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REVIEW

In vitro-based doubled haploid production: recent improvements

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Abstract The doubled haploid system is the fastest way of hybrid variety production and plays an important role in breeding programs and developmental studies. The most commonly used methods of haploid induction, leading to haploid plants in vitro through the plant tissue/cell culture, are named as the in vitro -based (IVB) methods. These methods have been established in many of the important crops, such as barley, pepper, rapeseed, rice, sugar beet, and wheat. There are ongoing researches to optimize and improve the efficiency of these methods by focusing on factors involved in induction and regeneration phases. These factors mainly include plant genotype, the surrounding environment of parental plants, components of culture medium, the developmental stage of initial gametophytic cells, physical treatments (cold pre-treatment, heat shock) of cultured gametophytic cells, and application of different additives and plant growth regulators. Stress treatment is one of the important prerequisites for stimulation of gametophytic cells to switch towards the sporophytic pathway. However, autophagy and programmed cell death, oxidative stress, and production of reactive oxygen species (ROS) are the major limiting factors in stress-induced embryogenesis. The positive effect of different additives, such as plant growth regulators, chlormequat, polyamines (putrescine, spermidine, and spermine), stress hormones (abscisic acid, jasmonic acid, salicylic acid), DNA demethylating agents and histone deacetylase inhibitors, cellular antioxidants, cell wall remodeling agents (arabinogalactan-proteins), and compatible solutes (proline and chitosan), has been proved on the efficiency of haploid induction through IVB methods. Different mechanisms have been reported through which the aforementioned additives can enhance tolerance to embryo-inducing stresses in plants, and subsequently increase the efficiency of induction phase of IVB methods of haploid induction. Finding the best combination/interaction of inductive stresses and their corresponding chemical enhancers is crucial for successful haploid induction through IVB methods. In the present review, we highlighted recently applied additives to enhance the efficiency of the major IVB methods of haploid induction in different plants. Other potentially applicable additives, those are involved in preventing ROS accumulation, ethylene inhibitors, activating of antioxidant enzyme activity,

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detoxification capacity, and defense response signal pathway, which could be useful in IVB haploid induction are also discussed. The presented information could be useful to improve the efficiency of developed IVB protocols and/or to develop new protocols in recalcitrant species/genotypes.

Keywords Androgenesis · Chemical enhancers · Gynogenesis · Haploid induction · Microspore

Abbreviations

AGPs	Arabinogalactan-proteins
BAP	6-Benzylaminopurine
CCC	Chlormequat chloride
CENH3	Centromere histone H3
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas	CRISPR-associated
DH	Doubled haploid
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IMC	Isolated microspore culture
IVB	In vitro-based
GWAS	Genome-wide association
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
PGR	Plant growth regulator
QTL	Quantitative trait loci
RB	Reverse breeding
ROS	Reactive oxygen species
SMC	Shed microspore culture
TALENs	Transcription activator-like effector nucleases
TDZ	Thidiazuron
TILLING	Targeting induced local lesions in genomes
ZFNs	Zinc-finger nucleases
2,4-D	2,4-Dichlorophenoxyacetic acid

Introduction

Biotechnology has been widely used in plant breeding to accelerate and complement the conventional breeding methods. The production of F_1 hybrid seeds is one of the most popular aspects of plant breeding, as has increasing importance for farmers and breeders (Ribarits et al. 2009). It is the hybrid vigor attracting plant breeders and commercial producers to create hybrid seeds. The production of inbred parental lines is the first prerequisite and one of the most difficult steps of F_1 hybrid variety production, because it takes several generations of selfing/inbreeding (reviewed by Germanà 2011). The conventional plant breeding programs are tedious, time consuming, and expensive to produce inbred parental lines (Khan et al. 2017). In addition, self-incompatibility is another problem of conventional inbreeding methods to produce homozygous parents, especially in woody plants (Germanà 2006). The fastest shortcut to create inbred lines is the doubled haploidy system. In self-pollinated crops such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.), the application of the doubled haploid (DH) methods reduces the time required for releasing a new cultivar by 3–4 years (reviewed by Shariatpanahi and Ahmadi 2016). In addition, the selection efficiency is also improved by exploiting the DH system.

The fast development of homozygous lines is the major advantage of haploid induction and subsequent DH production (reviewed by Ren et al. 2017). There are two main methods of haploid induction, including in vitro-based (IVB) and in vivo- based haploid induction systems (Fig. 1). The IVB system is based on the culture of immature male or female gametophytic cells, whereas the in vivo system is based on inter- or intraspecific hybridization and subsequent uniparental chromosome elimination (Ren et al. 2017). The IVB methods are including in vitro induced parthenogenesis (gynogenesis) and androgenesis (anther culture, isolated microspore culture, and shed microspore culture) (Fig. 1). Some valuable previous reviews addressed the principles of various methods of plant haploid induction (Forster et al. 2007; Wędzony et al. 2009; Dwivedi et al. 2015; Ren et al. 2017; Kalinowska et al. 2019).

