Among several media used for ovule culture, 1/2-strength MS medium supplemented with IAA allowed the induction of embryos at a similar frequency as on the media supplemented with kinetin, gibberellic acid, putrescine or thidiazuron, but restricted callus development. As shown by flow cytometry, the regenerants were haploids (20%) and diploids (79%). Moreover, a small portion of mixoploids (1%) was obtained.

3.7. CENH3-Mediated Haploid Induction

The newest approach resulting in haploid induction in plants is engineering in the centromeric region. Centromeres are chromosomal regions that mediate assembly and spindle attachment during cell divisions (mitosis and meiosis). When the centromeric region is disrupted, substantial abnormalities occur in spindle fiber attachment. The localization of the centromere is governed by the nucleosome carrying the variant histone *CENH3* (centromeric histone protein, centromere-specific histone H3). The *CENH3* region is present in all plants; thus, targeting the modification of this histone protein (defective kinetochores) enabled the elimination of one set of maternal or paternal chromosomes after fertilization to achieve haploid induction [97]. Such breakthrough technology has been presented for the first time by Ravi and Chan [98] in *Arabidopsis thaliana* (L.) Heynh. In transgenic experiments, they produced *cenh3* mutants, where the N-terminal tail of *CENH3* was replaced with a sequence from another H3 variant and was fused to the

green fluorescent protein (GFP) producing the "tailswap-*cenh3*" construct. When these mutants were used as male or female parents in a cross with the wild-type genotype containing unaltered *CENH3*, the genomes of the transgenic mutants were eliminated, and haploid offspring containing the genome of only one parent was generated with a frequency of 4–34%. Thus, manipulating a single centromere protein, the centromere-specific histone H3 variant *CENH3*, in one of the parents (either male or female) leads to the generation of 'haploid inducer lines'—a line with an altered centromeric gene [99,100]. Other studies in *Arabidopsis* utilizing alterations in the centromeric region *CENH3* showed that the frequency of haploids was on average 0–5% [101,102]. The employment of *CENH3* in mediating haploid induction is carried out in a few important crops, i.e., maize [103], wheat [104,105], bananas [106] and mustard [107]; however, the efficient introduction of this method to breeding has not been reported. Moreover, so far, the efficiency of haploid induction using this approach, except for *Arabidopsis*, is quite low (0.1–16.3%) [104,105, 108,109] and is comparable to classical methods of induction of haploids from female gametophytes [6,7,110].

The generation of doubled haploids using centromere-specific histone H3 variant *CENH3* has not yet been reported for *Daucus*, but initial studies towards this direction have been performed. The first report on the investigations of the structure and function of plant *CENH3* in different *Apiaceae*, including *Daucus carota* L., has been given by Dunemann et al. [111]. Based on mining the carrot transcriptome and PCR-based cloning, homologous coding sequences for *CENH3* in four *Daucus* species (*D. glochidiatus* Labill., *D. pussillus* Michx., *D. muricatus* L. and *D. carota* L.) were identified. Phylogenetic alignment of these *CENH3* forms with other dicots *CENH3* showed homology. To verify the location of the *CENH3* protein in the kinetochore regions of the *Daucus* chromosomes, immunostaining of mitotic root cells was performed. The chromosomal location of *CENH3* proteins in the centromere regions of the chromosomes were confirmed. The *CENH3* locus was mapped on the carrot chromosome 9. In the next study, Dunemann and co-workers [112] cloned the native *PgCENH3* gene from *Panax ginseng* used for *Rhizobium rhizogenes*-based co-transformation simultaneously with a CRISPR/Cas9 construct targeting *DcCENH3* CRISPR/Cas9-based mutations within the carrot *CENH3* gene. The mutations have been cytogenetically studied in transgenic carrot hairy root cultures and preliminary in subsequently regenerated plants. Recently, the same team [113] reported the continuation of these studies and the generation of *CENH3* knockout mutants, such as the 27-bp in-frame indel mutant (1-STEP approach) or the 3bp in-frame deletion mutant (2-STEP approach). They also described the discovery of a second *CENH3* locus in the carrot genome. Another experiment aiming at the induction of mutations in the *CENH3* region in carrot was reported by Meyer et al. [114]. They applied polyethylene glycol (PEG)-mediated transformation (targeting different locations within *CENH3)* of carrot protoplasts and evaluated the efficiency with which regenerated, edited plants could be produced. In total, they analyzed 374 regenerated plants and found that 13% carried targeted mutations within *CENH3.*

