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Review

Spontaneous and Chemically Induced Genome Doubling and Polyploidization in Vegetable Crops

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Abstract: Plant ploidy manipulation is often required for breeding purposes. However, there is no comprehensive review covering genome doubling in vegetable crops despite the abundance of data for a large number of vegetable species. Similar to other species, genome doubling is required in vegetable crops to obtain doubled haploids (DHs). It is also utilized for the production of polyploids to overcome interspecific hybrid sterility and improve agricultural traits. Spontaneous haploid genome duplication (SHGD) occurs in many Apiaceae, Brassicaceae, Cucurbitaceae, and Solanaceae crops, allowing for the laborious treatment with antimitotic agents to be bypassed. SHGD mechanisms are not fully understood, but existing data suggest that SHGD can occur via nuclear fusion, endoreduplication, or other mechanisms during microspore or ovule early embryogenic development. Other studies show that SHGD can occur at later developmental stages during extended plant growth in vitro or ex vitro, possibly due to the presence of phytohormones in the medium and/or diploid cell competitive advantage. For unresponsive accessions and species with rare SHGD, such as onion (*Allium cepa* L.) and beet cultivars (*Beta vulgaris* subsp. *vulgaris* L.), antimitotic agent treatment has to be applied. Antimitotic agent application efficiency depends on the treatment conditions, especially the agent concentration and exposure time. Also, plant developmental stage is critical for agent accessibility and plant survival. The existing methods can be used to further improve genome doubling methodology for major vegetable crops and other species.

Keywords: doubled haploid; DH technology; polyploidy; antimitotic compound; colchicine

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1. Introduction

Plant breeding aims to improve cultivar yield and valuable compound abundance, for instance, sugar, antioxidant, and essential oil content. Another important goal of breeding is developing cultivars resistant to diseases, pests, and abiotic stressors, such as extreme temperature, salinity, and drought. Genome doubling is one of the tools required to speed up breeding process and increase genetic diversity. It is required for doubled haploid (DH) production. It is also utilized for doubling genome in interspecific hybrids and obtaining polyploids with improved agricultural traits. The methods of chromosome doubling are covered in many reviews for cereal and industrial, medicinal, and ornamental crops, but vegetable crops have not been discussed extensively so far [1–8].

Conventional breeding requires self-pollination for multiple generations, which makes this method extremely time-consuming. Many species have severe inbreeding depression [9] or self-incompatibility [10], which additionally complicates the generation of pure lines.

DH technologies allow for the production of fully homozygous pure lines in one generation, which immensely accelerates breeding. DH lines are produced when haploid cells, microspores, or ovules are stimulated to switch toward a sporophytic developmental

route. The resulting haploid regenerants need to double their genome to become diploid plants with two identical copies of genetic material [11,12]. SHGD induction or doubling with antimitotic agents are required for the successful inclusion of regenerant plants in a breeding program because haploid plants are sterile or have low seed productivity [5]. Similarly, if mixoploids or polyploids are generated, they can be of limited value for breeding. Therefore, SHGD induction methods or antimitotic treatment protocols are crucial for DH technology's successful implementation. Detailed reviews provide protocol development guidelines for haploid genome doubling in many species [4–6]. However, there is no comprehensive review covering genome doubling in vegetable crops.

Vegetables play a crucial role in human nutrition, providing dietary fibers, vitamins, minerals, and other essential nutrients [13–15]. DH technologies are being developed and improved to facilitate vegetable crop breeding. Vegetable crops are less responsive to DH technologies compared to cereals and rapeseed (*Brassica napus* L.). Therefore, a lot of data on doubled haploids, including the putative mechanisms of SHGD, were obtained in cereals and rapeseed [6]. Recently, a large degree of progress was made in the development of DH technologies for vegetables [15]. However, problems with the induction of embryogenic growth in many vegetable crops, especially Solanaceae, and the limited SHGD, especially in Amaranthaceae and Amaryllidaceae, limited the production of sufficient numbers of DH plants for breeding programs [9,16,17].

Another problem faced by breeders is introducing new valuable traits. Polyploidization produces varieties with new characteristics that could not be achieved by other means. This method is exploited in ornamental crops [1,7,8], medicinal plants [1,2], and industrial crops [3] because polyploids often have increased plant size and vigor, improved decorative traits, or increased secondary metabolite synthesis. Polyploidization is used for vegetable breeding to improve agricultural traits as well. One of the commercial successes of this method is seedless watermelon hybrids produced from diploid and tetraploid parent lines [18]. Genome doubling is also applied to overcome interspecific hybrid sterility [19]. Broadening the genetic diversity of breeding material can be achieved via hybridization with related species. This method is commonly used to introduce disease, herbicide, and abiotic stress resistance, as well as cytoplasmic sterility genes and other traits from other species. However, the obtained hybrids are often sterile, but genome duplication with antimitotic compounds can restore fertility.

SHGD and colchicine treatments exert stress on the genome of treated plants. The skim sequencing of wheat (*Triticum aestivum* L.) showed that various chromosomal aberrations, including deletions, duplications, and aneuploidy, can happen due to genomic shock during DH production [20]. These alterations can affect plant fitness and agronomic performance. In addition, DNA methylation patterns change as a result of spontaneous and artificial doubling. Most differential DNA methylation occurred at random sites [21]. Colchicine treatment changed the expression of genes involved in hormone signal transduction, metabolism, cytoskeleton control, and others [22]. More studies are needed to identify how long these changes last and what their consequences are. Possibly, the development of less-stressful protocols of spontaneous and artificial genome doubling will be beneficial.

Genome doubling is an indispensable tool for a multitude of applications in plant breeding. In this review, we discuss SHGD incidence and mechanisms, as well as antimitotic treatment protocols for different vegetable crops. Emphasis was placed on the most recent papers and literature sources that provided well-described and statistically tested data with controls and a large number of tested plants. Very limited data were available for some species; therefore, any available information was analyzed for them. The critical treatment information was unified (concentrations in g/L, doubling rate in %) and summarized in a table (Table 1).

2. Spontaneous Genome Doubling

The term "spontaneous doubling" is used to differentiate from doubling induced by chemical treatment. SHGD is extremely advantageous for DH production as it allows for the laborious and costly genome doubling with antimitotic compounds to be omitted.

2.1. Spontaneous Haploid Genome Doubling in Vegetable Crops

SHGD occurs at different frequencies in different species. Moreover, it can vary greatly within a species between different cultivars. For instance, in cereal crops, the percentage of doubling in barley (*Hordeum vulgare* L.) is up to 87% [23], in rice (*Oryza sativa* L.) it is up to 72% [24], in wheat (*T. aestivum* L.) it is up to 50% [25], and in maize (*Zea mays* L.) genotypes it is up to 0–21.4% [26,27].

SHGD can be documented in vegetable crop plants generated by DH technologies (Figure 1). For the Amaranthaceae vegetable crop red beet (*Beta vulgaris* subsp. *vulgaris* L.), the SHGD rate varied in different studies. Some authors observed no incidence of SHGD [28], while others obtained up to 70% diploid plants [29] or tetraploid regenerants [30]. The close relative of red beet, sugar beet (*B. vulgaris* subsp. *vulgaris* (var. *saccharifera*)), had gynogenic regenerants with a haploid chromosome set, but the root tips had endopolyploid cells [31]. In another study, a 10% SHGD rate was observed for sugar beet [32].

For the Amaryllidaceae family, SHGD was reported in onion (*Allium cepa* L.) during gynogenesis in vitro, but the doubling efficiency was relatively low. When a large number of regenerants (about 100) were obtained, the doubling efficiency could be more accurately assessed, and it was determined to be about 10% [33–35].Various SHGD rates were reported in the Apiaceae family. In some studies, no doubling occurred, as reported by Hu et al., who showed that among 18 carrot (*Daucus carota* L.) plants obtained from the embryoids and calli, 16 plants were haploid (2n = 9), and the other 2 plants were aneuploids (2n = 10 and 11) [36]. In another study, regenerants obtained in a culture of isolated microspores had an SHGD rate ranging from 17.5% to 63.6% depending on carrot accession [37]. A total of 90% of carrot regenerants obtained from another culture were diploid [38]. The flow cytometric analysis of carrot plants obtained in an in vitro culture of unfertilized ovules in carrot showed that 97.7% of the regenerants were diploid [39].

About 50% of the dill (*Anethum graveolens* L.), caraway (*Carum carvi* L.), carrot (*D. carota* L.), fennel (*Foeniculum vulgare* Mill.), lovage (*Levisticum officinale* Koch.), and parsnip (*Pastinaca sativa* L.) regenerants obtained from microspore culture spontaneously doubled their genome. However, regenerant images and ploidy evaluation [40].

SHGD is widely observed in the Brassicaceae family. For instance, besides haploid plants, mixoploid, diploid, and tetraploid cabbage (*Brassica oleracea* var. *capitata* L.) regenerants produced from microspores can be observed as assessed by flow cytometry, guard cell chloroplast, and chromosome counting (Figure 1). SHGD occurred in 0–76.9% of cabbage (*Brassica oleracea* var. *capitata* L.) regenerants, in 52.2–100% of broccoli (*B. oleracea* var. *italica* L.) [41], in 50–70% plants of different *B. rapa* L. accessions [42–45], and in more than 60% of rapeseed (*B. napus* L.) regenerants [46].