Androgenesis is the most effective method to obtain DH plants (Wędzony et al. 2009). There are, however, some valuable genotypes of different crops, such as barley, maize (*Zea mays* L.), potato (*Solanum tuberosum* ssp. tuberosum), and rye (*Secale cereale* L), which are recalcitrant to this method (Maluszynski et al. 2003). Recently, different additives, including phytohormones—endogenous-produced organic compounds that are necessary for regulating plant growth and yield (Khan et al. 2020)—stress hormones, growth

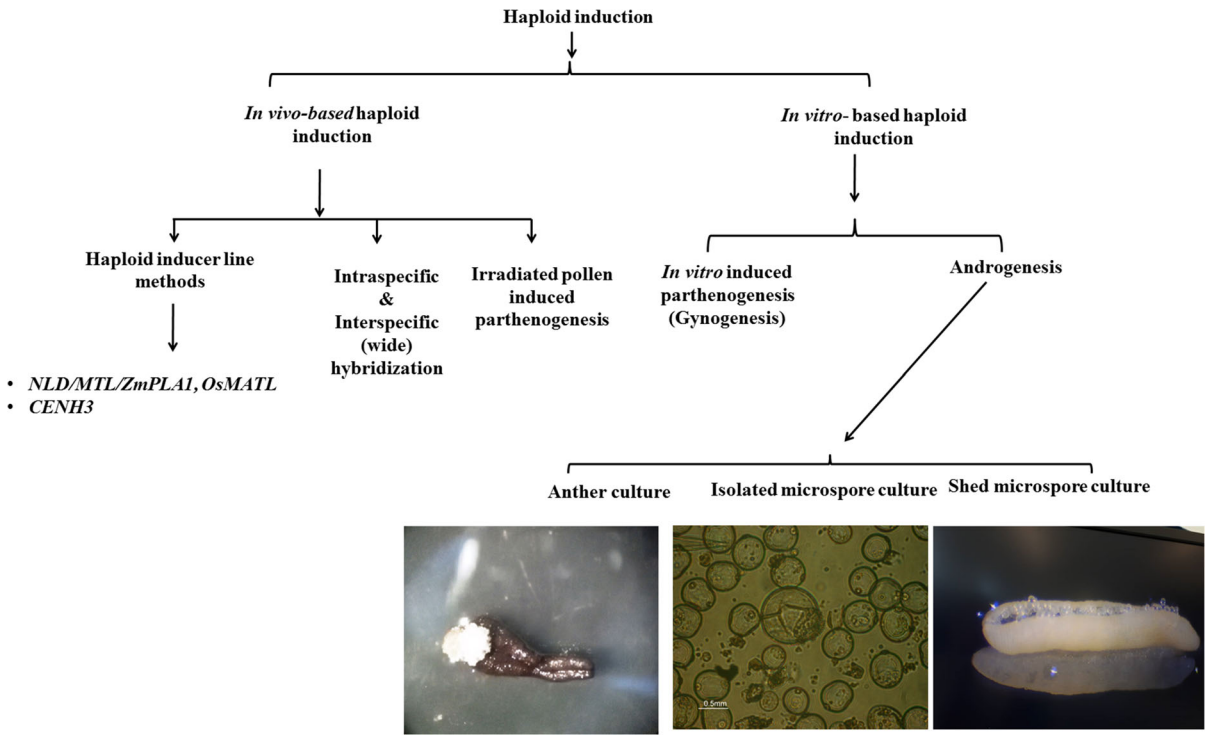

Fig. 1 The main methods of in vitro and in vivo haploid induction in different plants

retardant hormones, polyamines, compatible solutes, histone deacetylase inhibitors, cellular antioxidants, and arabinogalactan-proteins, have been used to improve the efficiency of IVB methods of haploid induction, through enhancing tolerance to embryo-inducing stresses. In the present review, we first presented a general view of major IVB methods of plant haploid induction and then highlighted the recently applied chemical enhancers (additives) to improve the efficiency of IVB methods. There are some in vivo- *in planta* haploid induction methods, such as centromere histone H3 (CENH3), which can lead to haploidy in IVB-recalcitrant genotypes and considered as universal haploid induction systems. These methods consist of one step of pollination of a line of interest, in a target plant, with a haploid inducer line (Fig. 1). A brief summary of in vivo methods of haploid induction is provided in the last sections of this review. Advantages and disadvantages of in vivo haploid inducer line methods, compared to IVB methods, are also discussed.

Applications of doubled haploid plants

Haploid refers to a sporophytic plant with gametophytic chromosome numbers (Kasha and Maluszynski 2003). Haploids are excellent examples of cellular totipotency because they are gametophyte-originated plants containing only half of the chromosome number of a zygote and/or a somatic cell (Gilles et al. 2017; Sood and Dwivedi 2015). Haploids/doubled haploids play an important role in agricultural and developmental studies.

The completely homozygous background of DHs can be used for stable gene transformation through electroporation, microprojectile bombardment, and *Agrobacterium*-mediated transformation methods (Shariatpanahi and Ahmadi 2016). Gametophytic and sporophytic pathways are the two main categories for gene transformation through isolated microspore culture (Resch and Touraev 2010). In the gametophytic route, the transferred DNA can be introduced to a mature pollen or stigma before pollination or to microspores and then in vitro matured transformed pollen grains can be used for pollination, so called male germ line transformation (Fig. 2a)

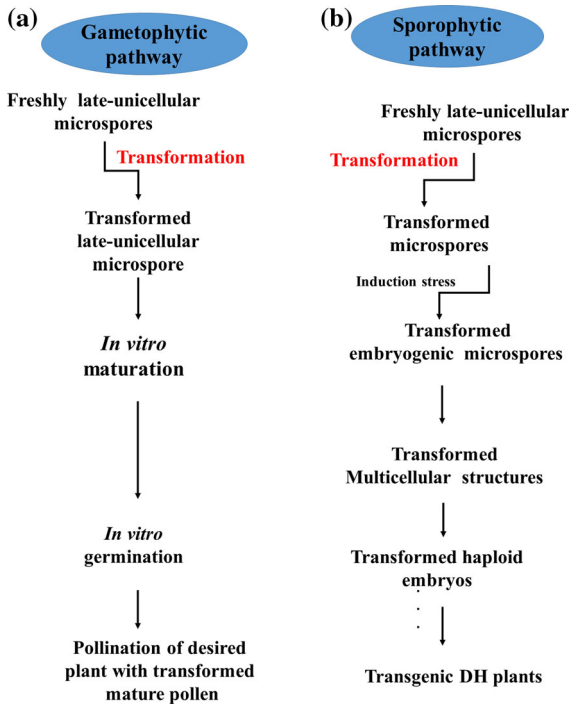

Fig. 2 The two main categories for gene transformation through isolated microspore culture. **a** The gametophytic pathway in gene transformation and, **b** the sporophytic pathway in gene transformation through isolated microspore culture

(Shariatpanahi and Ahmadi 2016). In the sporophytic pathway, embryogenic microspores are explants used for gene transformation, therefore genome doubling of transformed haploids can lead to completely homozygous transgenes (Fig. 2b) (Brew-Appiah et al. 2013). Microspore engineering, through genome editing methods of transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeats (CRISPR/Cas), has a great potential in plant breeding by producing doubled haploids (DHs) with desired characteristics in one season (Dwivedi et al. 2015).

