Oat (*Avena sativa* L.) In Vitro Cultures: Prospects and Challenges for Breeding

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Abstract: Plant in vitro cultures have been a crucial component of efforts to enhance crops and advance plant biotechnology. Traditional plant breeding is a time-consuming process that, depending on the crop, might take up to 25 years before an improved cultivar is available to farmers. This is a problematic technique since both beneficial qualities (such as pest resistance) and negative ones (such as decreased yield) can be passed down from generation to generation. In vitro cultures provide various advantages over traditional methods, including the capacity to add desirable characteristics and speed up the development of new cultivars. When it comes to oat (*Avena sativa* L.), the efficient method of plant regeneration is still missing compared to the most common cereals, possibly because this cereal is known to be recalcitrant to in vitro culture. In this review, an effort has been made to provide a succinct overview of the various in vitro techniques utilized or potentially involved in the breeding of oat. The present work aims to summarize the crucial methods of *A. sativa* L. cultivation under tissue culture conditions with a focus on the progress that has been made in biotechnological techniques that are used in the breeding of this species.

Keywords: androgenesis; callus; doubled haploids; embryogenesis; organogenesis; wide crossing

1. Introduction

In crop breeding practice, the development of new cereal cultivars takes from several to several dozen years and is mainly based on generating plants with a high degree of homozygosity through inbreeding crosses, followed by the selection of individuals with desirable traits. The use of biotechnological methods allows us to shorten this procedure by up to several years and involves the obtaining of haploid plants, followed by the generation of doubled haploid (DH) lines through in vitro culture methods. The obtained homozygous lines guarantee that subsequent generations will be genetically and phenotypically identical. Therefore, they are increasingly utilized in breeding programs. It is also worth noting that DH lines have a larger percentage of plants with breeders' target genes than the F_2 generation and subsequent generations acquired using conventional techniques [1]. Additionally, DH lines find applications in studies involving molecular markers by accelerating the derivation of mapping populations and genetic transformations, estimating recombination fractions, and detecting recessive mutants. They are also an effective means of genetic enrichment in plants, introducing more favorable alleles into the genome, i.e., those that carry traits such as resistance to biotic and abiotic stresses [2].

More than 20 species belonging to the genus *Avena* L. exist at the diploid, tetraploid, and hexaploid ploidy levels. *Avena sativa* L., the most widely cultivated plant, is a hexaploid (2n = 6x = 42), having three genomes: AA, CC, and DD. Common oat is grown worldwide in agricultural regions with a temperate climate, and its grains are primarily used for feed and food production [3]. Despite having lesser economic and commercial significance

compared to other grains, the scientifically proven health benefits of oat grains make it an interesting subject of both breeding and genetic research. However, using standard research methods, the large size and complexity of the oat genome are significant limitations, which has led to limited advancements in oat research [4].

Haploids are plants in the sporophytic development stage, but with the gametic number of chromosomes (n). They are produced without the involvement of fertilization by male gametophyte cells from in vitro cultures of anthers or isolated microspores (androgenesis) or via female gametophytic cells from in vitro cultures of ovaries or ovules (gynogenesis). Both methods involve the action of various factors leading to the reprogramming of the developmental pathway of haploid male and female cells, resulting in the formation of haploid androgenic or gynogenic embryos rather than gametes. Methods based on gynogenesis also include wide crosses, or wide hybridization, involving the forced pollination of plants belonging to different species or genera. The ground-breaking work on cereal haploidization was performed by Kasha and Kao [5], who pollinated *Hordeum vulgare* L. with the pollen of the wild species *Hordeum bulbosum* L. This technique, known as the "bulbosum method", proved to be highly efficient and found application in generating haploids not only of barley or common wheat but also of other plant species. Currently, the most common pollen donor in wide crosses of cereals is maize (*Zea mays* L.), followed by pearl millet (*Pennisetum glaucum* (L.) R. Br.), sorghum (*Sorghum bicolor* (L.) Moench), Job's tears (*Coix lacryma-jobi* L.), and cogon grass (*Imperata cylindrica*) [6]. These crosses result in hybrid embryos of wheat, oats, triticale, or barley, in which paternal chromosomes are eliminated during successive nuclear divisions, resulting in haploid embryos containing only maternal genetic material. Sometimes, however, elimination does not occur properly, and whole or fragments of donor chromosomes are incorporated into the recipient's genome. This most commonly occurs in crosses between plants belonging to the two subfamilies, *Pooideae* and *Panicoideae*, within the family Poaceae. Additional chromosomes from maize or pearl millet have been observed in both wheat haploids [7,8] and oat haploids [9]. Investigating the causes of this phenomenon, Mochida et al. [10] found incomplete attachment of the spindle apparatus to maize centromeres, while Ishii et al. [11] reported chromosome breaks in pearl millet. The presence of stable maize chromosomes in the oat genome was first described by Riera-Lizarazu et al. [8], and they were referred to as oat × maize addition (OMA) lines by Kynast et al. [12]. Since then, these OMAs have been used for physical mapping of the maize genome [13], studies on CENH3 centromere-specific histones [14], or gene expression analysis in the C_4 photosynthetic pathway [15].

The use of oat in vitro techniques still faces difficulties, even though many of the methodological issues have been resolved. Moreover, there is a lack of knowledge regarding the mechanism of oat haploid induction. This review paper focuses on oat in vitro organogenesis, embryogenesis, and haploidization via anther and microspores cultures, via wide crossing (chromosome elimination), and via the modification of centromere specific histone CENH3. The paper points out the recent advances in oat in vitro cultures which might be successfully incorporated in this crop breeding.

2. Callus Culture, Organogenesis, Somatic Embryogenesis, and Cell Suspension of Oat (*Avena sativa* L.)

The development of in vitro regeneration techniques is crucial for improving cereals biotechnologically. Like other Poaceae species, hexaploid oat (*Avena sativa* L.) can be regenerated from tissue culture via either organogenesis or somatic embryogenesis. Limited reports have been published on the development of effective plant regeneration systems from various tissues and organs in oat compared to the most common cereals, e.g., maize, rice, wheat, and barley.

2.1. Effect of Explant on Callus Production

Oat callus cultures might be induced from seeds, immature embryos, germination-stage seedling roots, and germination-stage embryo hypocotyls, as first described by Carter

et al. [16]. Nine years later, Lörz et al. [17] reported successful plant regeneration from non-friable calluses that had structured, green primordia. In the same year, Cummings et al. [18] used germinating immature embryos from 25 oat genotypes as explants for initiation of callus cultures on B5 [19] or MS [20] media containing from 0.5 to 3.0 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D). These cultures were maintained by subculturing to B5 medium with 1 mg L^{-1} 2,4-D every 4 to 6 weeks. The callus from the cultivar "Lodi" has been maintained for the longest time and retained the regeneration ability through 18 months. Maddock [21] then notes that morphogenetic oat callus does not appear to develop from the scutellum, which becomes necrotic, especially when older embryos are cultivated, but rather from the entire embryo or the mesocotyl area.

The very efficient regeneration method from leaf tissue of six different oat cultivars named "Coolabah", "Cooba", "Blackbutt", "Mortlock", "Victorgrain", and "HVR" was reported by Chen et al. [22]. Callus was produced using leaf base segments from seedlings on MS medium with 2 mg L^{-1} 2,4-D; moreover, explants grown in the light or the dark responded to callus induction similarly. The two-to-five-day old seedlings and the callus from the first leaf segment demonstrated a comparatively high potential for regeneration. Therefore, seedling age must be considered as a key variable for in vitro regeneration from leaf explants in oat. Calluses had been proliferating for more than eight months without substantial reductions in regeneration capacity.

Shoot apical meristems of *A. sativa* were also used to establish an effective microprop-agation method [23]. Explants were obtained from aseptically germinated oat seedlings and cultivated in vitro. After five weeks in culture on MS medium with various combinations of 2,4-D and N^6-benzyladenine (BA), the enlarged apical meristems and multiple adventitious shoots were produced. All tested oat cultivars formed seedlings at a high efficiency and fertile oat plants were produced. These in vitro multiplied shoots might serve as an alternate tissue of selection for oat genetic transformation. Cummings et al. [18] also obtained calluses from apical meristems capable of plant regeneration on B5 medium containing 2,4-D.

Nuutila et al. [24] enhanced the regeneration efficiency from oat leaf base cultures by altering the nitrogen composition and the concentrations of the sugar and auxins in the culture medium. The effectiveness of inducing embryogenic callus and plant growth was studied on MS and L3 [25] media. The callus-producing abilities of leaf base segments 1 through 6 were compared. Concerning all cultivars, the first three leaf base segments produced embryogenic callus, but segments 4–6 either produced very little or none. For both tested cultivars, "Aslak" and "Veli", the L3 medium turned out to be more efficient and produced more embryogenic calluses and plants, compared to the MS medium. Lower concentrations of ammonium (4.9 mM) and nitrate (29 mM) and high organic nitrogen (11 mM) in medium caused the highest regeneration of green plants in "Aslak". On the other hand, high ammonium (20.1 mM), high nitrate (46.8 mM), and low organic nitrogen (0.9 mM) concentrations resulted in the greatest green plant regeneration in "Veli". This study has additionally demonstrated that both sugar and auxins have a definite impact on the induction of embryogenesis. For oat leaf base in vitro culture, sucrose and maltose have been investigated as carbohydrate sources [22,26].