4. Identification of Haploids and Double Haploids

The techniques of haploidization differ in the rates of plants of gametic origin (haploids, spontaneously doubled haploids) and plants of somatic origin. It has been shown that the culture of isolated microspores is superior in that context, as the suspension of microspores is free of somatic tissues, and embryos develop from microspores exclusively. However, when the culture is initiated with other plants organs, such as anthers, flower buds, ovaries or ovules, undesired clones of donor plant can be obtained. Such plants can be regenerated fromthe somatic tissue of anther wall cells, tapetum, somatic cells of flower buds, ovaries or ovules. Therefore, it is essential to evaluate the ploidy level and genetic status (homo-, heterozygotes) of obtained regenerants.

The ploidy level can be evaluated by chromosome counting in the root tips of regenerants, or by flow cytometry. The latter method allows the screening of large populations and quickly assessing the ploidy status, as well as detecting different chromosomal

rearrangements, such as mixoploidy, euploidy or aneuploidy. The ploidy of regenerants resulting from haploidization procedures depends on the genotype and applied method of haploidization. The frequency of spontaneous genome doubling during androgenesis (particularly microspore culture) is 10–40% in oilseed rape (*Brassica napus* L.), 25–70% in wheat (*Triticum aestivum* L.), 50–60% in rice (*Oryza sativa* L.), 50–90% in rye (*Secale cereale* L.) and 70–90% in barley (*Hordeum vulgare* L.) [115,116]. Spontaneous duplication of the haploid genome occurs through endoreduplication and by nuclear fusion taking place at the initial stages of development, leading to the regeneration of homozygous doubledhaploids [117–120]. In contrast to androgenesis, development from female gametophytes through gynogenesis leads to predominantly haploid regenerants and spontaneous diploidization is very low [6,58,67].

In carrot, most of the plants obtained after various haploidization techniques were diploids (Tables 1 and 2). Similar results were reported for other members of Apiaceae family. Smykalova et al. [15] reported that 59% obtained androgenic caraway plants were diploids. A prevailing number of androgenic plants of *Bupleurum falcatum* L. also possessed diploid chromosome numbers [14]. Monahova et al. [121] studied androgenic and gynogenic regenerants of carrot and observed frequent changes leading to spontaneous diploidization during the development of haploid embryos into plants. After cytological analysis of the explants in the culture, they concluded that cell ploidy starts to change at the early stages (first mitoses) of primary embryoid development. According to their studies, plant regeneration is also accompanied by a change of ploidy. At this stage, they also observed some anomalies, such as multiple mitoses and disturbed cytokinesis. All these results suggest that the high percentage of diploids in umbellifers might be caused by a spontaneous diploidization during embryo/plant development.

In a prevailing number of studies on haploidization in carrot, the occurrence of secondary embryogenesis has been pointed at. Secondary embryogenesis was observed in anthers [24,27,121], isolated microspore cultures [33,34] and isolated ovule culture [75,76]. Secondary embryogenesis, on the one hand, increases the total number of regenerated plants, but on the other hand, the secondary embryos might develop from somatic tissues of anthers and ovules, which is undesirable in the context of haploidization, and it is another argument for molecular evaluation of the regenerants in carrot.

In carrot, apart from diploid and haploid regenerants, both in the studies on androgenesis and in isolated ovule cultures, the presence of 2 to 5% polyploids (3x, 4x, 6x), but also mixoploids (1x + 2x, 2x + 4x) or aneuploids (2n = 10 and 11) with a frequency from 2 to 5% [12,25,29,74–76], was recorded. The occurrence of euploids and aneuploids has been noted among regenerants from the anther/ovule culture in other species [4,46,47,115]. The development of euploids may be caused by spontaneous chromosome set multiplication, but also regeneration from the tapetum cells, which, by nature, undergoes extensive polyploidization and has been observed in caraway anther culture [122]. Aneuploids might be a result of numerical and structural chromosomal aberrations caused by stress treatments or culture medium composition [123,124].