Completely homozygous doubled haploid populations are worthwhile sources for molecular map constructs to analyze the quantitative trait loci (QTLs) of important quantitative agronomic traits, marker assisted selection (MAS), mutation and selection, reverse breeding (RB) (Shariatpanahi and Ahmadi 2016), and genome-wide association (GWAS) (Sanchez et al. 2018) studies.

In addition, validation of gene functions through the targeting induced local lesions in genomes (TILLING), gene overexpression, gene silencing, genome editing methods of Zinc-finger nucleases (ZFNs), TALENs, and CRISPR/Cas methods is much faster and easier by using modified homozygous genotypes that are valuable for phenotypic validation (Shen et al. 2015).

IVB methods of haploid induction

To date, two main IVB methods of haploid induction have been identified, including induced parthenogenesis and androgenesis. The induced parthenogenesis consists of in vitro rescue of parthenogenic embryos induced in situ through pollination with irradiated pollen, and gynogenesis (in vitro culture of ovaries or ovules) (Fig. 1). Female organs are the source of haploid induction in induced parthenogenesis, while the male cells/organs are responsible for haploid induction through the androgenesis pathway. The general principles of all IVB methods are discussed in the following sections.

In vitro induced parthenogenesis (gynogenesis)

In parthenogenesis, mitotic divisions of the egg cell, within the ovule, lead to the formation of an embryo that is genetically identical to the mother plant (Conner et al. 2017). Endosperm formation is required for development of the egg for the viable seed. There are two forms of induced parthenogenesis, including parthenogenesis induced by irradiated pollen and parthenogenesis induced by in vitro culture of female gametophyte (gynogenesis). In both forms, female organs are the sources of haploid induction. In the first method, pollination with defective pollen (pollen grains treated with radiation and/or chemicals) is stimulating. However, it is not well documented whether always such pollen fertilizes the egg cell, or whether it acts as not-fertilizing stimulus (pseudo-fertilization). Therefore, it is not an IVB method of haploid induction, but it is the in vitro rescue of in vivo induced embryos leading to haploid induction (Claveria et al. 2005). Although the efficiency of this category of induced parthenogenesis is lower than the androgenesis, the genetic stability of produced doubled haploids and also the absence of albino plants are the key advantages of this method (Sharma et al. 2018).

In gynogenesis, female gametophyte is responsible for haploid induction. In vitro culture of unfertilized (unpollinated) ovules or ovaries containing an unfertilized egg cell, or synergids, leads to development of haploid plants (reviewed by Bohanec 2009). This method is similar to the first form of induced parthenogenesis (induced by irradiated pollen), because female cells are the source of haploid induction but in gynogenesis, the stimulating role of the male part is not necessary. Intergeneric crossing is a useful pretreatment that can enhance gametophytic embryogenesis efficiency in the gynogenesis pathway. Piosik et al. (2016) reported the successful growth of haploid embryos in lettuce (*Lactuca sativa* L.) by culture of ovaries, ovules and embryo sacs after cross-pollination with *Helianthus annuus* and *H. tuberosus*. The selection of the proper flowers, containing responsive ovules, is a critical factor affecting the efficiency of gynogenesis. The genotype and surrounding environment of donor plants, physical treatments (cold pre-treatment, and heat shock), the developmental stage of female gametophyte, culture medium, plant growth regulators (PGRs), and culture conditions are the factors affecting successful haploid induction and subsequent DH production in the gynogenesis pathway (Fayos et al. 2015; Dong et al. 2016).

Androgenesis

Androgenesis is the most widely used IVB method of haploid induction (Kasha 2005). Androgenesis refers to the unusual development of cultured male reproductive units of plants, which switching their pathway from gametophytic to sporophytic (Seguí-Simarro and Nuez 2008; Makowska et al. 2017). This method can be done in three main forms, including anther culture, isolated microspore culture, and shed-microspore culture. All three methods have their own advantages and disadvantages; however, the second method (isolated microspore culture) is the most efficient one (Kasha et al. 2002; Szarejko 2003). The genotype and growth condition of donor plants are the factors that can affect the efficiency of the androgenesis pathway, similar to the gynogenesis. The developmental stage of microspores is another factor affecting androgenesis. It is mentioned that the beginning of the flowering period is the best time to harvest the anthers, while later stages will lead to the reduction of androgenesis efficiency (Dwivedi et al. 2015). The cultivation of donor plants in a controlled environmental condition can also lead to better results as compared to field cultivated plants (Datta 2005).

Anther culture

The in vitro culture of the whole immature anthers, containing microspores, is the simplest method of haploid induction through the androgenesis pathway. This method was first discovered by Guha and Maheshwari (1964).