Establishing tissue cultures in oat has frequently run into complications because of the strong dependency of the donor plant cultivar. The variations in cultivars' susceptibilities to genetic programming and the external reprogramming of embryogenically capable cells might be the cause of the discrepancies. Although it may be reasonable, from the practical point of view, to choose a medium in routine systems that elicits an average response from the majority of cultivars, these responses do not always reflect how well or badly the cultivars generally respond in generally. This genotypic dependency was described by Cummings et al. [18]. In their investigation, from 25 tested genotypes, 2 failed to initiate calluses, and 9 of them were able to produce callus but had no regeneration ability. Next, Rines and McCoy [27] noted that the frequency of callus development varied between oat cultivars and ranged from 5% to 75%. Studies of Chen et al. [22] showed that it is possible to

obtain the callus form of all tested genotypes in 100% or almost 100% frequency. However, the rate of regenerable callus formation obtained from leaf base segments was substantially higher in mature embryos [18,27]. Because they are less genotype dependent, leaf explants appear to be more appropriate donor materials to produce regenerable oat callus cultures.

2.2. Factors Affecting Organogenesis and Somatic Embryogenesis

In comparison to callus cultures only capable of organogenesis, those capable of somatic embryogenesis are more likely to show fast growth rates and very high levels of plant regeneration. Due to these, the production of friable and embryogenic callus has received most of the attention in the quest to create tissue cultures of monocotyledonous plants. As with all cereals, immature tissues must be used to initiate oat in vitro cultures since these differentiated tissues are typically unable to induce cell division and proliferation. Two different callus types, some of which can regenerate plants, are regularly generated during indirect somatic embryogenesis. Typically, the embryogenic callus is friable, compact, and yellowish-white, and this non-embryogenic is rough-looking, moist, non-friable, and transparent [28].

The formation of an embryogenic oat callus is reliant on the source of the explant, its physiological stage, genotype, and the composition of the culture initiation medium. Most of the work on this topic has utilized immature zygotic embryos as explants to initiate embryogenic oat cultures [21,27,29–32], cultured on MS medium and supplemented mostly with 2,4-D in different concentrations. Other explants exhibiting callus formation include mature seeds [16,17] and mesocotyl of germinated seedlings [30]. Embryogenic calluses from immature and mature embryos [33,34] and leaf segments [26] have also been used for gene transfer.

Avena sativa L., *Avena sterilis* L., and *Avena fatua* L. are three hexaploid oat species from which tissue cultures were started and plants were regenerated [27]. Immature embryos were used to start a variety of tissue cultures, with regenerable-type cultures distinguished by the presence of organized chlorophyllous primordia in a compact, yellowish-white, strongly lobed callus. The frequency of regenerable-type cultures was determined by the embryo size, species, genotype, growing conditions of the donor plants, and 2,4-D concentrations in the culture induction media. The highest rates of regenerable-type of cultures were consistently produced by the "Lodi" cultivar and 2 "Lodi"-related lines out of the 23 investigated *A. sativa* cultivars. For "Lodi", this frequency reached 80% in one test. Only 3 of the 16 *A. sterilis* lines produced regenerable-type cultures, but more than 20%. In 7 out of the 32 investigated *A. fatua* lines, regenerable-type cultures were generated at rates higher than 45%. The tissue cultures of all three species could regenerate plants after 9–10 subcultures and more than a year in in vitro culture.

Since genotype affects culture initiation frequency and culture type, genotype screening and selection, as well as the developmental stage of embryos, ought to be a successful strategy for enhancing oats' capacity for cell culture. The aim of King et al.'s [35] investigation was to determine the optimum size of immature embryos of 10 oat cultivars for callus induction and plant regeneration under in vitro culture. Plant regeneration was assessed after three months of culturing one hundred immature embryos on MS medium with different 2,4-D concentrations. No differences between cultivars were observed in the amount of callus produced, but the rate of regeneration from the different cultivars extended from 3 to 42%, suggesting that there are genotypic differences in the ability to regenerate plants from calluses. Scanning electron microscopy and light microscopy were used by Bregitzer et al. [30,31] to characterize the stages of development of somatic embryos in friable embryogenic callus. Following Bregitzer et al. [30], the cultivation of non-friable oat calluses produced from immature embryos on MS medium containing 20 mg L^{-1} sucrose and no hormones led to the production of separate somatic embryos that hatched into full-grown plants. During repeated culturing on a modified MS medium containing 2 mg L^{-1} 2,4-D, and 20 g L^{-1} sucrose, embryogenic sectors separated from non-friable calluses were visually selected to create friable callus. The maturation of somatic embryos was

stimulated by transferring friable calluses to a modified MS medium containing 60 g L^{-1} sucrose and no hormones. Some of these embryos were able to germinate after being transferred from this friable callus to a modified MS medium that included 20 g L^{-1} sucrose and no hormones. According to Bregitzer et al. [30], the culture of non-friable oat calluses derived from immature embryos on MS medium containing 20 mg L^{-1} sucrose and no hormones resulted in the development of distinct somatic embryos that germinated to form complete plants. Embryogenic sectors isolated from non-friable calluses were visually selected during repeated subcultures on a modified MS medium containing 2 mg L^{-1}, 2,4-D and 20 g L^{-1} sucrose to produce friable callus. After the development of friable calluses, plants continued to grow from those callus lines for more than 78 weeks. Additionally, immature embryos of three genotypes and seedling mesocotyls of two genotypes were used to directly generate friable embryogenic callus. There was also evidence of genotypic heterogeneity in this reaction. For the first time, calluses produced from oat seed developed root primordia, and the meristems of these primordia were sites of somatic embryo production, according to Chen et al.'s study [36]. The callus that was kept on MS medium with 1, 2, or 4 mg L^{-1} 2,4-D with underlying root or shoot parts was transferred to a new supply of the same medium after one month of culture. The callus induction frequency among the five cultivars studied was 93% for cv. "Risto", and 76% for cvs. 'Victory', 'Sang', 'Sanna' and 'Vital' respectively. Between 30 and 200 mg per seed were produced as fresh weight of each callus. The proliferating callus cells caused the seedling roots to swell during callus induction. Epidermal and cortical cells of the roots tended to be expelled and fall off. Differentiated pericycle cells became meristematic. When these roots were sectioned longitudinally or transversely, numerous lateral root primordia were seen originating from the pericycle cells along the vascular strand. Additionally, callus induction took place in the shoot's basal regions. Meristematic cells and solitary xylem cells developed from parenchymatic cells. The meristematic cells could directly generate root primordia and contained noticeable nuclei. After callusing in the vicinity of the shoot bases, groups of root primordia were also inadvertently generated. On various media, somatic embryos connected to underlying callus cells grew. The MS medium that proved best for germination contained 6% sucrose and 0.01 mg L^{-1} of abscisic acid (ABA). Single plantlets or clumps with 2–5 mm green leaves, with or without roots, were produced after 30–40 days on this medium. Ten-month-old embryogenic tissue may produce 200 shoots or plantlets per gram. After being transferred to media devoid of hormones, plantlets grew stronger and had more developed roots. In the soil, more than 90% of the green plantlets survived and matured. More than 30 months have passed since the preservation of embryogenic tissue.

In the next study, young seedlings of five oat cultivars—"Fuchs", "Jumbo", "Gramena", "Bonus", and "Alfred"—were tested for their regenerative abilities [26]. Two different basal media—MS medium and L3 medium [25]—were enriched with phytohormones in different concentrations for the callus induction, shoot proliferation, and regeneration of plants. Four-week-old culture-produced calluses were transferred to induction medium, and one week later, somatic embryos began to germinate. To develop further, shoots were placed in hormone-free media, and developed plants were morphologically healthy and fertile. From the base of the oat leaves, a callus was induced on all the tested media. However, certain phytohormones had better effects on plant regeneration. The highest regeneration frequencies were attained on media with 2.5 mg L^{-1} 2,4-D. In five oat genotypes, 25 plants on average could be grown per explant, and for the most responsive Jumbo, more than 50 regenerants could be produced per explant. Hence, the oat leaf bases are very promising as primary explants for micropropagation due to their strong capacity for regeneration.

To examine the effects of polyamines on somatic embryogenesis and plant regeneration oat genotypes Tibor (*Avena nuda* L. with low regeneration factor), GP-1 (*Avena sativa* L. with high regeneration factor) and their crosses, GP-1 × Tibor and Tibor × GP-1, were grown in in vitro cultures [37]. Somatic embryos were produced in large amounts from mature embryos of Tibor and Tibor × GP-1 on MS medium supplemented with 2.0 mg L^{-1}

2,4-D and 0.5 mM putrescin. Putrescin treatments induced plant regeneration from other genotypes in most cases, compared with the results of Somers et al. [33] obtained with the same regeneration media. This suggests that media enriched with putrescin can be used to screen other oat lines for regeneration efficiency. Moreover, the shoot proliferation medium containing low concentration of putrescin induced significant numbers of plants from usually recalcitrant cultivars.