If no spontaneous diploids occur in the regenerants obtained following the haploidization process in vitro, a diploidization procedure is necessary. The method most frequently used for genome duplication is colchicine treatment which, however, causes various abnormalities, such as chimerism, chromosomal aberrations, altered morphology in plants. Moreover, it is carcinogenic to humans [125,126]. Many herbicides are known to inhibit microtubule assembly and its organization, i.e., amiprophos-methyl, pronamid, trifluralin, oryzalin or chlorpropham, and their suitability as antimitotic agents has been tested, from which the most promising were amiprophos-methyl and pronamid [127]. In carrot, there are very few reports on genome doubling in haploid plants. In carrot, colchicine was applied both to the cultured microspores, and on regenerated plantlets. Colchicination of isolated microspores was previously reported for *Brassica napus* L., as a highly efficient method of doubling the number of chromosomes [128]. Górecka et al. [32] applied 0.5 mg/L colchicine to the medium for culturing microspores for 24 h, and after treatment, microspores were

cultured in the same medium without colchicine. The application of colchicine to carrot microspores did not show a negative effect on the embryoid development. As a result, they obtained only diploid regenerants. Similar results were reported by Voronina et al. [35]. They also applied 0.5 mg/L colchicine to the nutrient medium for microspore culture. The treatment was applied for 48 h. Colchicination increased the embryo development (3,3 embryos/dish) compared to the colchicine-free control (1,7 embryo/dish).Only diploid regenerants were observed in their study. Ferrie et al. [16] obtained single carrot embryos via microspore culture. Embryos were further regenerated into plants; however, no spontaneous chromosome doubling was observed. They treated whole plantlets with 0.34% colchicine and obtained diploid carrot plants, of which three DH lines were produced. In another study, haploid carrot plants obtained from isolated microspore cultures were subjected to diploidization at the in vitro culture stage. Roots of these plantlets were immersed in a solutionof 0.34% colchicine in water for 1.5 h, rinsed with distilled water, and then planted in soil. Such treatment was detrimental to most of the plants, and colchicine treatment was eliminated from the regeneration protocol [11]. Based on the reports above, it seems that for carrot, colchicination at the stage of microspore culture is more beneficial, compared to its application at later stages.

As it has been mentioned above, the application of haploidization techniques might result in a haploid plant that can undergo spontaneous chromosome doubling during the culture phase, but plants can also develop from somatic tissues. Sometimes, both processes occur at the same time and the obtained plants will be recognized as diploids or polyploids [129]. Therefore, it is necessary to evaluate the originof diploid and polyploid regenerants, especially those obtained from the techniques utilizing whole organs such as anthers and ovules. The obtained plants can be characterized with regard to theirhomozygosity using isozyme analysis. Isoenzymes (isozymes) are different forms of a polypeptide that catalyze the same chemical reaction. Isozymes are separated by gel electrophoresis of the proteins present in crude extracts of specific plant tissues. The gel is then exposed to a proper substrate for the enzyme. In regions of the gel where the appropriate enzyme is present, the substrate will produce a colorimetric reaction. Thus, isozymes are enzymes reacting with a specific substrate but differing in their migration pattern on the gel. Allozymes are special forms of isozymes. They generally differ due to the substitution of a single amino acid of different charge at a locus. This leads to a change in the electrophoretic mobility of the enzyme. Allozymes are generally inherited according to the codominant monogenic ratio, allowing the discrimination of homo- and heterozygotes. If an individual is heterozygous for two alleles each coding for structurally different variants of a polypeptide chain present in a particular enzyme protein, then more than one molecular form of that enzyme occurs and will be detectable after gel electrophoresis [130]. This system was utilized for the identification of the origin of regenerants in several different plants. Keller and Korzun [131] reported the use of malate dehydrogenase (MDH), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM) and galactosidase (GAL) systems on putative onion doubled-haploid regenerants. Bohanec et al. [132] and Jakše et al. [133] reported the use of an esterase (EST) system for donor plant selection for the induction of gynogenesis, and later for the verification of onion regenerants. Bartošová et al. [134] applied an analysis of acid phosphatase (ACP) and peroxidase (POX) isoenzymesto verify the gametic origin of flax plants obtainedfrom anther cultures and ovule cultures. Smykalova et al. [122] reported the use of esterase (EST) to study the homozygosity of caraway plants obtained from anther cultures.