In Cucurbitaceae species, for instance, in a summer squash (*Cucurbita pepo* L.) ovary/ovule culture, most regenerants doubled their genome. Although, occasional haploid, mixoploid, or polyploid regenerants were observed as well [47]. In styrian oil pumpkin (*Cucurbita pepo* ssp. *pepo* var. *styriaca*), most plants were also diploid, with rare occurrences of n, 3n, and 4n plants [48].

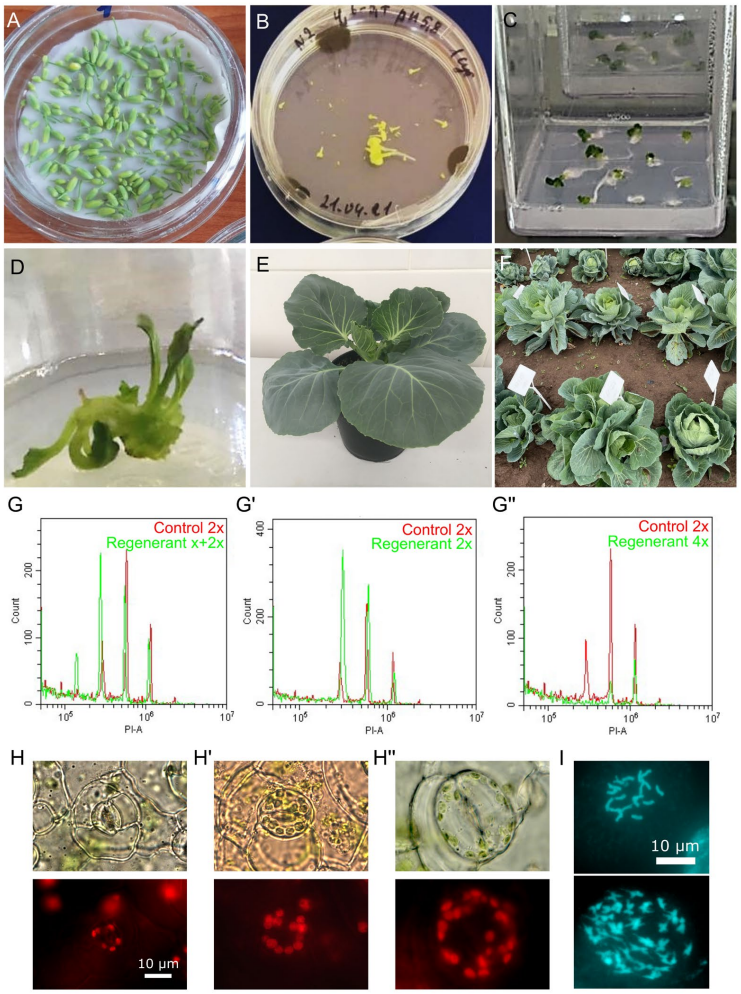

Figure 1. Obtaining cabbage (*B. oleracea* var. *capitata* L.) doubled haploids in microspore culture in vitro with subsequent regenerant ploidy analysis. (**A**) Microspores were isolated from the collected flower buds under sterile conditions. (**B**) Embryoids growing from microspores in liquid NLN medium. (**C**) Embryoids were transferred to solid MS medium. (**D**) Embryoids regenerated into plants. (**E**) Rooted plants were adapted to ex vitro conditions. (**F**) The adapted plantlets were grown under field conditions. (**G**–**G′′**) The ploidy was analyzed with a flow cytometer (Beckman Coulter, USA). Nuclei were isolated from plant leaves in Galbraith lysis buffer and stained with propidium iodide. The analyzed sample (green peaks) was compared with a control diploid cabbage (red peaks). Mixoploid (**G**), spontaneously doubled diploid (**G′**), and tetraploid (**G′′**) plants were observed. (**H**–**H′′**) The ploidy estimation by counting the chloroplast number in stomatal guard cells. Stomatal guard cells in phase contrast (top images) and chloroplast red autofluorescence (bottom images) were imaged. Haploid (**H**), spontaneously doubled diploid (**H′**), and tetraploid (**H′′**) plants can be distinguished by the number of chloroplasts that is higher in higher ploidy plants. (**I**) The direct counting of chromosomes in DAPI-stained spontaneously doubled diploid (2n = 2x = 18) and tetraploid (2n = 4x = 36) samples.

Solanaceae crops also demonstrate genome doubling in DH regenerants, although the doubling rate often differs greatly not only between different species but also within the same species between different genotypes or even between different plants within the same genotype [49]. The frequency of SHGD in bell pepper (*Capsicum annuum* L.) plants was 14%–51% [50], 14.3–65.8% [51], 30–55% [52,53], 32.6% [54], 33% [55], and 65% [56]. In hot pepper (*C. annuum* L.), the SHGD rate was 16.3% [57]. In Indonesian hot pepper (*C. annuum* L.), spontaneous doubling was 14–33% in four accessions and 47% and 51% in the other two pepper accessions [50]. The analysis of cayenne pepper (*C. frutescens* L.)

regenerants showed that about 40% of plants were diploids and 8% were mixoploids [58]. Interspecific hybrids, *C. frutescens* × *C. annuum* and *C. frutescens* × *C. chinense* had a lower haploid embryoid regeneration rate compared to *C. annuum*, but the SHGD was 50% and 80%, respectively [49]. In the genus *Physalis*, a high level of spontaneous diploidization (up to 72%) was observed in tomatillo (*Physalis ixocarpa* Brot.) (2n = 2x = 24) [10,59]. At the same time, for the tetraploid species cape gooseberry (*Physalis peruviana* L.) (2n = 4x = 48), a fairly low doubling level was documented (28%), and antimitotic treatment was required to double the genome in haploid regenerants [60].

Tomato (*Solanum lycopersicum* L.) is one of the most recalcitrant crops for DH technologies. Unlike most other members of the Solanaceae family, tomato is unresponsive to all the existing DH methods available in the literature. Therefore, it is difficult to make conclusions about SHGD for tomato. The largest number of plants was obtained in the work of Zagorska et al., 2003, who analyzed ploidy in 700 androgenic regenerants. They concluded that 21,5% were haploid, 11.3% were diploid, and the rest were mixoploid [61].

Diploid plants obtained from the anther or ovule cultures can originate from surrounding somatic tissues. In addition, some regenerant plants can come from unreduced gametes that formed via meiotic restitution [62]. Therefore, regenerant origin has to be validated by molecular markers to select only true DHs. The reported SHGD rate may be affected by the erroneous inclusion of plants obtained via the abovementioned mechanisms.

A large number of vegetable species described above undergo spontaneous genome doubling. However, some accessions have an insufficient SHGD rate, and some species, including onion and beet, are recalcitrant to spontaneous doubling induction. Despite the challenges of the DH technologies in vegetable crops, doubled DH lines are included in breeding programs. Examples of the successful use of spontaneously doubled DH lines include the F1 hybrid of cabbage 'Nataly', kohlrabi 'Dobryniya', sweet pepper 'Mila', 'Nataly' and 'Gusar', pumkin 'Vega', and a carrot variety 'Sonata' [63].

2.2. SHGD Mechanisms

A list of factors influence spontaneous doubling induction, including genetic factors and microspore or anther temperature stress treatments. In cereal crops, mannitol or 2-hydroxynicotinic acid can also be used as a stress treatment [62]. In addition, spontaneous doubling can occur after a long-term regenerant subculturing in vitro or ex vitro.

Four possible mechanisms of genome doubling were proposed: nuclear fusion, endoreduplication, C-mitosis, and endomitosis [6,27,62]. Endoreduplication is a process when one or several rounds of DNA replication happen without mitosis. It is widely observed in angiosperms at different stages of development [64]. One of the critiques to this plausible mechanism is that endoreduplication is generally seen as a terminal stage of cell differentiation served to obtain higher metabolic competences and/or cell size, which differs from the processes observed in developing haploids [27].

The nuclear fusion of two haploid nuclei is another possible mechanism. Nuclear fusion was experimentally documented in a barley microspore culture (*H. vulgare* L.) by live cell imaging [65]. More studies in different taxa are needed to further confirm this mechanism's applicability for SHGD.

Endomitosis happens when mitotic stages occur inside the nuclear membrane without spindle formation and daughter cell separation. However, endomitosis is rarely observed in angiosperms. C-mitosis is an artificially aborted cell division via the disruption of mitotic spindle by colchicine or other antimitotic agents [27,62], which is discussed in Section 2 of this review.