There are many factors affect the efficiency of anther culture, including plant genotype, growth condition of donor plants, developmental stage of immature pollen grain/microspores, basal culture medium, type and concentrations of PGRs, and type and intensity of stresses (Shariatpanahi et al. 2006a; Wang et al. 2018). The thickness of the anther wall and proper bud size are the other important factors that should be considered in anther culture studies. Younger anthers have thin walls facilitating the entry of effective factors leading to a better responsiveness of its internal microspores, whereas thick walls act like a barrier (Salas et al. 2012). The position of flower buds within the inflorescence can help to isolate younger anthers before maturation, because there is a maturity gradient within the inflorescence. The identification of suitable flower buds containing microspores at the appropriate developmental stage is also very important in anther culture studies. The bud size is a selection criterion helping to better identify anthers containing microspores in a proper developmental stage. However, it is variable in different plant species (Gu et al. 2014). Wang et al. (2018) reported that callus induction in the anther culture of kiwifruit (*Actinidia arguta* Planch.) at the late-uninucleate stage of microspores was significantly more than those with microspores in tetrad, early-uninucleate, and binucleate stages.

Isolated microspore culture (IMC)

The mechanical isolation of microspores from anthers, using magnetic stirring/blending, and transferring them to a culture medium is the second form of androgenesis, called isolated microspore culture. IMC is the most common and efficient method of DH production (Shariatpanahi et al. 2006b). In the anther

culture method, the presence of the anther wall can lead to somatic embryogenesis whereas IMC is free from this disadvantage (Ferrie and Caswell 2011). The negative effects of anther walls on microspores, production of diploid somatic callus originated from anther walls, more time-consuming and labor-intensive, preventing the availability of nutrients for developing microspores through anther walls, and limitations for tracking and studying the microspores maturation and embryo development, are the disadvantages of the anther culture method, that could be avoided in the microspore culture method (Ferrie and Caswell 2011). Rapid optimization of culture conditions, due to the direct observation of microspore embryogenesis, is another advantage of isolated microspore culture over anther culture (Lantos et al. 2009). However, external supplementing of embryogenesis factors provided by anthers, is necessary in IMC (Sood and Dwivedi 2015). Albinism is a major problem for haploid induction through androgenesis in cereals (Forster et al. 2007).

Morphological characteristics such as bud size, changes in the pattern of cytokinesis, and nuclear changes are the most common and easiest indicators for identification of the proper developmental stage of microspores (Seguí-Simarro and Nuez 2008). Nuclear changes, including decondensed chromatin pattern, forming a reticulum of small chromatin patches connected together through chromatin fibers, and chromatin condensation pattern similar to interphase nucleus of cycling cells, are the markers that can be used for early monitoring of pollen embryogenesis (Testillano et al. 2000). However, genetics also helps this process through the identification and isolation of genes and proteins involved in the embryogenesis process of isolated microspores. In rapeseed (*B. napus* L.), two genes, *SERK1* and *SERK2*, were identified as the two main genes involved in microspore embryogenesis and plant regeneration, as their expression level was significantly increased during the early steps of embryo formation (Ahmadi et al. 2016). Krzewska et al. (2017) analyzed the protein profile of four winter triticale (× *Triticosecale* Wittm.) DH lines, using mapping of sub-proteome of anthers by two-dimensional gel electrophoresis, to understand the physiological background of microspore embryogenesis and reported that protein species of responsive DH lines (31 protein species) was significantly more than other investigated DH lines.

Shed-microspore culture (SMC)

In SMC, microspores are non-mechanically shed to a medium, after initial culture of intact anthers on a liquid (single-layer) or semi-liquid (double-layer) medium (Wędzony et al. 2009). In fact, SMC is the interstitial mode of anther and isolated microspore culture that is created by a simple modification in anther culture method. Physical damages to microspores in SMC is lower than IMC, however the efficiency of haploid induction in SMC may be less than IMC. Supena et al. (2006) developed a shed-microspore culture protocol in Indonesian hot peppers (*Capsicum annuum* L.) through a double-layer medium where the liquid upper layer contained zeatin and indole-3-acetic acid (IAA) of PGRs and the solid under layer contained 2% maltose plus 1% activated charcoal. They used flower buds with more than 50% late unicellular microspores and reported 76.11% haploid plants from shed-microspore culture-derived embryos. Shariatpanahi et al. (2006b) applied heat shock and starvation stresses in shed-microspore culture of two spring wheat cultivars, Falat and Rasool, and compared the results with freshly isolated microspore culture method without any stress pretreatment and reported that regeneration frequency and the percentage of green plants in freshly isolated microspore culture was significantly higher than in the SMC method. Supena and Custers (2011) reported that a reduction of incubation temperature from 28 °C to 21 °C and adding abscisic acid to the solid lower layer led to a significant improvement of their previous shed-microspore culture protocol in Indonesian hot peppers (Supena et al. 2006) so that normal-looking embryos were more than 50%.

Inductive stresses and improving the efficiency of in vitro-based methods of haploid induction

Stress treatment, applied in a variety of ways, is a common embryogenesis trigger (Shariatpanahi et al. 2006a). There are three groups of stresses, resulting in embryogenesis induction, in isolated microspore/anther cultures, including: (1) widely used stresses such as temperature (cold and heat), carbon starvation, and colchicine, (2) neglected stresses such as gamma irradiation, ethanol stress, centrifugal treatment, reduced atmospheric pressure, and abscisic acid, and (3) novel stresses such as high medium pH,

carrageenan oligosaccharides, heavy metals stress, 2,4-Dichlorophenoxyacetic acid (2,4-D) pre-treatment, and chemical inducers (Shariatpanahi et al. 2006a). The positive effect of cold pretreatment (4 °C for 2 days) followed by heat shock (30 °C for 2–5 days) has been reported on microspore embryogenesis efficiency in SMC of tomato (*Lycopersicon esculentum* L.) (Ahmadi et al. 2015). Heidari et al. (2017) investigated the effect of cold pretreatment and heat shock on the efficiency of anther culture in sweet peppers (*Capsicum annuum* L.) and reported the positive effect of both cold (4 °C for 24 h) and heat (35 °C for eight days) shocks on the induction of microspore embryogenesis and regeneration of plantlets. Bhatia et al. (2016) studied the effect of two different heat shock treatments (30 and 32.5 °C) in microspore embryogenesis of cauliflower (*B. oleracea* var. *botrytis* L.) and reported the highest number of embryos per petri dish obtained by heat shock treatment at 30 °C for 24 h followed by maintenance at 25 °C.