Bregitzer et al. [30] showed that immature embryos gave the highest and most repeatable callus initiation frequency; however, it was shown that, generally, the frequency of embryogenic callus initiation of some of the elite germplasm lines is still quite low [31]. A significant contribution to the formation of embryogenic calluses in oat and the subsequent demonstration of plant regeneration via somatic embryogenesis, as well as variables controlling plant regeneration, were reported by Somers et al. [33]. This paper includes the methods used to manipulate oat cells and tissues in tissue culture, the constraints on their usage, and both planned and actual uses for improving oats. The genotype of the oats, the explant utilized to start the development process, and the tissue choice made during subculture can all affect the oat callus structure and its ability to regenerate. However, according to these authors, by seeing and picking out extremely transparent to opaque, compact, highly lobed tissues within early cultures, oat cultures with long-term preservation of plant regeneration potential can be produced. Borji et al. [38] used mature caryopses as initial material for somatic embryogenesis from oat cultivar "Meliane". Longitudinal sections of caryopses were plated on MS medium supplemented with 3 mg L^{-1} 2,4-D. Primary calluses were removed from explants after four weeks of growth and placed into the proliferation medium (MS medium with 1.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} 6-Benzylaminopurine (BAP)). For germination, somatic embryos were transferred to MS medium without plant growth regulators and then to MS medium containing 0.5 mg L^{-1} indole-3-acetic acid (IAA) to promote root system. The regenerated seedlings were acclimated to ex vitro conditions and were grown until maturity in a greenhouse.

2.3. Oat (Avena sativa L.) Cell Suspension Culture

The experiment by Gana et al. [39], among others, set out to evaluate the relative adaptability and plant regeneration of four oat genotypes in suspension cultures and to examine plant regeneration in 19 genotypes from three different oat species in three successive callus subcultures. Highly significant differences were found between 19 Avena genotypes for callus initiation, germination, and rhizogenesis in this study, in which the ability of "88Ab3073" to regenerate plants in suspension culture and the highly regenerable capacity of "GAF/Park" in both agar and suspension culture systems were also described. A highly significant genotype impact (32.1% variation), genotype subculture interactions (9.9% variance), and a non-significant subculture effect (0.3% variance) were all seen in the analysis of variance for plant numbers for genotypes in three subcultures. Genotypes with the highest callus production were selected to initiate liquid cultures. Two-month-old calluses from "GAF/Park", "Tibor", "88Ab3073", and "87Ab5932" genotypes were used for the suspension culture initiation. To better assess the regeneration potential of small and big cell clusters, the suspensions were divided into fractions of <3 mm or >3 mm. The "GAF/Park" and "88Ab3073" clusters that were 3 mm and bigger generated yellow friable callus. Clusters of "87Ab5932" developed slowly and finally ceased to multiply, whereas "Tibor" clusters began to form a rhizomorphic callus, a propensity that was also seen in its suspension cultures. Within two weeks, clusters of "GAF/Park" had quadrupled in size on the solidified medium. Plant regeneration from clusters larger than 3 mm was observed after three weeks in three of the four tested genotypes. Additionally, on the "GAF/Park" callus, many globular somatic embryos were observed. A total of 42 plants were regenerated from suspension clusters larger than 3 mm, and 50 plants were derived from the agar-based callus culture.

Wise et al. [40] used suspension cultures of *Avena sativa* L. cv. "Belle" for the biosynthesis of avenanthramides. Calluses of oat were initiated from the shoot apical meristem on

solid MS media in dark conditions. Liquid cultures were established from 1.0 g callus and 25 mL of MS media containing 2 mg L^{-1} 2,4-D. To stimulate avenanthramides production, chitin (poly-N-acetyl glucosamine) was added as elicitor, and two unique callus phenotypes, named "aggregate" and "friable", were identified. The more brittle aggregate tissue easily shed off and was easily separated from the friable tissue, which remained evenly scattered in the culture medium. Because the suspension cultures produced relatively large quantities of avenanthramides, these results point to the potential of oat suspension culture as a tool for future in-depth research into the processes that initiate their production, as well as the variables that determine the specific kinds of avenanthramides that are produced.

The summary of research on *A. sativa* L. callus induction, organogenesis, and somatic embryogenesis with the improved biotechnological potential of named species is presented in Table 1.

Table 1. Callus culture, organogenesis, and somatic embryogenesis of oat (*Avena sativa* L.).

A. sativa L. Genotype	Explant Used	Media/PGRs	Experimental Outcomes	References
cv. "Lodi", cv. "Moore", cv. "Lyon", cv. "Benson", cv. "Marathon", cv. "Dal", cv. "Stout", cv. "Tippecanoe", cv. "Lang", cv. "Victorgrain", cv. "Garry", cv. "Hudson", cv. "Terra", cv. "0A338", cv. "Victory", cv. "Black", cv. "Mesdag", cv. "Victoria", cv. "Selma", cv. "AJ10915", cv. "NP3/4", cv. "Karin", cv. "Rallus", cv. "Coolabah"	immature embryos	MS/B5 medium with 2 mg L^{-1} 2,4-D for initiation; MS/B5 medium with 1.0, 2.0 and 5.0 mg L^{-1} 2,4-D for embryo regeneration	Tissue cultures capable of plant regeneration after more than 12 months in culture	[27]
cv. "Victorgrain", cv. "Victoria" GAF (*A. sativa* L. cv. "Garland" × *A. fatua* L.) × *A. sativa* L. cv. "Victoria"	10- to 12-days old embryos	MS medium with 2 mg L^{-1} 2,4-D; MS medium with 1 mg L^{-1} 2,4-D and 5 units mL^{-1} victorin; MS medium with 2 mg L^{-1} NAA, and 0.2 mg L^{-1} BAP for regeneration	12 of 65 immature embryos of the cv. "Victorgrain" and 2 of 21 embryos of cv. "Victoria" developed regenerable callus; without tissue growth or survival on a victorin-containing medium	[29]
cv. "Trafalgar", cv. "Rollo", cv. "07408 in 111/2", cv. "Rhiannon", cv. "Dula", cv. "Avalanche", cv. "Caron", cv. "Pennal", cv. "Cabanna", cv. "Margam"	embryos	MS medium with 2 mg L^{-1} 2,4-D for callus initiation and growth; MS medium with 0.5 mg L^{-1} 2,4-D followed by PGRs free MS medium for regeneration	The highest level of regeneration from 4–4.5 mm long embryos with the genotyping differences of plant regeneration	[35]
GAF (*A. sativa* L. cv. "Garland" × *A. fatua* L.) × *A. sativa* L. cv. "Victoria" lines GAF-18, GAF-30, GAF-30, GAF-30/"Park" and GAF-30/Park//GAF-30	immature embryos	MS medium with 4 mg L^{-1} 2,4-D for callus initiation; MS medium with 2 mg L^{-1} 2,4-D for callus maintenance; MS medium with 2 mg L^{-1} NAA and 0.2 mg L^{-1} BAP for shoot differentiation; MS medium free of PGRs for rooting	Embryogenic cultures maintained the ability to regenerate plants for more than 78 weeks	[30]
cv. "Risto", cv. "Sang", cv. "Sanna", cv. "Vital", cv. "Sol"	Embryos	MS medium with 2 mg L^{-1} 2,4-D for embryos; MS medium with 0.01 mg L^{-1} ABA and 6% sucrose for germination	Suppressed root elongation, promoted secondary root initiation and proliferation of embriogenic cells with 2,4-D in the medium	[36]

Table 1. *Cont.*

A. sativa L. Genotype	Explant Used	Media/PGRs	Experimental Outcomes	References
cv. "Coolabah", cv. "Cooba", cv. "Blackbutt", cv. "Mortlock", cv. "Victorgrain", cv. "HVR"	Immature embryos, leaf segments	MS medium with 2 mg L^{-1} 2,4-D for callus induction and growth; N6 medium (Chu et al. 1975) with 2 mg L^{-1} KIN, and 2 mg L^{-1} NAA for shoot regeneration; MS medium with 0.3 mg L^{-1} KIN for root regeneration	callus formation from the leaf segments and plant regeneration are comparable to that of the immature embryos; plants were grown to maturity	[22]
line GAF, line GAF/Park	Immature zygotic embryos	MS medium with 2 mg L^{-1} 2,4-D for embryos; MS medium with 6% sucrose for embryo maturation, and sucrose reduction for bipolar plant development	Friable embryogenic callus inoculated into liquid medium will produce rapidly growing dedifferentiated suspension cultures	[31]
cv. "Corbit", cv. "Dark Husk", cv. "Winter Turf", cv. "Monida", cv. "SO87213", cv. "Dal"	Embryos	MS medium with 2 mg L^{-1} 2,4-D for callus initiation/proliferation; CIP medium with 0.5 mg L^{-1} picloram, and 5 mg L^{-1} KIN for plant regeneration	High level of plant regeneration	[39]
cv. "Prairie", cv. "Porter", cv. "Pacen", cv. "Ogle"	Apical meristems, leaf primordia, leaf bases	MS medium with 2,4-D (0 and 0.5 mg L^{-1}) and BA (0, 1.0, 2.0, 4.0, and 8.0 mg L^{-1})	Multiple shoot differentiation from shoot apical meristems on medium with 0.5 mg L^{-1} 2,4-D, and 2.0 or 4.0 L^{-1} BA	[23]
cv. "Fuchs", cv. "Jumbo", cv. "Gramena", cv. "Bonus", cv. "Alfred"	Leaf bases of young seedlings	L3 medium for callus induction; 2.5 mg L^{-1} 2,4-D for plant regeneration	for cv. "Jumbo" average of 50 regenerants per explant could be regenerated, whereas for cv. "Gramena", only 3–4 plants per explant could be regenerated	[26]
cv. "GP-1"	Mature embryos	MS medium with 2 mg L^{-1} 2,4-D for callus induction and shoot proliferation; after 6 weeks, 0.5 or 1.0 mM of putrescine was applied	Significant regeneration of plants in presence of 0.5 mM putrescine	[37]
cv. "Aslak", cv. "Velik"	Leaf based segments from 3- to 4-days old seedlings	L3 or MS medium for callus induction; L3 or MS medium with 0.2 mg L^{-1} for regeneration	Optimization of nitrogen, sugar, and auxin in media	[24]
cv. "Belle"	Shoot apical meristem	MS medium with 2 mg L^{-1} 2,4-D for liquid cultures	suspension cultures produced large quantities of aventhramides A and aventramides G in response to 0.25 mg mL^{-1} chitin (poly-N-acetyl glucosamine) elicitation	[40]
cv. "Meliane"	Mature caryopses	MS medium with 3 mg L^{-1} 2,4-D for callus induction; MS medium with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP for embryogenic callus induction and somatic embryos differentiation; MS medium with 0.5 mg L^{-1} IAA for rooting	Ultrastructural changes and cytological modifications of oat somatic embryogenesis	[38]