Besides haploid and doubled haploid plants, triploid and polyploid regenerants can often be observed in many crops, including *Brassica* species, *C. annuum* L., *D. carota* L. [4], and *C. pepo* L. [47,48]. Possibly, triploid plants can form due to the fusion of diploid and haploid nuclei. Tetraploid and higher ploidy plants are likely to result from more than one round of doubling [6]. As it was mentioned before, different cultivars can have a big difference in the incidence of SHGD. The genetic mechanisms that make some cultivars

more prone to SHGD are not clear, but the research conducted on maize showed one major and a few minor QTLs that are associated with spontaneous genome doubling. The possible candidate gene associated with the reported QTLs is the absence of the first division (*afd1*) gene that affects the first meiotic division, resulting in a single equational division. Another candidate is the formin-like protein 5 that affects actin cytoskeleton [66]. Studies of other species, including vegetable cultivars, are required for the elucidation of the genetic mechanisms controlling SHGD. The development of SHGD-associated markers would simplify the introgression of this valuable trait into elite germplasm and increase DH production efficiency.

2.3. SHGD Timing during Development

Spontaneous genome doubling can occur at different stages of microspore development [6,62]. The applied stress pretreatment leads to cytoskeleton perturbation. It disrupts mitotic spindle or cell wall formation that results in SHGD. It was demonstrated that to obtain the best results, the stress treatment has to be applied specifically at late uninucleate to early binucleate stages. Microspores isolated at early uninucleate stages predominantly resulted in haploid regenerants, while the binucleate microspores produced more doubled haploid and polyploid regenerants [6]. Stress application at later stages can increase the incidence of triploid and polyploid regenerants [62].

Spontaneous chromosome doubling can occur at later developmental stages as well. Yuan et al. reported that the long-term subculturing of cabbage or broccoli haploids on MS-2 medium with 0.1 mg/L NAA and 0.2 mg/L 6-BAP led to a gradual increase in the number of plants with a doubled chromosome set. After one or more years in tissue culture, most of the cabbage or broccoli haploids turned into DHs or mixoploid plants [41]. Similar results were observed in haploid pepper (*C. annuum* L.) plants grown ex vitro for 6 years. The plants were rejuvenated by cutting off the shoots and allowing young shoots to regrow. The authors reported that out of 12 plants, 1 plant had all diploid shoots, 7 plants had both haploid and diploid shoots, 2 plants remained haploid, and 2 plants died [67]. For plants that change their ploidy after long-term growth in vitro or ex vitro, it can be speculated that spontaneous endoreduplication or doubling via other mechanisms can happen in some cells. Subsequently, doubled haploid cells could outcompete haploid cells. However, this hypothesis requires experimental testing. Possibly, culture media hormones can affect the doubling rate. For instance, auxins are involved in the transition from the mitotic cycle to endoreduplication [68]. For instance, it was shown that NAA caused genome duplication in sugar beet [69]. 2,4-D is also known to increase plant ploidy, as shown, for example, in an orchid tissue culture [70]. The cucumber embryogenic callus established from immature embryos on media supplemented with 6-BAP, NAA, and 2,4-D regenerated not only into diploid (57%) but also tetraploid (18%), octoploid (4%), and mixoploid (2n/4n—4% and 4n/8n—17%) regenerants, as tested by flow cytometry [71].

Currently, the SHGD mechanisms are not fully understood. More research is needed to definitively demonstrate how SHGD occurs and what molecular mechanisms underlie these processes. Understanding the mechanisms of spontaneous doubling can vastly improve the genome doubling rate and the efficiency of DH technology.

3. Chemically Induced Genome Doubling Protocols

When SHGD does not occur or happens at a low frequency, genome doubling can be induced with antimitotic compounds. Currently, a number of antimitotic agents are used to induce genome duplication, including a natural alkaloid colchicine ($C_{22}H_{25}NO_6$) and herbicides, such as trifluralin ($C_{13}H_{16}F_3N_3O_4$), oryzalin ($C_{12}H_{18}N_4O_6S$), pronamide ($C_{12}H_{11}Cl_2NO$), and amiprofosmethyl (APM) ($C_{11}H_{17}N_2O_4PS$). Alcohol n-butanol (C_4H_9OH) is also utilized as an antimitotic agent. The listed antimitotic compounds have a similar mechanism of action. They perturb the microtubule cytoskeleton that prevents mitotic spindle formation and chromosome separation. As a result, the cell receives a doubled chromosome set [4,72,73].

The antimitotic compound choice, concentration and treatment time, supplementary compounds, and the explants used for treatment are critical for successful genome doubling. Different approaches for chemically induced genome doubling are covered below.

3.1. Antimitotic Agent Choice

Colchicine is one of the most widely used antimitotic agents (Table 1). Colchicine is typically applied at concentrations of 0.05 to 5 g/L, but in some cases, it may be outside this range. For example, Gurel S. et al. (2021) treated sugar beet (*B. vulgaris* subsp. *vulgaris* (var. *saccharifera*)) with 20 g/L colchicine, while Vasilchenko et al. (2018) used it at a 4000 times smaller concentration (0.005 g/L) on the same crop [16,74]. Hence, the antimitotic agent should be tested in a wide range of concentrations to determine the most effective and cost-efficient treatment protocol. Colchicine must be handled with caution with the use of protective clothing and gloves due to its toxicity [75]. In addition, colchicine is a light-sensitive compound; therefore, it has to be used under limited or no light conditions [76]. For instance, plant apices with applied cotton balls soaked in colchicine should be covered with foil to prevent colchicine degradation.

Trifluralin is another common substance used to increase the ploidy level. It is a pre-emergence herbicide that prevents seed germination. Trifluralin has low acute toxicity, but it has been classified as a group C, possible human carcinogen [77]. Similar to colchicine, it has to be protected from light due to its light sensitivity [72]. Trifluralin is typically used at 3.35×10^{-4} [78] to a 0.1 g/L concentration. However, concentrations close to 0.1 g/L or above have led to plantlet death [79,80]. Trifluralin was successfully used in a list of species, including onion (*A. cepa* L.) [81], rapeseed (*B. napus* L.) [78,82], sugar beet (*B. vulgaris* subsp. *vulgaris* (var. *saccharifera*)) [79,83], rice (*O. sativa* L.) [5], maize (*Z. mays* L.) [84], and others (Table 1).

Oryzalin is used to increase ploidy in many horticultural and ornamental species. For genome doubling purposes, oryzalin is used at concentrations ranging from 4×10^{-4} [79] to 0.1 g/L. Similar to trifluralin, high oryzalin concentrations (0.1 g/L or above) are also detrimental for plant survival [79,85]. Oryzalin was tested in rapeseed (*B. napus* L.) [82], sugar beet (*B. vulgaris* subsp. *vulgaris* (var. *saccharifera*)) [79], onion (*A. cepa* L.) [34,81,86], maize (*Z. mays* L.) [84], and other species (Table 1).

Amiprofos-methyl (APM) is an antimitotic agent that destroys mitotic spindle or induces multipolar spindle division [4]. Successful genome doubling using APM was reported for a list of species, including rapeseed (*B. napus* L.) [82], wheat (*T. aestivum* L.) [87], and maize (*Z. mays* L.) [84,88,89]. For vegetable crops, it shows promising results in onion (*A. cepa* L.) [81,86,90–93].

The use of herbicide pronamide is limited to a small number of studies. In maize, 0.0026 g/L pronamide resulted in about a 60% doubling rate [84]. In sugar beet and fodder beet, 0.004 g/L pronamide induced 2% doubling [79]. Rapeseed treatment with pronamide increased the number of plants with doubled genome (52%) compared to untreated control (36.4%), but it was less effective than colchicine treatment (69.6%) [94] (Table 1).

N-butanol is a primary 4-carbon alcohol that induces cortical microtubule depolymerization [95,96]. A drastic increase in the microspore embryogenesis rate after n-butanol treatment was observed in wheat (*T. aestivum* L.) [97] and certain barley cultivars (*Hordeum vulgare* L.) [98]. N-butanol has some positive effect on embryogenesis in maize (*Z. mays* L.) [95,99,100] and pepper (*C. annuum* L.) [101]. A further improvement in the n-butanol treatment regimen and testing of this compound in other species is still needed.

The efficacy of an antimitotic agent treatment critically depends on a number of factors that determine the toxic effect of the antimitotic compound, as well as the duplication and the aberrant ploidy rate. The agent concentration, supplementary components, the application method, and other contributing factors are discussed below.

Table 1. Vegetable crop and sugar beet (*Beta vulgaris* L.) genome doubling approaches.