Despite their vital role in guiding gametophytic cells towards embryogenesis, induced stresses have an adverse effect on the metabolism and growth of the regenerated plants (Pourabdollah Najafabadi et al. 2015). In addition, these stresses can lead to recombination events in nuclear genes or chloroplast genome, resulting in low regeneration and albino plants in DH plants (Shariatpanahi et al. 2006b). The selection of the suitable stresses and their concentrations/intensity and durations are the critical points that must be addressed in any target plant. Pourabdollah Najafabadi et al. (2015) compared the effects of heat shock (30 ± 0.5 °C for 14 days) and 2,4-D treatment (159.08 µM/L for 30 min) on morphological and physiological characteristics of microspores and microspore-derived DH plants of *B. napus* and reported that the total protein content of treated microspores was reduced by using both 2,4-D and heat shock, whereas heat shock treatment led to an increase in concentration of chlorophyll a and b of DH plants.

Although there are some internal responses to applied inductive stresses, such as increase the accumulation of proteins responsible for cell defense against oxidative stress, like L-ascorbate peroxidase and HSP70, (Krzewska et al. 2017). However, using some components that are able to reduce stress-induced cell death during microspore embryogenesis is an applicable strategy to enhance the efficiency of IVB haploid induction. There are various strategies to improve the efficiency of IVB methods of haploid induction. One of these solutions is co-culture strategy. The co-culture of isolated microspores with immature ovaries/pistils is a strategy to enhance the efficiency of microspore embryogenesis in some plant species (Żur et al. 2015). Ovary co-culture is an efficient strategy to enhance microspore embryogenesis efficiency of both monocots and dicots (Lantos et al. 2009). It seems that ovaries release active signaling molecules, which can increase microspore-derived embryo yield and improve plant regeneration efficiency or act like nurse agents (Żur et al. 2015; Lantos et al. 2009). Ovary or microspores–conditioned medium has also been applied to enhance microspore embryogenesis. The addition of conditioned medium from actively growing isolated ovaries or microspore cultures of other responsive plant genotypes can potentially enhance the embryogenesis efficiency of isolated microspore cultures in recalcitrant genotypes. Lantos et al. (2018) investigated the effect of ovary co-culture in isolated microspore culture of spelt wheat (*Triticum spelta* L.) and reported the positive effect of ovary co-culture on development of embryo-like structures.

It has been reported that addition of some chemical treatments to the culture medium can improve the efficiency of IVB method of haploid induction (Chen et al. 2019). The enhancement of tolerance to embryo-inductive stresses, using different chemical enhancers, is another important strategy to improve the efficiency of IVB methods of haploid induction. Here, we focused on recently applied chemical enhancers used as additive materials in IVB methods of haploid induction in different plant species.

Additives influencing IVB haploid induction

The induction phase of IVB methods of haploid induction is more important than the regeneration phase (Wędzony et al. 2009), therefore, working on this step is crucial for successful haploid induction. As aforementioned, application of some specific additives can improve tolerance to embryo-inducing stresses and subsequent lead to a significant increase in haploid induction efficiency. The main groups of these additives are i.e. PGRs, stress hormones, polyamines,

compatible solutes, cellular antioxidants, and histone deacetylase inhibitors. Some of these additives are discussed in the following sections.

PGRs: Plant growth regulators—synthetic versions of phytohormones—are key signaling molecules affecting the efficiency of in vitro studies, including IVB methods of haploid induction. These materials can affect gametophyic embryogenesis and final haploid/DH regeneration through biosynthesis, distribution, gene expression or signaling pathways steps (Żur et al. 2015). Besides regulating role in development and basic metabolism, PGRs play an important role in the acclimation of living organisms to changing environment or stresses (Malaga et al. 2020). Greater production of IAA has been reported in cucumber (*Cucumis sativus* L.) seedlings under chilling stress, and authors mentioned that IAA acts as a downstream signal mediating in tolerance to chilling stress in cucumber (Zhang et al. 2020). The type of PGRs and the ratio of auxin:cytokinin are the two most important points in androgenesis cultures (Abdollahi and Rashidi 2018). Although it has been reported that exogenous application of PGRs is not required for microspore embryogenesis through isolated microspore cultures (Żur et al. 2015), however, pretreatment with regulators or changing the hormone composition of the culture medium can increase the efficiency of microspore embryogenesis in recalcitrant and low-responding wheat and barley genotypes (Esteves and Belzile 2018). However, the efficiency of PGRs in IVB haploid induction depends on the plant species. Among PGRs, 2,4-D has unique ability to induce somatic embryogenesis in many species, maybe because of its allosteric/electrostatic binding effects (Phillips and Garda 2019). As the same basic cellular mechanisms, cell expansion and asymmetric cell division, regulate both somatic and gametophytic embryogenesis (Esteves and Belzile 2018), 2,4-D is important in gametophytic embryogenesis. However, 2,4-D is one of the PGRs that can lead to somaclonal variation in tissue culture studies through DNA mutation and stimulation of unorganized cell growth (Niazian et al. 2017a), therefore more care and attention are required for the application of 2,4-D in IVB haploid induction studies. The positive effect of a higher concentration of 2,4-D (45.44 µM/L) in anther cultures and haploid induction of chickpeas (*Cicer arietinum* L.) has been reported (Abdollahi and Rashidi 2018). The external treatment of donor plants with 2,4-D has also been reported as a positive treatment in microspore embryogenesis. Nowaczyk et al. (2016) treated six hot and sweet F_1 hybrids of peppers with 4.54 µM/L of 2,4-D, by applying water solution of 2,4-D on the whole plants, one day before transferring anthers into culture and reported that the number of embryos and plantlets were increased in three of the investigated hybrids. Mishra et al. (2017) reported that an equal ratio of 2,4-D: kinetin (5 µM/L), for callus induction in anther culture of *Camellia assamica*, and then transfer of induced calli to Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 10 µM/L 6-Benzylaminopurine (BAP) + 3 µM/L gibberellic acid (GA3) + 5474.20 µM/L L-glutamine + 1903.13 µM/L L-serine, resulted in 85% embryogenesis. The positive effect of cytokinin thidiazuron (TDZ) in combination with auxin dicamba (DIC) has been reported in gametophytic embryogenesis of cereal species (Esteves and Belzile 2018).