3. Androgenesis of Oat (*Avena sativa* L.)

In recent years, the production of doubled-haploid (DH) lines using methods involving male gametic lines for developing haploid plants has proven efficient for species belonging to the families Solanaceae, Brassicaceae, and Graminae. Consequently, in vitro induced androgenesis has become the most promising biotechnological method applied in breeding practice [41]. However, not all species respond equally to the induction of this process. There are model species that respond with high efficiency to the application of this method, but other species are more resistant to it. The largest group consists of species in which the induction of microspore embryogenesis is possible but not very efficient from a practical standpoint. Although about 250 protocols related to androgenesis have been described so far, only in a few species, such as barley (*Hordeum vulgare* L.), oilseed rape (*Brassica napus* L.), tobacco (*Nicotiana* spp.), wheat (*Triticum aestivum* L.), pepper (*Capsicum annuum* L.), or rice (*Oryza sativa* L.) has this method have been applied in breeding programs due to the high regenerative efficiency of the obtained plants [42].

The process of androgenesis is defined as an alternative developmental pathway of microspores, involving redirecting their natural gametophytic development, which leads to pollen grain formation towards a sporophytic pathway, along with their reprogramming and the initiation of embryo development [43]. By inducing zygotic embryo-like structures (ELS), followed by their regeneration, androgenic embryos with a haploid number of chromosomes (*n*) are obtained. The literature indicates that microspores in the late uninucleate or early binucleate stage, directly after division, are most susceptible to androgenesis induction, and the process of microspore differentiation occurs under the influence of abiotic stress in the period preceding culture initiation [44]. Among the most used stress-inducing factors are exposure to low or high temperatures, application of sugar- or nitrogen-free media, and treatments with colchicine, heavy metal ions, or mannitol [44]. In cereals, storing spikes at a low temperature can disrupt cytoplasm polarity and impair the direction of spindle formation, leading to a change in the developmental pathway of microspores towards embryo formation [45]. Additionally, subjecting spikes to cold treatment prolongs the viability of anthers, which promotes synchronization of nuclear divisions and inactivates substances that inhibit androgenesis. In practice, a combination of two or three of these factors is applied, and depending on the method, they are used on whole donor plants, cut shoots with spikes, isolated spikes from leaf sheaths, or anthers alone [46]. The main factors determining the androgenic response in in vitro cultures include the genotype of the donor plants, the physiological state and growth conditions of the plants, in vitro medium composition, and physical factors at play during tissue culture and their interactions [47].

In cereals, the process of androgenesis is a more commonly used method for obtaining homozygous plants, and in vitro production of androgenic embryos is more efficient than methods based on gynogenesis [2]. In vitro anther cultures enable the rapid and efficient production of haploid plants, primarily due to the abundance of male reproductive cells. Thousands of microspores present in each anther can potentially give rise to androgenic embryos and, subsequently, haploid plants [48]. However, the main challenge associated with the anther culture technique is the strong dependence of androgenesis not only on the species but also on the genotype of the donor plant [47]. In addition, albinism, i.e., the formation of plants with disrupted chlorophyll production, is a serious problem in anther and isolated microspore cultures. Such plants significantly lower the efficiency of the applied method, expressed in the number of regenerated, green plants with a doubled chromosome number [49].

3.1. Effect of Panicle Pretreatment and Media Composition on ELS Formation

Despite the progress that has been made in improving the effectiveness of methods based on microspore embryogenesis in cereals, the common oat is still considered a recalcitrant species in this process. The first oat regenerants using androgenesis were obtained by Rines [50], who acquired one haploid (n = 3x = 21) and one diploid (2n = 6x = 42) plants of

the cultivar "Stout" from around 65,000 isolated anthers. Before usual incubation at 22 °C and immediately following plating, these anthers were also heat-shocked at 35 °C for 24 h. MS medium without hormone, supplemented with 10% sucrose, had the highest anther callus initiation frequencies among all media tested. However, only from anthers which have been plated on a modified potato extract medium containing 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin (KIN) were seedlings produced. Subsequently, Kiviharju and Pehu [51] reported the unsuccessful regeneration of androgenic embryos in *Avena sativa* L. and the production of haploid plants in *Avena sterilis* L. Five days of heat pretreatment (32 °C) radically increased the embryos induction of *A. sterilis* L. (27.5 embryos/100 anthers), compared to three-day (3.8 embryos/100 anthers) and one-day (0.6 embryos/100 anthers) treatments. Embryo production of *A. sterilis* L. was better on high maltose concentrations than that of *A. sativa* L. The highest number of embryos was obtained on the medium with 14% maltose under both temperature pretreatments. For 10 weeks, 230 embryo-like structures were transferred onto differentiation media. Consequently, two haploid green plants survived transfer to the greenhouse, but these plants did not produce seeds. An attempt to induce androgenesis in Polish oat cultivars was made by Ślusarkiewicz-Jarzina and Ponitka [51,52], who tested the androgenic response of 15 genotypes on solid, liquid, and two-layer media. Oat panicles were harvested and cold-treated at 4 °C for a few days in an N6 mineral salt medium [53] with 2.0 mg L^{-1} 2,4-D. Of the 45,000 anthers plated in this experiment, 637 ELS (1.4%; in all three physical states) were generated on W14 media. Genotype had a significant impact on the frequencies of ELS and green plants production. Eight genotypes yielded ELS (average 1.4/100 anthers). Successful induction of ELS on W14 [54] and C17 media [55] from F3 generation of nine hexaploid oat hybrids was described by Ponitka and Ślusarkiewicz-Jarzina [56]. When compared to medium W14, which generated 137 ELS (from 0.6 to 3.3/100 anthers), medium C17 produced 409 ELS (from 0.6 to 12.1/100 anthers), achieving a greater induction efficiency for all genotypes. Crossing of Bohun × Deresz gave the best ELS induction rates on both media.

In the same year, Skrzypek et al. [57] analyzed the possibility of inducing androgenic ELSs depending on the genotype, the length of the panicle cooling period, the density of anthers in a Petri dish, and the type and physical properties of the media. These studies have shown that pretreatment of oat panicles at a low temperature (4 °C) for 1–2 weeks stimulated induction of ELS the most on W14 and C17 media. Thus far, the highest efficiency of this method has been achieved by Kiviharju et al. [58], resulting in 30 green plants per 100 anthers from the crossing of Aslak × Lisbeth. In this study, the cut tillers were pretreated for 7 days at 4 °C, and the isolated anthers were followed by treatment for 5 days at 32 °C on a double-layer induction medium. "Lisbeth" naked-type oat was used to examine the effects of cytokinins, amino acids, reducing and ethylene-increasing agents and light and temperature conditions. For cv. "Aslak" (2.1/100 anthers) and "Lisbeth" (5.3/100 anthers), the induction medium comprising 2,4-D, BAP, ethephon, cysteine, and myo-inositol produced noticeably higher rates of green plant regeneration than the media containing simply 2,4-D and KIN. In comparison to other treatments, the conversion rate of ELS to green plants was also noticeably greater for the cv. "Aslak" (33%) and much better for the cultivar "Lisbeth" (13%), demonstrating that the 2,4-D and KIN applied together enhance the quality of ELS. Regeneration rates between these two induction media did not significantly differ when weak light was utilized for induction, most likely because of a reduced entire response.