The Crop	Ploidy before Treatment	The Best Doubling Efficiency	Application Method	Antimitotic Agent	Treatment Time	Growth Conditions after Treatment	Practical Results	References
				Amaranthaceae				
Sugar beet (*Beta vulgaris*)	n	–	Seedlings with 3–7 leaves	5 g/L colchicine	5 min 2 times	MS, 10 g/L sucrose, 6.5 g/L Gelrite, 0.002 g/L kinetin	–	[16]
	n	91.3%	Microclones	0.0005 g/L colchicine	48 h	MS, GA, 6-BAP, kinetin—0.0002 g/L each	Selection of new lines with sterile cytoplasm (confirmed by PCR and RFLP analysis). Four lines were selected for breeding purposes (the plant qualities are not described)	[74]
	n	29.1% 20.7%	Shoots higher than 1 cm with roots removed	0.05, 0.1, 0.15 or 0.5 g/L colchicine 0.017, 0.0034 or 0.005 g/L trifluralin	12, 24, 36 or 48 h	MS, 0.001 g/L 6-BAP, solid or liquid media	–	[83]
	n	4.7% (0.03 g/L) 2.8% (0.00035 g/L) 2.0% (0.003 g/L) 2.0% (0.003 g/L)	Ovules. Antimitotic agent with 1.5% DMSO	0–0.09 g/L APM 0–0.1 g/L oryzalin 0–0.1 g/L pronamid 0–0.1 g/L trifluralin	5 h	Liquid culture medium	–	[79]
	n	60% (4 g/L, 2.5 h)	Ovules after 7 days in culture	4–60 g/L colchicine	0.08—5 h	Induction medium	–	[102]
	n	64% (0.046 g/L APM, 5 h)	Ovules after 10 days culture	0.006, 0.046, 0.092 g/L APM + 15 g/L DMSO in liquid induction medium	2, 5, 18 h	Culture medium	–	[103]
	n	19%	Roots of regenerant plants	3 g/L colchicine	24 h	Plants were planted in soil	Three lines that exceeded diploid control for yield and sugar concentration were produced (for instance, BTS 40 DH line—377.7 g and 19.4%, control diploid—204.2 g and 18.4% root weight and sugar content, respectively)	[104]
	n	8.4% 2n, 3.4% 4n, 1.1% 8n, 33% mixoploids	Meristem of plants at the 6–8-leaf stage	1 g/L colchicine with 20 g/L DMSO	72 h	–	–	[105]

Table 1. *Cont.*

The Crop	Ploidy before Treatment	The Best Doubling Efficiency	Application Method	Antimitotic Agent	Treatment Time	Growth Conditions after Treatment	Practical Results	References
				Amaryllidaceae				
Garlic (*A. sativum*)	2n	–	Basal plates in liquid B5 with 20 g/L DMSO and colchicine	2.5–7.5 g/L colchicine	36–72 h	B5, 0.0008 g/L 2iP, 0.0001 g/L NAA	4n plants leaves had 3 times larger area. Allicin content increased by 30%	[106]
Onion (*Allium cepa*)	n	–	Basal explant in MSO (MS based) medium with colchicine	0.4 g/L colchicine	48 h	1/2 BDS, 30 g/L glucose and 7.0 g/L agar, pH 6.0	–	[9]
	n	36.7%	Embryos in medium with APM	0.015 g/L APM	48 h	1/2 BDS, 30 g/L glucose, 7.0 g/L agar, pH 6.0	–	[93]
	n	36%	Intact or cut longitudinally into halves basal explants in BDS medium with antimitotic agents	0.003; 0.006; 0.009 g/L APM	72 h	1/2 BDS or M4, 30 g/L glucose and 0 or 7.0 g/L agar, pH 6.0	Fertility/fecundity recovered in some genotypes, but not others. Lines with uniform bulb shapes were produced	[90]
		40–46% 2n, 18–30% mixoploids (0.2–0.4 g/L colchicine in liquid medium, 48 h		0.01–0.4 g/L colchicine	24, 48, 72 h			
	n	32.8% (0.4 g/L)	Whole basal explants	0.3–0.4 g/L colchicine	–	MSO, 30 g/L sucrose	Fertility/fecundity recovery	[86]
		15.8% (0.03 g/L)		0.017–0.051 g/L oryzalin				
		25% (0.03 g/L, 0.045 g)		0.015–0.045 g/L APM				
	n	100% (0.25 g/L, 48 h)	in vitro plants	0.25 or 0.5 g/L colchicine	24 and 48 h	1/2 MS, 40 g/L sucrose, 7.5 g/L agar, pH 5.8	–	[92]
		57.7% (0.015 g/L 48 h)		0.015–0.06 g/L APM				
	n	46% (0.01 g/L, 72 h)	Root tips, shoot apex	0.01; 0.1 g/L colchicine	24 and 72 h	M3 medium	–	[91]
	n	up to 65.7% (1 g/L)	in vitro plants	0.25–5 g/L colchicine	24 h	MS	–	[34]
		up to 57.1% (0.0175 g/L)		0.0035–0.07 g/L oryzalin				
	n	44%	Two-month-old haploid plants on BDS basic medium with colchicine	0.015 g/L colchicine	72 h	The plants were grown in peat blocks	–	[107]

Table 1. *Cont.*

The Crop	Ploidy before Treatment	The Best Doubling Efficiency	Application Method	Antimitotic Agent	Treatment Time	Growth Conditions after Treatment	Practical Results	References
Onion (*Allium cepa*)	n	47.1% 2n, 29,4% 29,4% another ploidy (0.017 g/L 72 h)	Gynogenic embryos were plated on the media with antimitotic agents in the dark	0.0017 g/L; 0.017 g/L trifluralin in acetone	24 and 72 h	Medium R1: 1/2 BDS, 30 g/L glucose and 7.0 g/L agar, pH 6.0; medium R2: BDS, 40 g/L sucrose, 7.0 g/L agar, pH 6.0	–	[81]
		47.1% 2n, 8.8% other ploidy (24 h)		0.012 g/L oryzalin in acetone				
		34.8% 2n, 17.4% other ploidy (72 h)		0.015 g/L APM in methanol				
		35.3% 2n, 5.9% other ploidy (0.05 g/L 72 h)		0.001 g/L; 0.05 g/L colchicine with 20 g/L DMSO				
	n	Up to 38% 2n (0.015 g/L, liquid media, 24 h)	Embryos in liquid or solid media with APM	0.008 g/L; 0.015 g/L APM	24 and 72 h	1/2 BDS, 15 g/L glucose	–	[108]
Onion (*A. fistulosum* × *A. cepa*)	2n	51.4% 4n (10 g/L)	Callus in liquid BDS on a shaker at 60 rpm	5–20 g/L colchicine	36–72 h	solid BDS medium with 8 g/L agar	Five 4n, likely amphidiploid plants were obtained and adapted to field conditions for future breeding	[109]
Onion (*A. cepa* × *A. vavilovii*, *A. cepa* × *A. nutans*, *A. cepa* × *A. schoenoprasum*)	3n *	–	Meristems in vitro	0.01 g/L colchicine	–	–	An increased vegetative mass (no quantitative data) and resistance to downy mildew was observed in interspecific hybrids	[110]
Persian shallot (*A. hirtifolium*)	2n	died after treatment	Basal plates in liquid MS with 10 g/L DMSO and antimitotic agent on a shaker at 100 rpm	3–7 g/L colchicine	24–48 h	MS, 30 g/L sucrose, 8 g/L agar, 0.001 g/L 6-BAP, 0.0005 g/L NAA	Increased total phenolic compound and allicin content by 27 and 15%, respectively[111]	[111]
		died after treatment		0.01–0.04 g/L oryzalin	4–8 h			
		35% 4n (5 g/L, 36 h); 31.82% mixoploids (3 g/L, 36 h)	Basal plates on solid MS with 8 g/L agar with antimitotic agents	3–7 g/L colchicine	24–48 h			
		45.45% 4n (0.01 g/L, 8 h); 16.9% mixoploids (0.04 g/L)		0.01–0.04 g/L oryzalin	4–8 h			

Table 1. *Cont.*

The Crop	Ploidy before Treatment	The Best Doubling Efficiency	Application Method	Antimitotic Agent	Treatment Time	Growth Conditions after Treatment	Practical Results	References
				Apiaceae				
Ajowan (*Trachyspermum ammi*)	2n	11.53% 4n (0.5 g/L, 24 h)	Seeds	0.24–0.5 g/L colchicine	6–48 h	liquid MS	Thymol in essential oil increased by 39% in 4n plants. The increase was observed in plant organ sizes with the largest difference in plant height (over 2 times)	[112]
American wild carrot (*Daucus pusillus*)	n	–	Cut umbrellas before flowering in the green bud phase	1 g/L colchicine with 20 g/L DMSO	20 h	MSm, 0.0002 g/L 2,4-D, 6–8 g/L agar	–	[110]
Caraway (*Carum carvi*)	n	–	Root system of in vitro plants	0.4 g/L colchicine with 10 drops/L DMSO	24 h	Containers with soil, plants were covered to maintain high humidity	–	[113]
Carrot (*Daucus carota* L.)	n	–	Microspores	0.5 g/L colchicine	24 h	B5, 0.0001 g/L 2,4-D, 0.0001 g/L NAA, 100 g/L sucrose	–	[114]
Carrot (*D. carota* L.)	n	–	Microspores	0.5 g/L colchicine	48 h	NLN, 0.0001 g/L 2,4-D, 0.0001 g/L NAA, 130 g/L sucrose	–	[115]
Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*)	n	–	Root system and crowns	3.4 g/L colchicine	1.5 h	Containers with soil, plants were covered to maintain high humidity	–	[116]
Parsley (*Petroselinum crispum* L.)	2n	about 30% (0.5, 24 h)	Seeds ex vitro on a rotary shaker at 120 rpm	0.25, 0.5, 1, 2 g/L colchicine	8–48 h	Seeds were planted in pots with soil mixture	Plant height is increased by 42% and leaf length is increased by 64% in 4n plants	[117]
		100% (1 g/L, 24 h)	Plant nodes in vitro on a rotary shaker at 120 rpm			MS with 0.001 g/L 2,4-D		