Stress hormones: stress hormones are another category of additives that can enhance somatic/gametophytic embryogenesis. These are hormone-like endogenous regulators, mainly produced in response to biotic and abiotic stresses, which can manage various growth and developmental process in plants such as cell division (Ahmadi et al. 2014a). These components can increase the accumulation of nitrogen and sulfur in plant cells and subsequently maintain cell membranes and photosynthetic ability under stressful conditions (Gupta and Huang 2014). Figure 3 shows the possible pathways and mechanisms through which stress hormones can enhance the efficiency of somatic/gametophytic embryogenesis. Our results showed the positive effect of stress hormones on the microspore embryogenesis of *B. napus* (Ahmadi et al. 2014a). In three independent experiments, different concentrations of stress hormones, including abscisic acid (ABA) (0.75, 1.89, 3.78, 7.56, and, 18.91 µM/L), jasmonic acid (JA) (0.95, 2.37, 4.75, 9.51, and 23.77 µM/L), and salicylic acid (SA) (1.44, 3.62, 7.24, 14.48, and 36.20 µM/L) were applied in different incubation periods (6, 12, and 24 h). In final, 1.89 µM/L of ABA for 12 h, 4.75 µM/L JA for 24 h, and 1.44–3.62 µM/L of SA for 6 h, were chosen as the best concentrations and incubation durations of applied stress hormones for embryogenesis in the microspore culture of *B. napus* (Ahmadi et al. 2014a).

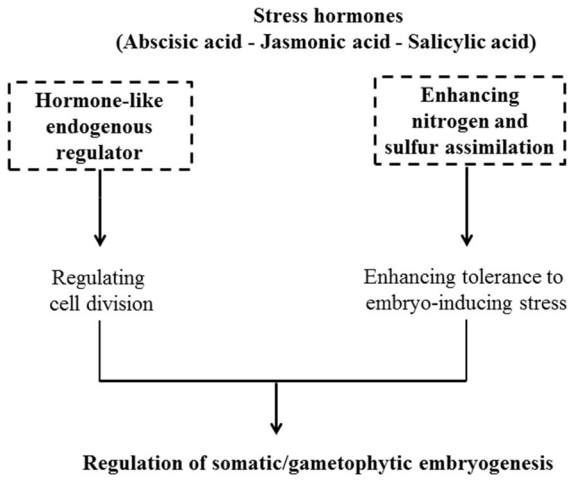

Fig. 3 The possible mechanisms of involvement of stress hormones in somatic and gametophytic embryogenesis of plants (The dashed frames indicate to the main ways that additives affect the plant responses to inductive stresses)

Cycocel (CCC): chlormequat chloride or cycocel (CCC) is another kind of PGRs in which significant effects on stem elongation, induction and improvement of flowering, and somatic embryogenesis have been reported (Agustí and Primo-Millo 2020; Shariatpanahi et al. 2018). CCC is a growth retardant that inhibits gibberellin metabolism by blocking cyclases copalyl-diphosphate synthase and *ent*-kaurene synthase (Chen and Chang 2003). The CCC-mediated disrupted metabolism of gibberellin can subsequently lead to reduced stem elongation, improvement of flowering, the enhancement of multiple buds per shoot, and the enhancement of somatic/gametophytic embryogenesis (Fig. 4). Our results also showed the positive effect of CCC in in vivo haploid induction (Ebrahimzadeh et al. 2018). Different concentrations of CCC, including 407.77, 4077.77, and 40,777.71 µM/L, were applied in the parthenogenesis pathway of cucumbers and haploid embryos were significantly increased when mother plants were sprayed with 407.77 µM/L of cycocel (Ebrahimzadeh et al. 2018).

Polyamines: polyamines are the another group of additives that can be used to improve gametophytic embryogenesis. Putrescine, spermidine, and spermine are the polyamines that are present in all living organisms and are considered as key modulators of plant growth and development. This category of materials acts like PGRs and therefore can be involved in the embryogenesis process, through interaction with nitric oxide (Tiburcio and Alcázar 2018). In addition to a hormonal role, these materials can act as carbon and nitrogen reserves and therefore can control many critical developmental processes. The cationic nature of putrescine, spermidine, and spermine give them the ability of interaction with phosphate groups of DNA, anionic components of phospholipids, and also cell wall components (Kakkar and Sawhney 2002; Sakhanokho et al. 2005; Tiburcio and Alcazer 2018). Polyamines are ethylene inhibitors that can improve the efficiency of anther culture experiments by delaying the senescence of cultured anthers (Sarao and Gosal 2018). Figure 5 shows the possible mechanisms of involvement of putrescine, spermidine, and spermine in somatic and gametophytic embryogenesis. Our results showed the positive effect of polyamines in androgenesis of *B. napus* (Ahmadi et al. 2014b) and sweet pepper (Heidari-Zefreh et al. 2018). The effect of different concentrations of putrescine, including 2.26, 5.67, 11.34, 22.68, and 56.72 µM/L, for 12, 24, and 48 h on the microspore embryogenesis of *B. napus* was investigated and it has been discovered that the application of 2.26 µM/L putrescine for 48 h increased microspore embryogenesis three-fold. In addition, a normal plantlet regeneration of 92% was achieved by the application of 5.67 µM/L putrescine for 48 h. In isolated microspore culture of sweet pepper, application of 6.67–11.34 µM/L putrescine, during the mannitol starvation treatment, led to the significant increase in multicellular structures, cotyledonary embryos, and haploid regenerants (Heidari-Zefreh et al. 2018). In addition to IVB methods of haploid induction, the positive effect of putrescine and spermidine in in vivo haploid induction of cucumber (irradiated pollen induced parthenogenesis), through the spraying of mother plants, has also been reported (Ebrahimzadeh et al. 2018).