3.2. The Developmental Stage of Microspores Affects ELS Formation

Microspores' competence for androgenesis varies not only among species or cultivars; it is primarily limited temporally and has been referred to as the "developmental window" by Pechan and Smykal [59]. During this short period, it is possible to redirect microspore differentiation from the gametophytic to the sporophytic pathway by applying appropriate physicochemical factors known as stress factors. In addition, by manipulating the composition of the induction media, especially the content of auxins or their analogs, it

is possible to effectively induce callus formation and subsequently embryogenic structures from microspores [60]. The architecture and morphology of oat panicles contribute to the non-linear maturation of anthers, which significantly complicates the identification of microspores at the appropriate developmental stage and likely accounts for the low efficiency of androgenesis in this species. Research conducted by De Cesaro et al. [44] has confirmed that the developmental stage of microspores depends not only on the genotype and age of the plant, but primarily on the position of the anthers in the inflorescence, which results in their uneven maturation. It has also been observed that microspores within a single anther often differ in their embryogenic competence due to slight differences in their developmental stage. The aim of the experiments presented in the work of Warchoł et al. [61] was to determine which external stimuli should be used to arrest the gametophytic pathway of the microspores and direct their development towards embryo formation. The optimization of media composition for the initiation of embryo-like structures was also performed. In addition, the distance from the base of the flag leaf to the penultimate leaf of the panicle was measured to correlate the developmental stage of microspores with shoot morphology. In this way, four distances were determined, i.e., (i) 0.0–4.0 cm, (ii) 4.1–8.0 cm, (iii) 8.1–12.0 cm, and (iv) 12.1–16.0 cm, thereby selecting panicles based on the competence of their microspores for androgenesis. In the first stage of the experiment, the cultivars "Akt", "Bingo", "Bajka", and "Chwat" were tested for their susceptibility to androgenesis induction. In the latter experiment, a significant impact of oat cultivar and the distance from the base of the flag leaf to the penultimate leaf of the inflorescence on the formation of ELS was observed. ELS formation was observed in all cultivars, but the highest number of structures was recorded in the cultivars "Chwat" and "Bingo" (3.6% and 1.6%, respectively). In addition, the highest ELS production was observed on anthers isolated from the youngest panicles, i.e., when the measured distance did not exceed 4.0 cm. The second stage of the experiment aimed to increase the efficiency of androgenesis in the cultivars "Bingo" and "Chwat" by changing the length and type of thermal stress, as well as modifying the composition of the induction media. For the first time, the induction of oat ELS was carried out using a combination of low temperature (4 °C) followed by high temperature (32 °C). The anthers were plated on C17 [62] and W14 [54] media, which were supplemented with the following auxins: 2,4-D, picloram, dicamba and NAA; and cytokinins: KIN and BAP. More ELS were obtained from the anthers of the cultivar "Chwat" compared to the cultivar "Bingo". Differences in androgenesis response depending on the hormones in the induction medium were manifested in the number of obtained haploid plants and DH lines. Based on the results, it was shown that treating oat panicles for 14 days with a low temperature of 4 °C and a high temperature of 32 °C for 24 h before anther isolation increased the efficiency of androgenesis in the cultivar "Chwat". The most susceptible to this process were anthers isolated from panicles where the distance from the base of the flag leaf to the penultimate leaf did not exceed 4 cm. The best medium for induction of ELS and haploid plants was W14 with the addition of 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} KIN.

3.3. Impact of Cu^{2+}, Zn^{2+} or Ag^+ Ions on ELS Formation

The literature suggests that increasing the concentration of Cu^{2+}, Zn^{2+}, or Ag^+ ions in the induction medium not only stimulates haploid embryogenesis of microspores but also regulates numerous physiological and biochemical cellular processes. Cu^{2+} and Zn^{2+} ions stimulate the normal division of chloroplasts, while Ag^+ ions act as an inhibitor of ethylene biosynthesis in in vitro cultures, preventing the aging of microspores [63–65]. Warchoł et al. [66] studied the efficiency of induction of embryonic structures in oat anther cultures depending on the concentration of $CuSO_4 \times 5\,H_2O$ (10 and 20 μM), $ZnSO_4 \times 7\,H_2O$ (90 and 180 μM), and $AgNO_3$ (25 and 50 μM). Copper, zinc, and silver ions were added to the media at two stages of androgenesis: during pretreatment of panicles of donor plants and as an addition to the induction medium. Ions added to the medium during the pretreatment of panicles had a significant effect on the formation of embryonic structures. The highest number of ELS was obtained when oat panicles were treated with 50% Hoagland medium

supplemented with $CuSO_4 \times 5 H_2O$ at a concentration of 10 or 20 μM (2.1% and 1.8%, respectively). The introduction of Cu^{2+}, Zn^{2+}, or Ag^+ ions into the W14 induction medium had no significant statistical effect on the number of ELS. When comparing the cultivars, it was observed that the highest number of ELS (0.7%) was obtained from the cultivar "Chwat", resulting in the production of haploid plants only in this cultivar. The present results demonstrated that the treatment of panicles with $CuSO_4 \times 5 H_2O$ at a concentration of 10 or 20 μM increased the efficiency of androgenesis in the tested cultivars. Table 2 summarizes recent progress in androgenesis in various cultivars of *A. sativa* L.

Table 2. Androgenesis of oat (*Avena sativa* L.).

A. sativa L. Genotype	Culture Conditions	Experimental Outcomes	Reference
cv. "Clintford", cv. "Stout"	4 or 8 °C cold pretreatment	The highest anthers callusing initiation on MS medium with 10% saccharose and no hormones	[50]
Line WW 18019, cv. "Stout"	4 °C in the dark cold pretreatment for anthers from the main culm; 4 °C in the dark for cold pre-treatment for tillers, and MS medium with no PGRs; 32 °C heat pre-treatment for anther cultures	The pretreatment of isolated anthers for 5 days at 32 °C, before culture at 25 °C, is the key point	[51]
44 genotypes	4 °C in the dark cold pretreatment for anthers from the main culm; MS medium with or without 5 mg L^{-1} 2,4-D for ELS induction;	Callus growth, ELS * production rates and plant regeneration differed between naked oat, wild oat, and crosses	[67]
Line WW 18019, cv. 'Kolbu'	4 °C in the dark cold pretreatment for anthers from the main culm; MS medium with 2,4-D and KIN for anthers; MS medium with 1 mg L^{-1} KIN for embryo structures; 32 °C heat pre-treatment for anther cultures	High 2,4-D concentrations enhanced embryo induction with or without heat pre-treatment	[68]
cv. "Lisbeth", cv. "Virma", cv. "Cascade", cv. "Kolbu", cv. "WW 18019", cv. "OT 257", cv. "Stout", cv. "Sisu", cv. "Katri", cv. "Yty", cv. "Sisko", cv. "Talgai", cv. "Roope", cv. "Salo"	tillers pretreated at 4 °C for 7 days; double-layer induction medium MS or W14 with 10% maltose and PGRs; 32 °C heat pre-treatment for anther cultures	Regenerable-type embryos from heat-pretreated anthers on media containing 2, 3 or 5 mg L^{-1} mg 2,4-D and 0.2 or 0.5 mg L^{-1} KIN	[69]
cv. "Lisbeth"	4 °C for 7 days for the tillers; 32 °C heat pretreatment; W14 medium with 10% maltose and PGRs for anthers, W14 medium with 2 mg L^{-1} NAA, and 0.5 mg L^{-1} KIN for ELS and regeneration; MS with 0.2 L^{-1} NAA for rooting	Improved number of derived plants via application of W14	[58]
Oat hybrids 1705/05, 1717/05, 1725/05, 1780/05, 2038/05, 1889/05, 1893/05, 1903/05, 1944/05, 1954/05, 956/05, 1967/05, 1985/05, 1989/05, 1997/05	4 °C for 6–9 days for the tillers in N6 medium with 2 mg L^{-1} 2,4-D; liquid, solid or double-layer W14 salts and vitamins, 5.0 mg L^{-1} 2,4-D, and 0.5 mg L^{-1} BAP for ELS induction;	Development of ELS after 6 weeks of culture on liquid medium, and between the 7th and 8th weeks on solid and double-layer medium	[52]

Table 2. *Cont.*

A. sativa L. Genotype	Culture Conditions	Experimental Outcomes	Reference
cv. "UPF 7", cv. "UPF 18", cv. "UFRGS 14", cv. "Stout"	Samples were collected when the distance between the flag leaf and the last node was one third of the distance between the last node and flag leaf	The use of anther size for the identification of microspore developmental stage is inefficient selection criterion	[44]
Cross combination of hexaploid oat: Lisbeth × Bendicoot, Flämingsprofi × Rajtar, Scorpion × Deresz, Aragon × Deresz, Deresz × POB7219/03, Bohun × Deresz, Krezus × Flämingsprofi, Krezus × POB10440/01, Cwał × Bohun	4 °C for 6–9 days for the tillers in N6 medium with 2 mg L^{-1} 2,4-D; C17 induction medium with W14 salts and vitamins, 5.0 mg L^{-1} 2,4-D, and 0.5 mg L^{-1} BAP for ELS induction; 190-2 regeneration medium	The highest number of ELS on C17 medium; incubation at 22 °C in the dark for the first two weeks for the highest rate of green plants per 100 ELS	[56]
Genotype 2000QiON43 (LA9326E86)	0.3 M mannitol pretreatment of the tillers for 7 days; W14 medium and continuous incubation at 28 °C; W14 medium for embryos observed; 0.2% colchicine for 4 h for DH	Protocol for the production of microspore-derived embryos of oat, 80% of the plants were converted to DH	[46]
cv. "Akt", cv. "Bingo", cv. "Bajka", cv. "Chwat"	for tillers: 2 and 3 weeks at 4 °C, or 2 and 3 weeks at 4 °C followed by 32 °C for 24 h; for ELS induction: C17 medium with 0.5 mg L^{-1} picloram, 0.5 mg L^{-1} dicamba, and 0.5 mg L^{-1} KIN, or W14 medium with different concentrations of 2,4-D, NAA, and BAP	Cold pretreatment and high temperature enhanced the technique efficiency; W14 medium with 2 mg L^{-1} and 0.5 mg L^{-1} KIN for the highest number of ELS	[61]
cv. "Bingo", cv. "Chwat"	2 weeks at 4 °C for tillers pretreatment in liquid medium alone or with Cu^{2+}, Zn^{2+}, or Ag^+ ions followed by 32 °C for 24 h	ELS formation depended on cold pretreatment combined with Cu^{2+}, Zn^{2+}, or Ag^+	[66]

* ELS—embryo-like structures.