Table 1. *Cont.*

The Crop	Ploidy before Treatment	The Best Doubling Efficiency	Application Method	Antimitotic Agent	Treatment Time	Growth Conditions after Treatment	Practical Results	References
				Brassicaceae				
Broccoli (*Brassica oleracea* var. *italica*)	n	50% (2 g/L, 6 h), 66.7% (1 g/L, 12 h)	In vitro seedling roots were trimmed to 1–2 cm length and immersed in colchicine	0.5, 1, 2, 4 g/L colchicine with 20 g/L DMSO	6–12 h	Containers with soil, plants were covered to maintain high humidity	Genome doubling recovered fertility (fertile DH and partially fertile mixoploids), high colchicine toxicity for broccoli was shown (from 50 (1 g/L, 6 h) to 100% (4 g/L, 9–12 h)	[41]
White cabbage (*B. oleracea* var. *capitata* L.)		50% (2 g/L, 9 h)			3–12 h			
B. oleracea × leaf mustard *B. juncea*	2n ***	Best results for 1.5, 2 g/L	Rooted ex vitro cuttings axillary meristems were soaked in colchicine solution and covered with foil	0.5, 1, 1.5, 2, 2.5 g/L colchicine	Two treatments/day for 3 days	–	Genome doubling recovered fertility (7–84% pollen fertility), an increased main stem thickness and leaf size, more compact inflorescences, different leaf texture and margins compared to ABC hybrids and parents (no quantitative data)	[118]
Chinese cabbage (*B. rapa*) × white cabbage (*B. oleracea*), rapeseed (*B. napus*)/leaf mustard (*B. juncea*) × Chinese cabbage (*B. rapa*)	2n **	–	Plantlets in vitro	1 g/L colchicine	4 h	Plants were planted in coco-peat	The white cabbage with orange/yellow inner leaves (no quantitative data) and Chinese cabbage with an increased anthocyanin content (increased from 0 to 4.7 mg/g)	[119]
				Cucurbitaceae				
Cucumber (*Cucumis sativus*)	n	–	Cuttings with 2 axillary buds in E20H8 medium with colchicine	0.2 g/L colchicine	48 h	–	Genome doubling recovered fertility. A 40–80% mortality rate from colchicine treatment.	[120]
Cucumber (*C. sativus*)	n	24%	Haploid plants were on CBM basic medium with colchicine	0.2 g/L colchicine	96 h	The roots were rinsed in water. Then, the plants were grown in peat blocks	–	[107]
Melon (*Cucumis melo*)	n	–	The main apical stem	colchicine	2 h	–	–	[121]

Table 1. *Cont.*

The Crop	Ploidy before Treatment	The Best Doubling Efficiency	Application Method	Antimitotic Agent	Treatment Time	Growth Conditions after Treatment	Practical Results	References
Pumpkin (*Cucurbita pepo*)	n	–	Plantlets on a shaker at 120 rpm	5 g/L colchicine	12 h	–	–	[122]
			Apical shoot	10 g/L colchicine	3 1 h treatments per day			
Watermelon (*Citrullus lanatus*)	2n	Over 60% (colchicine, ethalfluralin, oryzalin at the highest concentration, 9 days)	Shoot buds of in vitro plants in MS with antimitotics on a shaker	0.2, 0.4, 0.6, 0,8 g/L colchicine. Ethalfluralin, oryzalin, cobex, amex (25, 50, 75, 100 μM/L)	3, 6, 9 days	MS, 30 g/L sucrose, 7 g/L agar with 0.00225 g/L 6-BAP	–	[123]
	2n	–	Seedlings were immersed in colchicine aqueous solution	2 g/L colchicine	6 days	–	4n rootstocks are more tolerant to salt stress (minor withering at 300 mM NaCl, no quantitative data) due to lower Na+ /K+ ratio, higher photosynthetic capacity, antioxidant enzyme activity, and osmoregulatory gene expression	[124]
Solanaceae								
African nightshade (*Solanum nigrum ssp. villosum*)	2n ****	about 10% (0.1 g/L)	Ex vitro seedlings at cotyledonary stage were sprayed with colchicine solution and covered with polyethylene sheets	0.1, 0.5, 2.5 g/L colchicine in 1 mL DMSO and 0.1 mL Tween-20	7 d, sprayed once a day	–	–	[125]
Cape gooseberry (*Physalis peruviana*)	n ****	over 60% (2 g/L, 2 h)	Excised axillary buds were immersed in colchicine solution in the dark	2, 4, 6 g/L colchicine with 20 g/L DMSO	2 h, 4 h, 6 h	MS, 0.1 mg/ L IBA	Recovery of fertility and seed production	[60]
Chili (*C. annuum);* Ancho chili × habanero chili (*C. annuum* × *C. chinense*)	n	–	Seedlings in vitro/ex vitro. Each seedling was immersed to the base of the stem in a bottle with colchicine solution	5 g/L colchicine	8 h	Pots with sterile substrate	Maintainers of cytoplasmic male sterility resistant to *Phytophthora capsici* Leo. and to the geminiviruses PepGMV and PHYVV were obtained	[126]

Table 1. *Cont.*

The Crop	Ploidy before Treatment	The Best Doubling Efficiency	Application Method	Antimitotic Agent	Treatment Time	Growth Conditions after Treatment	Practical Results	References
Chili (*C. annuum*)	2n	–	Seedlings at the 4-leaf stage	3 g/L colchicine	9 or 12 h for 2 days or 8 h for 3 days	–	4n plants exhibit gigas characters when compared to the 8n (leaf area 1.8 times larger; fruit diameter 2.7 times larger; flower diameter 1.6 times larger), except for the anthers, which are large, thick, sometimes deformed and coalesce with the corolla in the 8n plants. 8n plants were less vigorous.	[127]
Eggplant (*Solanum melongena*)	n	–	Plants in vitro	5–10 g/L colchicine	2 h (5 g/L); 1 h (10 g/L)	–	–	[17]
		25% more compared to SHGD	Plant axillary buds ex vitro	5 g/L colchicine in lanolin paste	48 h			
	n	50–70%	Remove the apical and axillary buds from plantlets. Apply colchicine to secondary axillary buds in the dark	5 g/L colchicine in lanolin paste	48 h	Greenhouse conditions; remove shoots produced by untreated buds	–	[128]
	n	35%	2–3 leaf plantlets on 1/2 MS with colchicine	0.6 g/L colchicine	72 h	The plants were grown in peat blocks	–	[107]
	n	100%	Colchicine solution was applied to axillary buds with a piece of cotton in the dark	5 g/L	2 h	–	–	[129]
				10 g/L colchicine	1 h			
	n	–	Plantlets in vitro	5 g/L	2 h	Hormone-free medium R	–	[130]
	n	–	Plantlets roots in vitro/ex vitro	0.1 g/L colchicine	4 h	The plants were acclimated and transferred to the greenhouse	–	[131]
Paprika (*C. annuum*)	n	95% on average	In vitro plantlets at 2–3-leaf stage in plain V3 medium with colchicine	10 g/L colchicine	1 h	Plastic pots containing 1:1 mixture of non-sterilized peat and sandy soil mix	–	[107,132]
Pepper (*C. annuum*)	n	50–95%	In vitro plantlets in V3 medium with colchicine	0.05–0.4 g/L colchicine	96–144 h	–	–	[133]

Table 1. *Cont.*

The Crop	Ploidy before Treatment	The Best Doubling Efficiency	Application Method	Antimitotic Agent	Treatment Time	Growth Conditions after Treatment	Practical Results	References
Pepper (*C. annuum*)	n	up to 100%	Axillary buds of ex vitro plants were covered with cotton soaked in colchicine and wrapped with foil	5 g/L colchicine	12 h	Colchicine applications were continued until chromosome doubling was achieved	–	[134]
Pepper (*C. annuum*)	n	–	Axillary buds of ex vitro plants were covered with a piece of cotton soaked in colchicine in the dark	0.5 g/L colchicine	2 h	–	–	[135]
Pepper (*C. annuum*)	n	57.6% and 47.3% 33% SHGD	Anthers	3 g/L colchicine	30 days	MS with supplements	Addition of colchicine to culture medium resulted with positive effects on viability of embryo, regeneration, and growth into full developed plantlets; haploid plants are smaller, plant viability, in vitro regeneration, development, and growth is lower compared to diploid donor plants	[55]
Pepper (*C. annuum)*	n	25–27% 2n, 29–55% mixoploids	The apical shoot fragment with 2–3 leaves in vitro	0.2, 0.4 g/L g/L colchicine	6–9 days	MSm, 30 g/L sucrose, 8 g/L agar, pH 5.8	Four weeks after the second colchicine treatment the growth disorder was observed, which proved to be directly proportional to the time of explant incubation on the colchicine-containing MS medium; after six days of colchicine treatment, 38% of the plants regenerated, and after nine days, their number decreased to 33%	[136]
Spice and bell pepper (*C. annuum*)	n	75%	4–6 leaf stage in vitro plantlets	3 g/L colchicine with 1.5 g/L DMSO	3 h	The roots were rinsed in water. Then, the plants were grown in peat blocks	–	[137]