In carrot, as shown in Tables 1 and 2, in the majority of reports on the induction of androgenesis or gynogenesis, the genetic status of the regenerants was not verified, and authors assumed that all diploids resulted from spontaneous diploidization [12,23,69–71,121]. In carrot, only two research groups reported the use of isozyme analysis for the determination of the homozygosity of obtained regenerants. The very first study was by Adamus and Michalik [25], who evaluated donor plants with PGI isozyme, and selected only heterozygotic individuals at the PGI locus as a donor for the in vitro culture. Next, they analyzed

328 diploid plants obtained from carrot anther culture with PGI isozyme and found that 99% were heterozygous. Later, a similar approach was reported by Górecka et al. [27] and Kiszczak et al. [29], who showed the use of PGI and aspartate aminotransferase (AAT) for testing the homozygosity of carrot plants obtained from anther cultures. They also evaluated donor plants first and selected heterozygous plants as anther donors. Their results showed that the obtained regenerants of the cultivar 'Narbonne F1' were 100% homozygous with respect to the AAT isoenzyme and 96% homozygous with respect to PGI. Similar results were also presented for regenerants from anther cultures of other carrot cultivars: 'Berjo' and 'Splendid F1'. The results showed that 100% of those plants were homozygotes with respect to both PGI and AAT, proving their gametic origin. In another experiment, the obtained regenerants of the heterozygous donor plant of cultivar 'Narbonne F1'were found to be 94% homozygotic with respect to PGI and 100% homozygotic in the case of AAT. In the case of the cultivar 'Kazan F', 96% of the regenerated plants were homozygous with respect to AAT, but only 4% with respect to PGI, proving that they were not of gametic origin. Such a result showed that enzyme polymorphism depends on the genotype, and testing based on a single locus may be insufficient for a reliable verification of the origin of the regenerants.

In the experiments on parthenogenesis in carrot induced by foreign pollination, the isozyme system was also applied both for selecting the donors for the culture, as well as for screening the progeny. Fifteen open-pollinated carrot cultivars were evaluated for their response to the induction of the development in the culture of an unfertilized ovule. Only heterozygous plants at the PGI locus were used as donors for ovule culture. Results showed that 49% of diploid regenerants were homozygous based on a PGI isozyme assay [74,75].

Another method for homozygosity detectionand the assessment of plant origin involves the use of DNA molecular markers. DNA markers are particularly useful if they reveal differences between individuals (polymorphic markers). Polymorphic markers are described as codominant or dominant, meaning that they can discriminate between homozygotes and heterozygotes. Codominant markers indicate such differences, whereas dominant markers do not. The most commonly used marker technologies to characterize DHs are dominant RAPDs (random amplified polymorphic DNA) and codominant SSRs (simple sequence repeats, microsatellites) markers. RAPDs were used to screen homozygosity of diploid regenerants in asparagus (*Asparagus officinalis* L.) [135] and pomelo (*Citrus grandis* L.) [136]. Analysis of the genetic origin of haploid/diploid regenerants using SSR markers has been applied in various fruit crops, including clementines (*Citrus clementina Hort. Ex Tan.*) [137], apples (*Malus* × *domestica Borkh.*) [138], pears (*Pyrus communis* L.) [139], *Camellia* spp. [140], coconuts (*Cocos nucifera* L.) [141] and other crops, such as wheat (*Triticum aestivum* L.) [142] and maize (*Zea mays* L.) [143].