4. Wide Crossing of Oat (*Avena sativa* L.) with Chosen Species from Poaceae Family

Obtaining DH lines of oat is very challenging compared to other cereals, and both breeding and biotechnological research conducted by research groups from Poland, the USA, Finland, or Japan unanimously confirm the recalcitrance of this species to haploidization. Since the techniques used successfully in other plants are still not very effective in oat, a commercially viable and efficient method of obtaining DH lines in this species has not yet been developed. The reasons for the low efficiency of the methods used, which typically yield between 0.5% and 10.0% of haploid embryos per emasculated floret [70], are attributed to the presence of numerous pre- and postzygotic barriers. Prezygotic barriers include all factors that hinder the successful fertilization of the ovum, i.e., the formation of a zygote. The most listed prezygotic barriers include the inability of pollen to germinate on a foreign stigma, inhibition of pollen tube growth, or rupture of the pollen tube [71]. On the other hand, postzygotic barriers impede the development of the zygote after fertilization [72] and are often a result of genetic incompatibility between the parental plants in wide crosses. Hence, developing a thorough understanding of these barriers and overcoming them can contribute to the development of an effective and universal method for obtaining oat haploids and subsequently DH lines. From a practical standpoint, this opens new possibilities for improving haploidization methods not only for oat but also for other plants recalcitrant to this process, such as legumes or woody plants. In addition, the production of

new cultivars based on homozygous DH lines is becoming increasingly important in crop breeding programs and represents one of the key opportunities for adapting agriculture to ongoing climate change.

Pioneering work on obtaining DH oats through wide crosses with maize was conducted by Rines and Dahleen [73]. Pollen from maize (*Zea mays* L.) was applied to previously emasculated oat florets in a series of experiments. Extracted caryopses and the embryos formed from them were then cultured on an MS medium with 7% sucrose and amino acids supplements. Recovered plantlets were raised in soil-filled pots until they were fully developed. Following the pollination of maize pollen from around 3300 emasculated oat florets, 14 haploid oat seedlings were successfully produced via the embryo rescue technique. Subsequently, in studies conducted by Matzk [74], eastern gamagrass (*Tripsacum dactyloides* L.), pearl millet (*Pennisetum americanum* L.), and maize (*Zea mays* L.) were used to pollinate five varieties of oat. Postzygotic obstacles appeared while using early colchicine-mediated chromosomal doubling, exogenous auxins, and embryo rescue media. The embryo frequencies ranged from 0.4% in maize to 9.8% in pearl millet, depending on the type of pollinator. Although many plantlets in the embryo rescue process perished, the beginning of growth usually occurred. Four viable plants were formed overall, including hybrids with pearl millet and for the first time using eastern gamagrass. One to four chromosomes from pollinator species were discovered in oat root tip cells during the tillering stage. The authors stated that while the efficiency of haploid formation (0.1%) was too low to use in plant breeding programs, crossings of oat with maize and pearl millet looked promising for the transfer of genes or chromosomes. In 2015, Nowakowska et al. [75] conducted research aimed at developing an effective method for obtaining oat DH lines and demonstrated a significant influence of individual steps of the procedure on the efficiency of haploid production. In these experiments, the optimal timing between emasculation of florets, pollination with maize, treatment of ovaries with auxin, as well as the appropriate timing for the isolation of haploid embryos was determined. The highest number of haploid embryos and plants was obtained by pollinating donor plants with maize pollen 2 days after emasculation, when auxins were applied 2 days after pollination, and when embryos were isolated 3 weeks after pollination.

4.1. Induction of Haploid Embryos

The treatment of oat ovaries after pollination has also been the subject of many experiments. Initially, Rines et al. [76] negated the need for auxin application to oat ovaries to increase the efficiency of wide hybridization. However, Sidhu et al. [77] emphasized that growth regulators not only prevent the degeneration of ovaries; most importantly, they stimulate and sustain embryo development until its isolation from the ovary. Currently, to facilitate the formation of oat haploid embryos, pollinated flowers are most often treated with the following synthetic auxins: 2,4-D, dicamba, picloram, or gibberellic acid (GA_3) [70,75,77,78], or a combination of 2,4-D and GA_3 [12]. Only in a few cases were pollinated oat panicles cut and placed in a solution containing sucrose and 2,4-D [6]. Research by Smit and Weijers [79] has shown that auxins play a key role in the early stages of embryogenic plant development, mediating the formation of zygotic embryos. Exogenous 2,4-D application alters the levels of endogenous auxins, such as IAA, thereby modifying their intracellular metabolism, which leads to the establishment of proper embryonic symmetry [80]. Warchoł et al. [81] described the process of determining which auxin to apply at 100 mg L^{-1} to the ovary after removal of the anthers and pollination with maize pollen to induce the development of haploid embryos plants and the production of fertile DH lines. It was determined that the tested auxins did not affect the number of enlarged ovaries (83.4%—dicamba; 83.9%—2,4-D, calculated based on emasculated flowers), nor did they affect the number of resulting haploid embryos. However, the applied auxins significantly differentiated the capacity of embryos to germinate, thus affecting the production of haploid plants and DH lines. Nearly half of the generated embryos (48%) germinated when placed on 190-2 medium [62], but only 22% of them developed into haploid plants.

The final number of haploid plants was 45 (0.64%, based on emasculated florets) when using dicamba, and 104 plants (1.37%, based on emasculated florets) when 2,4-D was applied. The same concentration (100 mg L^{-1}) of the auxin analogues 2,4-D, dicamba, and picloram, as well as GA$_3$, were tested by Sidhu et al. [77]. A specific growth regulator was applied to emasculated oat florets of the AK-1 and F1 hybrid genotypes on the 2nd and 3rd post-pollination days after being pollinated with maize pollen. The ability of each hormone to promote caryopsis development varied significantly between the two genotypes. The largest proportion of caryopses were produced by the dicamba treatment, 94.5% for AK-1 and 94.1% for 01095, respectively. Following 2,4-D and GA$_3$, picloram stimulated caryopses development. There was no discernible difference between genotype and growth regulator interaction. Kynast et al. [12] used a phytohormone mixture (50 ppm 2,4-D + 50 ppm GA$_3$) and sprayed them 24 or 48 h after application of fresh pollen of maize Mo17 on emasculated oat panicles of Starter and Sun II to induce the growth of the haploid embryos.

As mentioned above, the application of synthetic auxins to pollinated ovaries is a required step in the process of oat haploidization because it leads to the proper distribution of endogenous auxins necessary for establishing embryogenic patterns. The studies conducted by Nowakowska et al. [75] and Mahato and Chaudhary [82] have emphasized that the efficiency of this process is influenced not only by the timing and method of hormone application but primarily by their concentration. However, it is important to remember that using high concentrations of 2,4-D in in vitro cultures is toxic to plants and can result in tissue necrosis [83] or the inhibition of embryo germination, as observed by Bronsema et al. [84] in maize. Considering that synthetic auxins applied at high concentrations exhibit strong toxic properties, which could consequently result in low survival rates of oat haploid embryos, the aim of the experiments published by Juzoń et al. [85] was to determine how two different 2,4-D concentrations affected the conversion of embryos into haploid plants and the subsequent development of fertile DH lines. Treating the ovaries with 50 mg L^{-1} 2,4-D yielded 27 haploid plants (8.5%, based on emasculated flowers), while using 100 mg L^{-1} of 2,4-D increased their number to 49 (16.3%, based on emasculated flowers). The higher concentration of 2,4-D led to the survival of all haploid plants from 17 genotypes after colchicine treatment (approx. 58% of obtained plants), resulting in twice as many DH lines (44 plants) compared to the lower concentration of 2,4-D (22 plants). Oat florets from genotype AK-1 which had been emasculated and pollinated with maize pollen were exposed to four different doses of dicamba (5, 25, 50, and 100 mg L^{-1}) [77]. With the increasing dicamba concentration, the proportion of caryopses per floret grew considerably, reaching a maximum at concentration of 50 mg L^{-1}. Caryopsis development and embryo formation at 50 and 100 mg L^{-1} did not significantly differ. Kynast et al.'s [12] studies, the phytohormone combination (50 ppm 2,4-D + 50 ppm GA$_3$) was proven to be more effective for embryo formation than the 100 ppm 2,4-D solution without GA$_3$.