Table 1. *Cont.*

The Crop	Ploidy before Treatment	The Best Doubling Efficiency	Application Method	Antimitotic Agent	Treatment Time	Growth Conditions after Treatment	Practical Results	References
F_2 hybrids *C. annuum* L. (cv. Zdorovie) × *C. chinense* Jacq; BC_2 of *C. annuum* L. (cv. Zdorovie) × *C. chinense* Jacq × cv. Zdorovie × cv. Zdorovie	n/2n	-	Apical meristem ex vitro plants	5 g/L colchicine with Tween-20 (1 drop/100 mL)	-	Plants were covered with polyethylene film to reduce evaporation	Fast generation of homozygous lines combining *C. annuum* L. and *C. chinense* Jacq traits. 17.5% were fertile and set seeds; fruit mass was 3 times larger than in *C. chinense*	[138]
Bell pepper, capia, charleston, and green types (*C. annuum*)	n	-	Ex vitro plants	5 g/L colchicine	2 h	Self-pollination was performed in at least 3–4 flowers. Three or four fruits were harvested from each self-pollinated plant, and their seeds were removed. Then, the seeds were labeled and packed.	Three homozygote pure lines with agronomically valuable traits, including Me1, Me3, N and Me7 nematode resistance genes, were obtained. Also, one homozygote pure line containing Me1 was found	[139]
Tomatillo (*Physalis ixocarpa*)	n	50% 2n, 50% 4n	Excised apical axillary buds from in vitro regenerants were inoculated on MS medium with colchicine	0.5, 1 g/L colchicine	2–6 days	MSD medium	–	[59]

Notes: * *A. cepa* 2n = 2x = 16; *A. vavilovii*, *A. nutans*, *A. schoenoprasum* are natural tetraploids 2n = 4x = 32. ** *B. rapa* 2n = 2x = 20, *B. oleracea* 2n = 2x = 18, *B. napus* 2n = 2x = 38, *B. juncea* 2n = 2x = 36. *** *B. oleracea* 2n = 2x = 18, *B. juncea* 2n = 2x = 36. **** the species is a natural tetraploid 2n = 4x: *S. nigrum* ssp. *villosum* 2n = 4x = 48; *Physalis peruviana* L. 2n = 4x = 48.

3.2. Antimitotic Agent Application Method

Various modifications of antimitotic agent treatment were used in the literature. Antimitotic agents can be added at different stages of plant development that can affect plant survival and the efficiency of genome doubling (Table 1). Antimitotic agents can be added to isolated microspores [78,82,94,101,140,141] and ovules [79,83,102,103] with subsequent transfer to antimitotic free medium. If doubling occurs at one of the first of the microspore mitotic divisions, mixoploids may not form [6,141]. However, antimitotic treatment can negatively affect microspore survival and normal development [4]. Regenerated embryoids [81,142,143] or entire plantlets [17,92,122,144], apices [91,122], or roots [41,91,113,116] of regenerated plants can be used for treatment too.

Alternatively, plants can be treated via the injection of antimitotic agents into the plantlet stems. However, this protocol is laborious and can lead to the formation of mixoploids, low survival, and seed production [41,140,145–147].

For polyploidy induction from diploid plants, the antimitotic treatment of seeds, especially pre-germinated seeds with emerging roots, can be the most effective way to double the genome [7,112,117].

3.3. Antimitotic Agent Supplementary Compounds

Antimitotic treatment efficiency can be improved with the addition of supplementary compounds. For instance, to increase the antimitotic compound solubility, stock solutions are prepared in different solvents. Trifluralin is dissolved in acetone, APM is dissolved in methanol, and oryzalin is dissolved in 1 M NaOH, 70% ethanol, or acetone. Colchicine can be dissolved in 96% ethanol, water, culture medium, or 2% dimethyl sulfoxide (DMSO) [1,148]. DMSO is used to increase cell permeability for antimitotic compounds. However, it also exerts a toxic effect on the cells. The application of colchicine with the addition of 2 or 4% DMSO decreased the survival rate compared to colchicine dissolved in water, but the doubling rate increased [144,149]. The addition of surfactants such as Tween 20, Teepol, or other detergents also increases cell penetration by antimitotic compounds [1,144,150].

Cotton balls, lanolin paste, glycerol, or agar can be used to localize the antimitotic solution [7,151]. Caffeine can be a promising supplement to reduce the number of albino regenerants, as shown in wheat. A 0.5 mM caffeine treatment for 24 h significantly increased the fraction of normal regenerants in two of the six spring wheat crosses [152]. Caffeine could be tested in other species, including vegetable crops, to study if it can improve survival or reduce the number of albino plants.

3.4. Antimitotic Agent Exposure Time

Antimitotic exposure time is one of the key factors determining the success of genome doubling. It depends on the cell cycle length and the accessibility of the compound. If the treatment is too short, only a small fraction of cells that enter cell division during the treatment will double their genome. As a result, no ploidy changes occur or mixoploid plants form. For better results, actively growing plants [6] and the sufficient treatment time to have most cells enter mitosis must be used. On the other hand, an excessive antimitotic treatment is toxic and leads to cell death [123]. Since the cell cycle time and sensitivity to the antimitotic compound depend on genotype and a number or external factors, the treatment time is determined empirically in different studies. The most commonly used exposure time range is 3–72 h (Table 1). After the treatment, the plant material is transferred to antimitotic-free medium, or the antimitotic is washed off from the roots or apices.

4. Chemically Induced Haploid Genome Doubling in Vegetable Crops

The successful genome doubling protocol depends on multiple factors and has to be adjusted for the species and genotype of interest (Figure 2). However, published data can be a good starting point for the experimental design (Table 1). Genome doubling protocols for vegetable crops are discussed below.