For carrot, only one study employing DNA markers for the analysis of the genetic origin of diploid regenerants has been reported. In the protocol of carrot induced parthenogenesis through pollination with parsley pollen and ovule culture, the donor plants selected for the experiments were heterozygous at three independent loci: PGI isozyme, and two variants in genomic DNA—chs2 (chalcone synthase) and ipi3 (isopentenyl diphosphate isomerase) loci [76]. The same set of markers was used for homozygosity analysis in diploids obtained as a result of the culture. Results showed that a total of 72.6% of regenerated diploid plants were of gametic origin, demonstrating the importance of markers to confirm the origin of regenerated plants in crops with a high regeneration potential from somatic tissue [76,144].

5. Perspectives

The high efficiency of the haploidization method is the main factor enabling its application in plant breeding. The generation of doubled haploids is a very efficient tool for the production of completely homozygous lines in a relatively short time. As has been shown above, numerous studies have been undertaken on carrot and progress in the research on haploidization is noticeable; however, so far, no easily applicable and repeatable protocols of haploidization exist. Studies on haploidization in carrot have been carried out in several

different laboratories all over the world. Results showed that the development of embryos from haploid cells in carrot, from both female and male gametophytes, is possible; however, it depends on many factors, i.e., the genotype of the donor plant, the developmental stage of the microspore or embryo sac, the culture medium, explant pre-treatments. Other limitations include the flower morphology—the isolation of anthers or ovules in carrot is very laborious due to a small flower size. On the other hand, a very high rate (70–80%) of spontaneous duplication of the genome, irrespective of the applied technique of haploid induction, and high levels of embryo regeneration in carrot are advantageous.

The studies on induced parthenogenesis showed the usefulness of this method in doubled haploid plants production in carrot; however, considering the application to breeding, this method brings some issues. Most of all is very laborious—it demands the synchronization of the flowering of donors and pollinators, then performing a hand pollination on very small flowers, and finally ovule isolation. Moreover, ovules contain somatic tissues, and, therefore, both donors and the regenerants must be verified with molecular markers.

In the view of that, for breeding purposes, two approaches seem to be promising. Since there are much less egg cells than microspores in a flower, androgenesis appears to be an alternative. In carrot, due to the small size of flower buds and anthers, anther cultures can be considered as technically difficult; however, to the best of our knowledge, the single carrot cultivar 'Sonata' based on doubled haploid lines registered in the Russian Federation was established on androgenic haploids obtained via anther cultures [17]. Considering above, it seems that androgenesis is a promising method for haploid induction in carrot; therefore, the isolated microspore culture technique could be of interest for increasing the effectiveness of androgenesis. Firstly, this technique eliminates the somatic tissues, and secondly, if the protocol is elaborated and completed, it will be easy applicable to the breeders. Moreover, because the embryos develop from microspores directly, there will be no necessity of verifying the homozygosity of regenerants. In carrot, significant progress has been made in regard to enhancing the efficiency of the microspore cultures protocol allowing for embryo development. Further research should be focused on increasing the efficiency of embryo production.

Lastly, great hopes are associated with theCENH3-mediated haploid induction. Research on carrot has already been initiated, and hopefully stable haploid inducer lines will be generated in the near future. Although this approach is currently a top interest of scientists working on developing effective methods of haploid production and breeders, it also raises serious legal concerns. In some countries (i.e., European Union countries (16 members), Africa (Algeria, Madagascar), Asia (Turkey, Saudi Arabia, Kyrgyzstan, Bhutan) and Latin America (Ecuador, Peru, Venezuela, Belize), genetic engineering in agricultural applications is either limited or restricted by laws [145–147] and, therefore, the commercialization of the product breed on the base of uniparental genome elimination depends on whether these will be regulated as genetically modified organisms (GMOs). In the case of the centromere-mediated haploid induction technique, the final haploid would not possess any transgenic elements, because the chromosomes of the inducer line are eliminated upon crossing. Now, the question of how such a process will be regulated under GMO legislation in the above-mentioned countries remains to be resolved.

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