4.2. Embryo Rescue Technique

Achieving approx. 10% of haploid embryos per emasculated flowers in the first stage of wide hybridization does not confirm a high efficiency of haploid plants or fertile DH lines production. This is because the embryos formed after fertilization have a very low viability, and most of them die in the early developmental stages. Rines [70] has reported that the rate of embryo germination and their regeneration into plants typically falls below 20%. This was also confirmed by other studies, e.g., Warchoł et al. [81] and Juzoń et al. [85], who isolated a relatively high number of embryos (683 and 619, respectively) but obtained only 149 and 76 haploid plants, respectively. In addition, as pointed out by Rines [70], the low regenerative capacity of embryos hampers the conducting of experiments that would allow for a statistical comparison of factors influencing their germination effectiveness; thus, in assessing the reproducibility of the applied method in oat, the haploid embryos resulting from wide crosses with maize are most often devoid of endosperm, or else this tissue is rudimentary. In consequence, the lack of access to nutrients leads to their death, and the in vitro culture stage where suitable conditions for their growth are provided is referred to as the

embryo rescue technique [86,87]. The first attempts at cultivating plant embryos outside their maternal tissues to obtain an interspecific cross of *Linum perenne* × *Linum austriacum* were conducted by Laibach [88]. Analyzing his research, it can be seen that the smaller the embryo, the more complex the medium required to continue its growth and development. In practice, this means that the regeneration medium closely mimics the composition of the maternal endosperm, thereby providing the appropriate nutritional components for the specific developmental stage of the embryo. The literature data indicate that the concentration of carbohydrates depends on the developmental stage of the embryo; the younger the embryos, the higher the concentration of sugars in the medium should be (even up to 12%). Sugars added to the medium at the appropriate concentration serve not only as a carbon source for heterotrophic embryos; they also ensure a suitable level of osmotic pressure [89]. The necessity of overcoming postzygotic barriers, including the selection of an appropriate regeneration medium that serves as an endosperm substitute for developing embryos, makes germination of embryos a critical stage in the method for obtaining oat DH lines.

Since each species requires the development of a detailed procedure concerning both culture conditions and the appropriate selection of regeneration medium components, Warchoł et al. [90] optimized the composition of the medium for embryo germination under in vitro conditions. This experiment, for the first time, analyzed the germination capacity of embryos on media with varying maltose concentrations and pH values. The resulting haploid embryos were plated on 190-2 agar medium [91] enriched with KIN and NAA at a concentration of 0.5 mg L^{-1}. Maltose was added to the medium at two concentrations, 6% and 9%, and the pH was set at 5.5 and 6.0. The medium with a pH of 6.0, compared to pH 5.5, increased the efficiency of embryo germination, similarly to the increased maltose content (9%) in the medium. Previous studies on obtaining oat DH lines have demonstrated high efficiency in inducing haploid embryos but unsatisfactory conversion of these embryos into plants [75,77,78]. The most frequently indicated reasons involved not only the lack of endosperm but also disrupted hormonal balance and a range of deformations visible at various stages of their development [92]. Moreover, the development of haploid oat embryos is not synchronized in time. Despite the fact that maize ovary pollination and auxin treatment occurred at the same time, the embryos transferred onto regeneration media differ in size and level of differentiation. When establishing in vitro cultures of embryos isolated from immature seeds, known as pseudo-seeds [93], it is important to remember that the establishment of the axis of symmetry is possible only when the embryo's development, at least up to the early globular stage, occurs in its natural environment, i.e., in the ovary. On the contrary, the initiation of cultures must occur before the critical point of developmental arrest, namely, the cotyledon formation stage. Additionally, in immature embryos, a phenomenon called "premature germination" is observed, typically occurring before the embryo axis formation. Since this type of germination is characterized by the elongation of cells and low intensity of divisions, the resulting haploid plants are weak and usually die back [94].

The experiments conducted by Noga et al. [95] aimed to increase the conversion efficiency of haploid embryos into haploid plants and to analyze correlations between the germination capacity of oat embryos at different developmental stages and the type of growth regulators. Although the isolation was performed at the same time, the embryos plated on the media exhibited differences in morphological structure. As a result, they were divided into four size classes: <0.5 mm, 0.5–0.9 mm, 1.0–1.4 mm, and ≥1.5 mm. Subsequently, they were cultured on a 190-2 regeneration medium [91], containing 9% maltose, 0.6% agar, and the following growth regulators: KIN, NAA, zeatin (ZEA), dicamba, and picloram. Microscopic observations revealed that embryos smaller than 0.5 mm were spherical, those ranging from 0.5 to 1.4 mm were elongated without distinct basal and apical parts, while embryos larger than 1.5 mm had a visible coleoptile and embryonic root. The conducted analysis of oat embryo germination capacity concerning their developmental stage showed that the largest embryos germinated at nearly 80%, while the smallest ones lacked regenerative capacity and died after plating on the medium. Furthermore,

it was observed that the size of haploid embryos and their germination capacity varied significantly among different oat genotypes. A similar observation was made by Sidhu et al. [77]. Compared to self-pollinated embryos, the white, embryo-like structures (ELS) of the four oat genotypes—AK-1, Carrolup, Dumont, Mortlock, and S093658 obtained by crossing oats with maize—differed in size and shape. The type of growth regulators added to the regeneration medium did not exert a significant effect on the regeneration of haploid embryos into plants. Nevertheless, the highest percentage of haploid embryos (19%) germinated on a medium with 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} KIN, and the smallest (11%) on a medium with 1 mg L^{-1} dicamba, 1 mg L^{-1} picloram and 0.5 mg L^{-1} KIN.

Skrzypek et al. [96] examined the role of light intensity applied in vivo to initiate haploid embryos and in vitro to regulate their development. For the growth of donor plants, the light intensity of 800 μmol m^{-2} s^{-1} more effectively stimulated the formation of haploid embryos (9.4%) compared to the light intensity of 450 μmol m^{-2} s^{-1} (6.1%). Light intensity during in vitro cultures of embryos also had an impact on their conversion into plants. Light intensity of 110 μmol m^{-2} s^{-1} during culture most optimally stimulated embryo germination (38.9%) and plant development (36.4%) compared to light intensities of 20, 40, and 70 μmol m^{-2} s^{-1}. In a previous study by Sidhu et al. (2006), it was amply shown that temperature has no influence on the caryopsis development. Despite this, the authors observed higher embryos production at 24 °C. However, this difference was not statistically significant, most likely as a result of the few repetitions (one donor plant per treatment).

To understand the slow rate of oat embryo germination, research was conducted to investigate the phytohormone content in ovaries during embryo development. Additionally, the hormonal profiles of zygotic and haploid embryos were analyzed. Dziurka et al. [97] compared ovules with embryos (OE) and ovules without embryos (OWE). The latter study analyzed the phytohormone content and found significantly higher concentrations of IAA, trans-zeatin (tZ), and KIN in OE compared to OWE. It was also demonstrated that an excess of cytokinins in OE was detrimental to embryogenesis, while reduced cytokinin levels increased the efficiency of obtaining DH lines. The presence of IAA was detected only in OWE, indicating its role in plant aging processes. Although both haploid and zygotic oat embryos were isolated at the same time, the extremely low levels of endogenous auxins, larger amounts of cytokinins, and a ten-fold higher cytokinin/auxin ratio in the haploid embryos may indicate an earlier developmental stage for the former. It was also shown that inadequate germination of haploid embryos could rsult from an excess of reactive oxygen species, raising levels of low-molecular-weight osmoprotectants and stress hormones in addition to hormonal modulation of embryogenesis [92]. The summary of progress in the wide crossing of *A. sativa* with various species from the Poaceae family is presented in Table 3.

Table 3. Crossing of oat (*Avena sativa* L.) with chosen species from the Poaceae family.

Plant Material	Culture Conditions	Experimental Outcomes	Reference
Oat × maize Oat: cv. "Stout", cv. "Starter", cv. "Steele", cv. "Black Mesdag" Maize: A188, B73, Honeycomb, A619 × W64A	Haploid plants recovered via embryo rescue following field-grown maize pollen application to emasculated florets of growth chamber-grown oat	Recovered haploids were from a different oat cultivar and different source of maize pollen—the process is not genotype unique	[73]
Oat × maize Oat: genotypes AK-1, S093658, Carrolup, Dumont, Mortlock Maize: early extra sweet F1, and Kelvedon Glory F1 varieties	100 mg L^{-1} GA$_3$, 2,4-D, 3,6-dichloro-o-anisic acid (dicamba) or 4-amino-3,5,6,-trichloro-picolinic acid (picloram) applied after pollination; four different temperature regimes (32/24, 24/20, 21/17 and 17/14 °C day/night) applied before flowering	The highest number of caryopses produced with dicamba, but without effects on embryo production; genotype dependent temperature effects	[77]