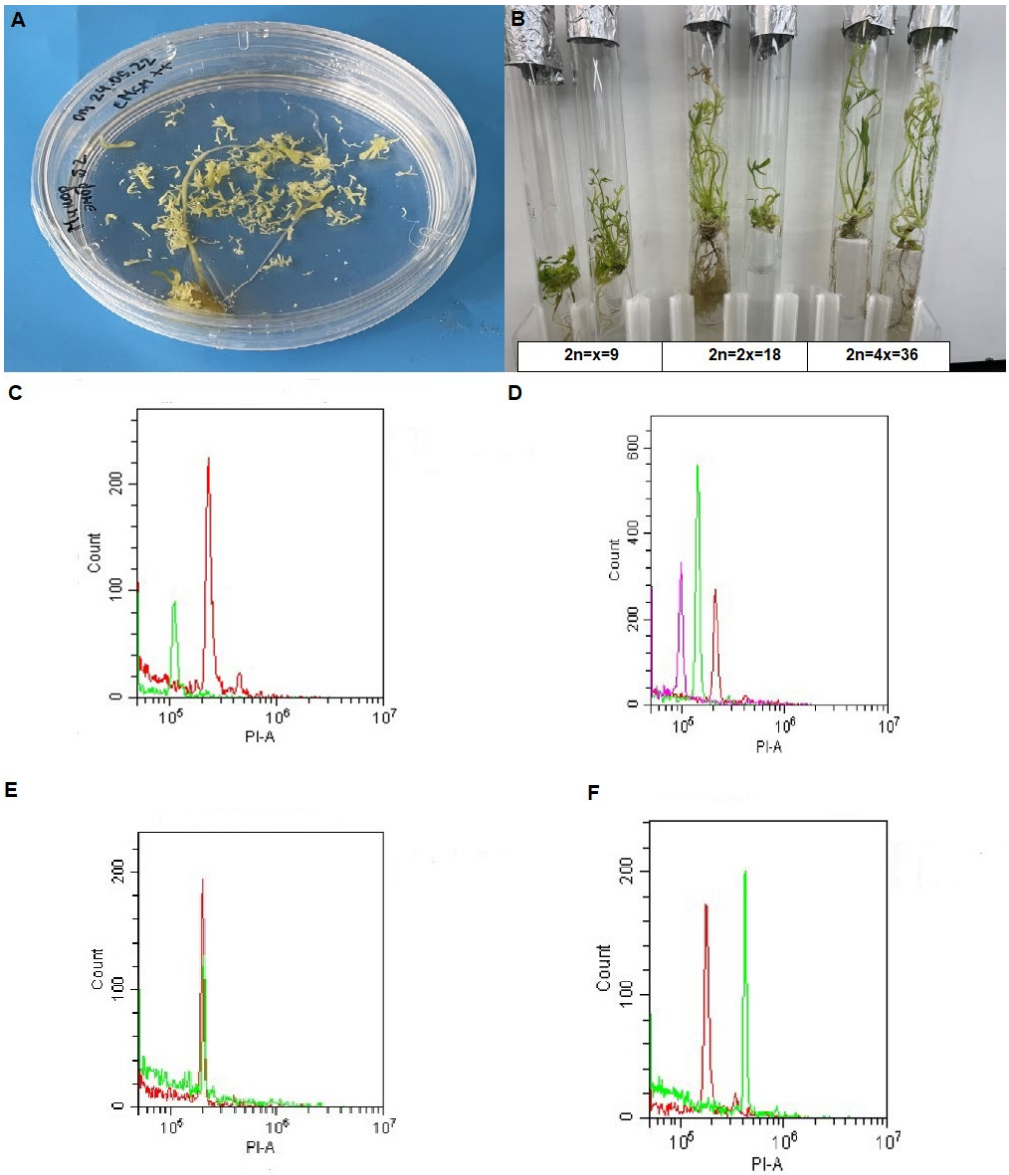

Figure 2. Carrot (*D. carota* L.) haploid regenerant artificial genome doubling with colchicine. (**A**) Carrot embryoids growing from microspores in liquid MSm medium. (**B**) Plants regenerated from embryoids were treated with 0.5 g/L colchicine for 48 h. Untreated haploid control tubes are shown on the left, the colchicine-treated plants that underwent one or two rounds of genome duplication are shown in the center and on the right, respectively. (**C**–**F**) The cytometric analysis of nuclei isolated from carrot leaves and stained with propidium iodide. The analyzed sample (green peak) was compared with a control diploid plant (red peak) or a control haploid plant (purple peak). (**C**,**D**) Haploid plants (**C**) were used for colchicine treatment; aneuploid plants (**D**) were discarded. (**E**,**F**) The colchicine-treated plants were analyzed by flow cytometry 6 weeks after treatment. Out of 8 treated plants, 2 (25%) became diploid (**E**) and 6 (75%) became tetraploid (**F**).

There are no data on the efficiency of red beet (*B. vulgaris* subsp. *vulgaris*) (the Amaranthaceae family) colchicine treatment. However, there are a number of papers on sugar beet (*B. vulgaris* subsp. *vulgaris* (var. *saccharifera*)) genome doubling. APM treatment yielded 64% genome doubling [103]. In other studies, treatment with different antimitotic compounds produced 2–4.7% doubling [79], 8.4% doubling [105], 19% [104], or doubling efficiency was not reported [16,74,83,102].

Low rates of spontaneous doubling in onion (*A. cepa* L.) (the Amaryllidaceae family) make an antimitotic treatment a necessary step. The application of an antimitotic agent

was attempted at different stages of development, including embryos [81,93], plantlets in vitro [34,86,90,92], shoot apices, and root tips [91]. The least laborious approach was the antimitotic treatment of embryos [153]. Trifluralin, oryzalin, and amiprofos-methyl (APM) were used successfully in many studies [34,81,86,90,92,93] (Table 1). Genome doubling efficiency was ranging from over 30% [81,86,90,93], 46% [91], 57.1%, and 65.7% [34] to 100% [92].

To date, there are few studies on the efficiency of induced chromosome doubling in the Apiaceae family. The most commonly used method for genome doubling in Apiaceae species is colchicine treatment (Table 1). For carrot (*D. carota* L.), a 0.5 g/L colchicine treatment was used for the in vitro culture of isolated microspores for 24 h [114] or 48 h [115]. In caraway (*Carum carvi*) [113], fennel (*Foeniculum vulgare*) and dill (*Anethum graveolens*) roots were treated with colchicine [116]. No data on the doubling efficiency were presented by the authors. In our preliminary studies, we treated eight carrot haploid plants produced from microspores with 0.5 g/L colchicine for 48 h (Figure 2A,B). Two plants (25%) became diploid, and six regenerants became tetraploid, as assessed by flow cytometry (Figure 2C–F).

Brassicaceae crops often have a high spontaneous doubling rate and do not need antimitotic treatment. If needed, antimitotic treatment can be applied. For instance, an over 50% doubling rate was observed when the haploid seedling roots of cabbage (*B. oleracea* var. *capitata*) and broccoli (*B. oleracea* var. *italica*) were treated with colchicine. However, colchicine negatively affected plant survival [41].

In *Cucurbita* spp., Kurtar (2018) doubled winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) haploid regenerants obtained in the anther culture using repeated 1% colchicine one-hour treatments of ex vitro shoot tips. The best reported efficiency was 93.3%. However, the actual data showing plant regeneration from microspores and ploidy measurement data were not shown [154]. Colchicine doubling protocols were tested in melon [121], pumpkin [122], and cucumber [120], but the doubling efficiency was not reported.

In Solanaceae crops, antimitotic treatment protocols were tested in a number of species. In pepper (*C. annuum* L.), the colchicine treatment of anther culture increased the doubling rate by 14.1–17.3% compared to SHGD. In addition, in the presence of colchicine, the embryo formation increased by 36.8% or 89.5%, depending on the media, compared to the control [55]. In another study, pepper (*C. annuum* L.) haploid plant treatment with colchicine led to a 25–27% incidence of diploids and 29–55% rate of mixoploids [136]. The obtained DH lines are successfully used for breeding. For instance, DH pepper (*C. annuum* L.) lines resistant to bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*) [155], *Verticillium dahliae* Kleb. [156], or carrying nematode resistance genes (Me1, Me3, N, and Me7) [139] were obtained. Elite homozygous sweet pepper [156,157], long sweet pepper [158], and four minipaprika [159] lines recommended for variety testing or new approved varieties with improved fruit qualities and productivity were created using DH technology.

In eggplant (*Solanum melongena* L.), an in vitro antimitotic treatment was more effective, yielding from 35% [107] to 100% plants with doubled genome [128,129]. In vitro treatment also saved time, allowing for earlier doubled haploid plant development and blooming [128]. The colchicine treatment of ex vitro plants in eggplant was also used in some studies. Axillary bud ex vitro treatment increased the doubling rate by 25% compared to SHGD in the untreated control [17]. In another study, eggplant ex vitro plant axillary buds were treated with colchicine in lanolin paste to minimize the evaporation of the antimitotic compound. A total of 50–70% of treated plants became diploid [128]. However, a similar treatment of tomatillo (*P. ixocarpa* Brot.) was considered unsuccessful. The best protocol was to excise the apical or axillary buds from the regenerants and place them on MS medium with 0.05 or 0.1% colchicine. The treatment yielded 50% diploids and 50% tetraploids [59].

5. Artificial Polyploidization for Breeding Purposes

Polyploidization is a common event in the evolutionary history of angiosperms. Polyploidy was documented in over 80% of plant species. It is attributed to 2–4% of angiosperm speciation [160]. The mechanisms of how polyploidy affects phenotype are not fully understood, but it is likely to be a multitude of factors, including a larger cell size, increased heterozygosity level, gene dosage, and new epigenetic and genetic interactions [7].

Polyploidy can give an evolutionary advantage to a species. It can also be useful for plant breeders since polyploidy can improve plant agronomic traits. Many domesticated crops, including durum wheat (*Triticum turgidum subsp. durum*), oat (*Avena sativa* L.), millet (*Panicum miliaceum*), potatoes (Solanum tuberosum L.), cotton (*Gossypium hirsutum*), and sweet potato (*Ipomoea* batatas), are polyploid organisms [160]. Polyploidy can occur naturally due to cell division failure or the fusion of unreduced gametes or via artificial polyploidization by antimitotic agents. Polyploidy can increase the organ size, useful substance content, tolerance to stressors, diseases, insects, and other traits [7].

5.1. Chemically Induced Polyploidization in Vegetable Crops

Polyploidization is often utilized in vegetable crops. Polyploidy induction in *Cucurbitaceae* crops plays a major role in obtaining varieties with superior qualities. Triploid hybrids are produced by crossing diploid and tetraploid watermelons. This method was first developed by Kihara in 1951. Triploid watermelons produce seedless fruits with an increased yield and other favorable traits [161]. The colchicine, oryzalin, or ethalfluralin treatment of watermelon in vitro shoots yielded over 60% tetraploids [123].

In other species, polyploidization is also attempted with the goal of improving the agricultural value. Colchicine treatment of Katokkon pepper (*C. annuum* L.) seeds produced 50% of mixoploid plants [162]. Genome doubling in Apiaceae species was performed with the goal of increasing the size and improving the essential oil content. Parsley (*Petroselinum crispum* L.) [117] and ajowan (*Trachyspermum ammi*) [112] treatment with colchicine produced 100% and 11.53% tetraploids, respectively. Tetraploid parsley and ajowan had larger plant size (1.4 and 2 times larger, respectively). The stomata size was increased, while the stomata density decreased in both studies [112,117]. Tetraploid ajowan had a 39% larger thymol content in essential oil [112].

Garlic and shallot are propagated vegetatively; therefore, breeders have a high need to increase the genetic diversity of these crops. One of the approaches that is being taken into account is the production of tetraploids. Persian shallot (*Allium hirtifolium*) tetraploids produced with colchicine or oryzalin treatment had increased the total phenolic compound and allicin content by 27 and 15%, respectively [111]. Garlic (*Allium sativum* L.) tetraploid plants obtained by colchicine treatment had a three times larger leaf area, and their allicin content increased by 30% [106].