Compatible solutes: compatible solutes are another category of additives that can enhance embryogenesis in IVB methods of haploid induction. These are low molecular and highly soluble organic compounds (sugar, amino acid, nitrogen and sulphur containing compounds) accumulated in large quantities when plants are exposed to stress (Ahmadi and Shariatpanahi 2015; Handa et al. 2018). These are osmoprotectant components that can interact with crucial macromolecules, with both hydrophobic and hydrophilic domains, such as plasma membranes,

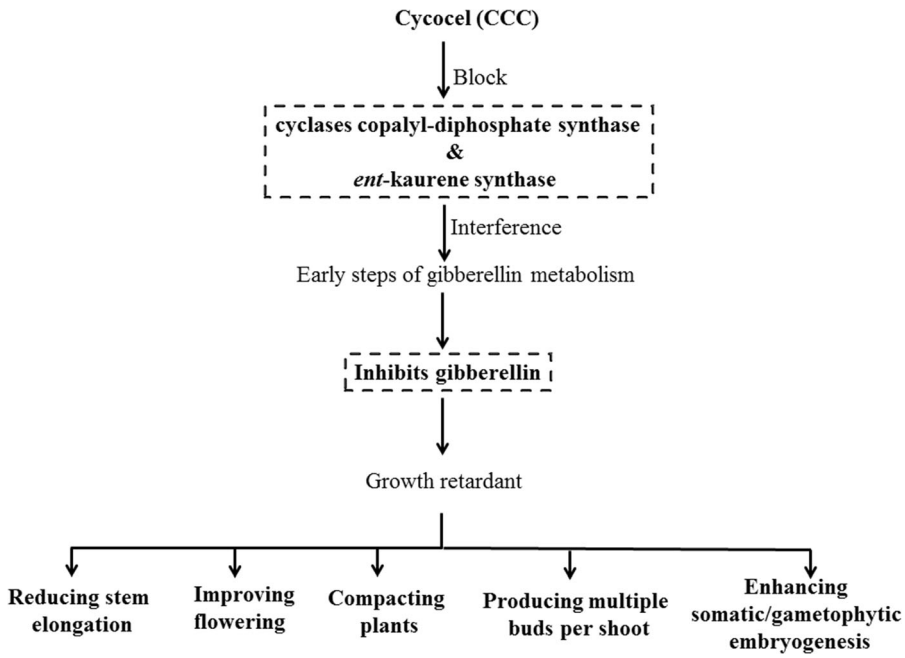

Fig. 4 The possible mechanisms of involvement of cycocel in somatic and gametophytic embryogenesis of plants (The dashed frames indicate to the main ways that additives affect the plant responses to inductive stresses)

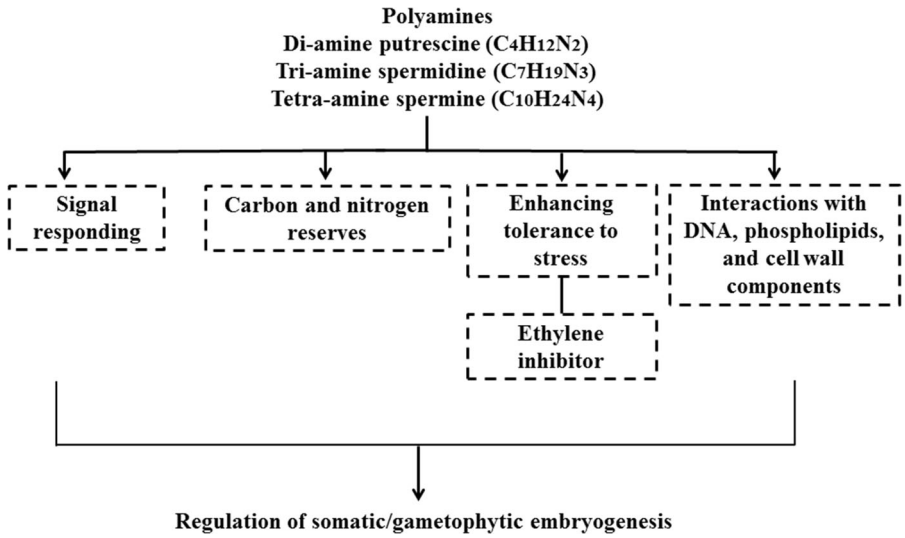

Fig. 5 The possible mechanisms of involvement of putrescine, spermidine, and spermine polyamines in regulation of somatic and gametophytic embryogenesis of plants (the dashed frames indicate to the main ways that additives affect the plant responses to inductive stresses)

antioxidative enzymes, photosynthetic elements, stabilized proteins, and critical elements of both electron transport chains (Gupta and Huang 2014). This property gives compatible solutes the ability to modulate biological processes such as embryogenesis. In addition, these reactive oxygen species (ROS) scavenging materials can enhance tolerance to embryo-inducing stress in plants (Ahmadi and Shariatpanahi 2015). All possible pathways, through which compatible solutes can enhance somatic and