Table 3. *Cont.*

Plant Material	Culture Conditions	Experimental Outcomes	Reference
Oat × pearl millet Oat: cv. "Best Enbaku" Pearl millet: *Pennisetum glaucum* cv. "Ugandi"	100 ppm 2,4-D dropped onto each floret 12 h after pollination; 100 ppm 2,4-D and 4% sucrose for the spike culture	Retention of all seven pearl millet chromosomes in embryos from the crosses with oat; oat haploid developed to a fertile adult plant	[6,11]
Oat × maize Oat: lines Black Mesdag, GAF- Park, Kanota, MN97201-1, Preakness, Starter, Steele, Stout, Sun II, and F1 (MN97201-1 × MN841801-1) oat hybrid Maize: lines Seneca60, *bz1-mum9*, A188, B73, Mo17, and the F1 (A188 9 W64A) maize hybrid	50 ppm 2,4-D and 50 ppm GA$_3$ for embryo formation delay of endosperm collapse	Euhaploid plants with complete oat chromosome complements without maize chromosomes; aneuhaploid plants with complete oat chromosome complements and different numbers of retained individual maize chromosomes; uniparental genome loss during early steps of embryogenesis causing the elimination of maize chromosomes in the hybrid embryo	[98]
Oat × maize Oat: genotypes 80022, 80031, 81711, 81350, 81384, 81524, 81559, 82072, 82091, 82230, 82266, 83200, 83207, 83213, 83421, 83430, 85924, and 85931 Maize: Waza, Dobosz, and Wania	Oat florets pollinated with maize pollen after 0, 1 or 2 days; 100 mg L^{-1} 2,4-D or 100 mg L^{-1} dicamba placed on the floret pistils 1, 2-, 3-, 4-, and 5-days following pollination	Genotype-dependent haploid embryo formation and plant regeneration; 2nd-day pollination together with auxin treatment was the most effective	[78]
Oat × maize Oat: 80031—(Deresz × Szakal), 81350 (Krezus × STH 454), 82072 (Bajka × STH 454), 82091 (Bajka × STH 7706), 83213 (Flamingstern × Chwat) Maize: *Zea mays* L. var. *saccharata*, **Oat × sorghum** Sorghum: *Sorghum bicolor* (L.) Moench **Oat × common millet** Common millet: *Panicum miliaceum* L.	100 mg L^{-1} dicamba one day after pollination; enlarged ovaries collected at 2, 3 and 4 weeks after pollination cultivated on 6 or 9% of maltose	2.5—6.9% of HE * for genotypes pollinated with maize, 1.3% for sorghum, and 1.2% for millet; the highest frequency of HE germination and number of plants 3 weeks after pollination; 9% maltose for embryo formation, germination, and haploid plants development	[75]
Oat × maize Oat: STH 4.8456/1, STH 4.8456/2, STH 4.8457/1, STH 4.8457/2, STH 5.8421, STH 5.8422, STH 5.8423, STH 5.8424, STH 5.8425, STH 5.8426, STH 5.8427, STH 5.8428, STH 5.8429, STH 5.8430, STH 5.8432, STH 5.8436, STH 5.8440, STH 5.8449, STH 5.8450, STH 5.8458, STH 5.8460 Maize: Waza	<0.5 mm HE, 0.5–0.9 mm HE, 1.0–1.4 mm HE, and ≥1.5 mm HE on 0.5 mg L^{-1} KIN and 0.5 mg L^{-1} NAA, or 1 mg L^{-1} ZEA and 0.5 mg L^{-1} NAA, or 1 mg L^{-1} dicamba, 1 mg L^{-1} picloram, and 0.5 mg L^{-1} KIN	Germination of HE ≥ 1.5 mm on medium with 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} KIN	[95]
Oat × maize 32 oat genotypes were pollinated with *Zea mays* L. var. *saccharata* (maize) genotypes: MPC4, Dobosz and Wania	Different light intensity during the growing period of donor plants and in vitro cultures	9.4% HE formed in a greenhouse, 6.1% in a growth chamber; 38.9% of embryo germination, 36.4% conversion into plants, and 9.2% DH ** line production with 110 µmol m^{-2} s^{-1} light intensity	[96]
Oat × maize Oat: F1 progeny of thirty-three oat genotypes Maize: *Zea mays* L. var. *saccharata* (maize) genotypes MPC4, Dobosz and Wania	Immersion of haploid plants for 7.5 h in a 0.1% colchicine, 40 g L^{-1} DMSO, 0.025 g L^{-1} GA^3 at 25 °C and 80–100 µmol m^{-2} s^{-1} light intensity for chromosome doubling procedure	From 149 haploid plants 61 survived chromosome doubling procedure, 52 (85%) were fertile and produced seeds	[81]
Not specified	Colchicine solution with DMSO for chromosome doubling	Detailed description of a method for DHs generation	[1]

Table 3. *Cont.*

Plant Material	Culture Conditions	Experimental Outcomes	Reference
Oat × maize 80 oat genotypes pollinated with maize cv. "Waza"	Colchicine solution applied on HP roots for chromosome doubling	from 138 oat lines, the presence of maize chromatin was indicated in 66 lines from which 27 OMA lines were fertile and produced seeds	[99]
Oat × maize Oat: F1 progeny of twenty-two oat genotypes pollinated with *Zea mays* L. var. *saccharata* (maize) genotypes MPC4, Dobosz and Wania	For chromosome doubling HP roots were immersed in a 0.1% colchicine with 4% DMSO, 0.025 g L^{-1} GA^3, and 20 μL of Tween 20, left for 7.5 h at 25 °C and 80–100 μmol m^{-2} s^{-1} light intensity	591 HE formed, 48 fertile DH plants producing in all 4878 seeds	[90]
Oat × maize Oat: F1 progeny of twenty-nine oat genotypes pollinated with *Zea mays* L. var. *saccharata* (maize) genotypes MPC4, Dobosz and Wania	9465 florets were pollinated with maize pollen 2 days after emasculation and treated with 2,4-D at 50 mg L^{-1} and 100 mg L^{-1}; colchicine solution applied on HP roots for chromosome doubling	Higher 2,4-D concentration is more efficient in obtaining haploid/DH plants with better vitality and fertility	[85]

* HE—haploid embryos; ** DH—doubled haploids.

5. Conclusions and Future Directions

Many variables, including screening practices, tolerance bases and mechanisms, gene function and inheritance, and linkages to agronomical traits, all have an impact on the choice of an appropriate breeding strategy for the creation of cultivars that are of interest to us. As new cultivars have been created mostly using conventional breeding methods, the typical approach involves recombining DNA by distinct chromosomal assortment and crossing-over.

In conventional breeding, the number of generations is needed to produce stable variations via natural segregation from the heterozygous progeny of the original crosses. The in vitro methods could eliminate the necessity for back-crosses or repeated self-pollination. Although plant tissue culture techniques have developed significantly since the first publication on oat regeneration from calluses in 1967, the plant regeneration effectiveness is still low and strongly genotype dependent. Oat plants must be grown year-round for the isolation of immature embryos, which is costly, requires elaborate equipment, and may subject donor plants to physiological fluctuation that could influence how frequently tissue cultures are started. Furthermore, it takes a lot of effort to isolate immature embryos from oats since the panicle's fertilization is not as synchronized as it is, for example, in wheat or barley. As a substitute, mass-producing mature seeds is affordable and offers reliable explants for starting in vitro cultures, potentially removing the variability in cultures from different explants. On the other hand, by manipulating gamete development, it is possible to regenerate fully homozygous plants in just one generation.

Since the beginning of the 20th century, when the theories of totipotency and naturally occurring sporophytic haploids were discovered, the production of haploid and DH plants has been introduced in the breeding several crop plants. Research has resulted in a better understanding of the mechanisms of haploid formation, the identification of factors influencing haploid induction, and the increase in genetic benefits through the application of DH technology in plant breeding. The recent finding of a very efficient centromere-mediated genome deletion approach for haploid production has sparked intense interest in its application in plant breeding.

For oat species, the generation of DH has become an essential tool in advanced plant breeding. Combining the use of DH methods with applied genomics opens novel possibilities for maximizing genetic benefits in selection and for developing new, more cost-effective, and efficient massive techniques, as well as for minimizing the time needed for cultivar production. However, the study of oat molecular genetics remains substantially behind that of other grains mainly due to the genome size, and that the oat DNA sequence

was not fully available. Since the publication of the first quantitative trait loci linkage map in oat, there have been constant attempts to enhance the density of the map via different kinds and numbers of markers. The recently sequenced hexaploid *Avena sativa* L. genome, although its size is expected to be over 11 Gb and it consists of two ploidy species: *Avena longiglumis* (AA) (3.7 Gb) and *Avena insularis* (CCDD) (7.3 Gb), should help to accelerate the process of enhancing oats for numerous features [100]. The most important objectives of oat breeding and modification of genes are to improve tolerance to diseases and environmental stressors, as well as yield and other important agronomical features [101].

Despite all these advancements, there are still DH line utilization difficulties that have yet to be resolved. There is a need for greater DH production efficiency (especially in anthers and microspore cultures compared with wide crossing), as well as improved germplasm control and a greater awareness of the molecular mechanisms that regulate the formation of haploid plants. Moreover, an important issue is the elaboration of the chromosome doubling method without using harmful chemicals. Recently, the production of haploids using centromere-mediated genetic engineering seems to have been of key importance. As described by Karimi-Ashtiyani et al. [102], point mutation in the histone H3 variant CENH3, which is specific to centromeres, may be utilized to create haploid plants. Plants with this single-point mutation in CENH3 are haploid inducers. Due to the high degree of conservation of the recognized mutation site and the fact that point mutation can be achieved via mutagenesis or genome editing, the disclosed method has the potential to be applicable to numerous crops.

The findings of this review may encourage the spread of this technology's application in accelerating and creating new oat breeding opportunities. We expect that this review will also assist molecular scientists in the construction of DH segregating populations in oat species, which are necessary in order to produce genetic maps employing molecular markers.

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