5.2. Chemically Induced Polyploidization in Interspecific Hybrids

Chromosome doubling is also used to restore the fertility of interspecific hybrids. Interspecific hybridization is widely used for the introgression of valuable traits, including resistance to diseases, herbicides, salinity, extreme temperature, and others. Interspecific hybrids are obtained by embryo rescue or natural seed setting [19]. However, most interspecific hybrids are sterile, and overcoming their sterility can be a non-trivial task. However, this is a necessary step to reproduce hybrid forms and perform backcrossing. One of the approaches to overcome sterility is genome doubling [163]. For example, colchicine treatment was used to restore fertility in Japanese bunching onion (*Allium fistulosum*) × onion (*A. cepa* L.) interspecific hybrids [110,164]. The genome doubling in *Allium cepa* × *A. fistulosum* hybrids was necessary for those plants to produce seeds. Plants grown from those seeds demonstrated robust shoots and inflorescences that were larger than their parent plants. They tolerated adverse environmental conditions well. They also produced 1.5 times more green mass that contained more sugars and vitamin C than *A. fistulosum* samples [110].

Interspecific onion hybrids (*A. cepa* L. (2n = 2x = 16) × *A. vavilovii* (2n = 4x = 32), *A. cepa* L. (2n = 2x = 16) × *A. nutans* (2n = 4x = 32), and *A. cepa* L. (2n = 2x = 16) × *A. schoenoprasum* (2n = 4x = 32)) meristems were treated with 0.01 g/L colchicine, which recovered fertility in over 60% of hybrid onion plants. By doubling the chromosome set of triploid forms (2n = 3x = 24), hexaploids (2n = 6x = 48) with fertile pollen were obtained. Hexaploid plants *A. cepa* L.× *A. nutans* (6x) were subsequently used as a mother plant for crossing with *A. cepa* L. (2x) to obtain fertile tetraploid interspecific hybrids. The resulting hybrid forms were perennial, wintered well, had an increased vegetative mass, and were resistant to downy mildew [110]. A similar approach was utilized in Brassicaceae crops. , crosses were treated with colchicine by soaking the axillary meristems, and resulting plants with a doubled genome were able to set seeds in contrast to the original plants. The best outcome was observed for the 1.5–2 g/L colchicine treatment. As a result, allohexaploid (AABBCC) crosses that do not exist in nature were obtained. These plants can be useful for combining valuable traits from A, B, and C *Brassica* genomes [118]. In another study, Chinese cabbage (*B. rapa* subsp. *pekinensis*) × white cabbage (*B. oleracea* var. *capitata*) and rapeseed (*B. napus*)/leaf mustard (*B. juncea*) × Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) crosses grown in vitro were submerged in 0.1 g/L colchicine for 4 h. The treated plants were fertile and were used for subsequent backcrossing. This study allowed for plants with new traits to be produced; the white cabbage with orange/yellow inner leaves trait (probably due to increased β-carotene levels) was transferred from Chinese cabbage. Also, Chinese cabbage with increased anthocyanin content (4.7 mg/g) was obtained by interspecific hybridization with rapeseed or leaf mustard [119].

Interspecific hybridization in *Solanaceae* also allows for the transfer of valuable traits. For instance, *C. annuum* L. × *C. chinense* crosses allowed for the transfer of the Tomato spotted wilt virus (TSWV) resistance gene from *C. chinense.* In total, 40 DH regenerants were obtained from both donor plants. F2 *C. annuum* L. (variety Zdorovye) × *C. chinense* and BC2 *C. annuum* L. (variety Zdorovye) × *C. chinense* × Zdorovye × Zdorovye were generated. The colchicine treatment recovered fertility. The fruit shape was intermediate between the two species. The weight of fruits in plants obtained from F2 *C. annuum* L. (Zdorovye) × C. chinense was three times larger than in the parental form of *C. chinense* and almost two times smaller than the fruits of the parental form of *C. annuum* L. (Zdorovye). Plants obtained from BC2 *C. annuum* L. (Zdorovye) × C. chinense × Zdorovye × Zdorovye had fruits with a weight that was equal or slightly inferior to the fruits of the Zdorovye variety [138].

Altogether, polyploidization can be a useful tool to tackle a list of problems faced by a breeder, including improving cultivar characteristics and overcoming interspecific hybrid sterility.

6. Ploidy Determination Methods

The analysis of ploidy is essential for genome doubling experiments and for the determination of spontaneously doubled haploid regenerants. Plants with different ploidy often differ in terms of size and morphology, as well as the development of reproductive organs. Haploid plants usually have smaller flowers, abnormal ovary development, irregular and uneven anther development, as well as smaller leaves. Seed setting is used as a marker to distinguish DHs from haploid plants since the latter ones are mostly sterile [4].

The most accurate ploidy determination methods are chromosome counting and the flow cytometry of isolated and stained nuclei since they allow for a direct genome size assessment.

The flow cytometry of cell nuclei is one of the best options for ploidy assessment since it allows for the fast and precise examination of samples. Tens and even hundreds of samples per day can be tested, which is advantageous for large-scale breeding programs. Also, this method allows for ploidy determination at any stage of plant development in vitro and ex vitro. Flow cytometry is the only method that provides information about thousands of cells in the sample, allowing for mixoploid plant documentation [165].

For flow cytometric analysis, plant tissue (mostly leaves) is minced with a razor blade. The releasing nuclei are filtered to remove debris. Then, they are stained with a DNA-specific fluorescent dye, such as propidium iodide. The cytometer detects the fluorescence brightness of the nuclei. Since the dye stoichiometrically binds DNA, it allows for the differences in the DNA content between the standard and the analyzed sample to be distinguished (Figure 1G–G″, Figure 2C–F). Although rapid processing samples have made flow cytometry the most efficient approach for determining the ploidy, its use in many laboratories is still limited due to the high cost of equipment and challenges in mastering quality sample preparation and instrument management [165,166].

The classical method of ploidy determination is chromosome counting in stained cytological preparations (Figure 1I). The root tips or developing flower buds are fixed, macerated with enzymes, and stained with DNA dyes for chromosome visualization. Direct chromosome counting is a very reliable method for ploidy determination, but this method is extremely time-consuming and technically challenging [166,167].

Also, the number of stomata guard cells in the field of view, the size of the stomata, and the number of chloroplasts in the guard cells of the stomata can be used to distinguish plants with different ploidy. The smaller the number of stomata, the larger the stomata, and the larger the number of chloroplasts in stomatal guard cells, the more they are associated with higher ploidy (Figure 1H–H″). This method is widely used since it is fast and inexpensive, but it can be applied only to ex vitro plants raised under the same conditions [166,167].

7. Conclusions

Increasing plant ploidy is a critical step for many biotechnological methods used for new breeding material production. In this review, we discussed genome doubling in vegetable crops for different applications, including DH technologies, obtaining polyploids and overcoming interspecific hybrid sterility.

Vegetable crops are a challenging subject for DH technologies. During DH production, microspores or ovules switch their development to sporophytic growth. At the second step, genome doubling is induced in haploid regenerants. Vegetable crops often pose difficulties at one or both stages of DH production. Brassicaceae crops can produce a largely varying number of haploid embryoids depending on a genotype, but SHGD is very common for them [41]. As a result, spontaneously doubled regenerants from responsive genotypes can be included in breeding programs. In Apiaceae, Brassicaceae, Cucurbitaceae, and Solanaceae crops, one or both stages of DH technologies are still challenging [9,17,113,116,122]. Improving both stages would allow for the implementation of DH technologies in vegetable crop breeding on a routine basis.

Multiple factors have to be adjusted to maximize the genome doubling rate. The published protocols can be used as a basis for experimental design, but different treatment regimens should be tested for the best results. The antimitotic agent and supplementary compound choice and concentration, exposure time, and the method of application should be tested on a small population of the plants of interest or on plants that are readily available. For instance, plants from a similar cultivar grown from seeds or micropropagated in vitro can be used. If no doubling occurs, the increased antimitotic agent concentration, exposure time, or a different application method can be tested. If mixoploidy or higher-than-needed ploidy is achieved, the plants can be unusable for future breeding purposes. To avoid this, the antimitotic agent concentration or exposure time should be adjusted. Excessive concentrations of antimitotic agents or DMSO can lead to plant death. The genotype, the quality of the reagents, and the researcher technique can also affect the genome doubling results. When the best regimen is found, it can be applied to the limited breeding material.

Unfortunately, many published DH protocols do not report the antimitotic agent application details and the efficiency of the treatment (Table 1). Interspecific hybrid genome doubling protocols are also often omitted or not covered in full. The assessment of the chemically induced genome doubling efficiency can be complicated by spontaneously doubled plants. The best design for experiments targeted to determine the best antimi-

totic doubling protocols should include an untreated control, but this is often lacking in published studies. In addition, the number of treated plants is often insufficient for the protocol efficiency statistical assessment or comparison with other treatment regimens. This can happen because researchers often have a practical goal of obtaining plants with a doubled genome from a limited amount of breeding material. Well-designed genome doubling experiments would provide valuable information for other researchers for the use and further development of genome doubling protocols.

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