gametophytic embryogenesis, are presented in Fig. 6. Our investigations showed the positive effect of proline and chitosan compatible solutes in the SMC of tomato (Ahmadi et al. 2015) and in the IMC of *B. napus* (Ahmadi and Shariatpanahi 2015). We found a positive effect of an application of 32.76 µM/L chitosan on callogenesis and shoot regeneration in the SMC of tomato (Ahmadi et al. 2015). The effects of different concentrations of proline (434.29, 868.58, 173,716, and 4342.91 µM/L) and chitosan (6.55, 13.10, 32.76, and 65.53 µM/L) were assessed on microspore embryogenesis efficiency of *B. napus* and the application of 868.58 µM/L of proline and 6.55 µM/L of chitosan led to significant increase in microspore embryogenesis (Ahmadi and Shariatpanahi 2015).

DNA demethylating agents and histone deacetylase inhibitors: DNA methylation and histone methylation and acetylation are the important factors that control functional status of chromatin and subsequent regulate gene expression during cell proliferation and differentiation. Epigenetic reprogramming, mainly a global DNA methylation decrease, is one of the cellular processes that happen during stress-induced embryogenesis (Testillano 2018). The positive correlation of low levels of H3K9 methylation with microspore reprogramming and initiation of embryogenesis has been proved (Testillano 2018). In *B. napus*, high levels of acetylated histones H3Ac and H4Ac has been reported in vacuolated microspores (Rodríguez-Sanz et al. 2014). Therefore, using DNA demethylating agents and histone deacetylase inhibitors can improve the efficiency of IVB haploid induction. Application of a DNA demethylating agent, 5-Azacytidine (AzaC) increased embryogenesis induction in microspore culture of *B. napus* and barley (Solís et al. 2015). Zhang et al. (2016) investigated the effect of three different histone deacetylase inhibitors, including trichostatin A, suberoylanilide hydroxamic acid, and sodium butyrate on microspore embryogenesis of pak choi (*Brassica rapa* ssp. *chinensis* L.). They reported that the largest embryo yield and the highest frequency of plant regeneration were achieved from NLN-13 medium supplemented with 0.05–0.1 µM of suberoylanilide hydroxamic acid. Application of BIX-01294, a small molecule that inhibits H3K9 methylation, enhanced microspore reprogramming and embryogenesis initiation in *B. napus* and barley (Berenguer et al. 2017). Wang et al. (2019) applied epigenetic chemicals of trichostatin A, scriptaid, BIX-01294, and sodium butyrate, as histone deacetylase inhibitors, in microspore culture of wheat and reported that only trichostatin A could enhance both embryogenesis and green plant regeneration.

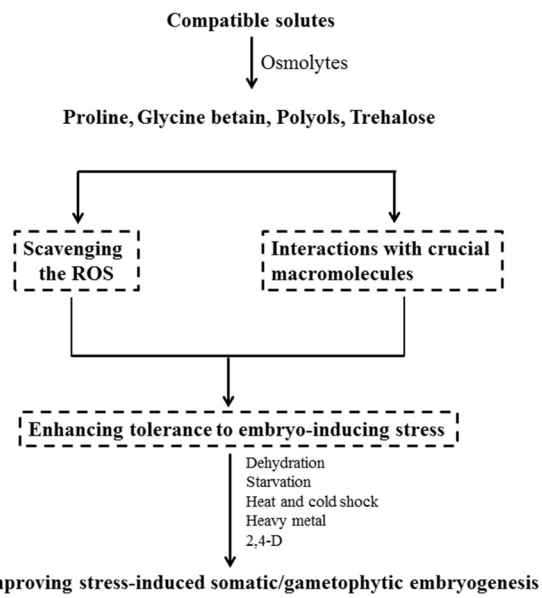

Fig. 6 The possible mechanisms of involvement of compatible solutes including proline, glycine betain, polyols, and trehalose osmoprotectant in somatic and gametophytic embryogenesis of different plants (The dashed frames indicate to the main ways that additives affect the plant responses to inductive stresses)

Cellular antioxidants: Maintaining the balance of ROS is very important for plant cell proliferation and differentiation. Antioxidants are one of the most important components to maintain ROS balance, by scavenging cellular ROS accumulation (Chen et al. 2020). The positive effect of low-molecular weight antioxidant of glutathione on microspore embryogenesis and increase the number of embryo-like structures has been reported in isolated microspore cultures of triticale (Żur et al. 2019). Other materials with antioxidant characteristics, such as L-ascorbic acid, can increase the antioxidant enzyme activities and antioxidant contents of treated cells (Chen et al. 2020). Heidari-Zefreh et al. (2018) reported that application of optimum concentrations of ascorbic acid (20 and 50 mg/L) under the mannitol starvation and heat shock treatment (32 °C) led to the significant increase in the number of cotyledonary embryos produced in isolated microspore culture of sweet pepper. In

isolated microspore culture of broccoli (*Brassica oleracea* L. var. italica), the embryogenesis efficiency, under heat shock stress (32.5 °C for 24 h) was 1.2-fold and 2.5-fold increased by application of 10 mg/L of reduced ascorbate and 20 mg/L of reduced glutathione, respectively (Zeng et al. 2017). In microspore culture of Zengcheng flowering Chinese cabbage (*Brassica campestris* L.), a 10.33-fold increase of frequency of embryogenesis was reported when L-ascorbic acid sodium salt (0.2 μM) was added to NLN-13 medium (Niu et al. 2019). Methylene blue is another kind of antioxidants whose positive effect has been reported in microspore embryogenesis of ornamental kale (*Brassica oleracea* var. acephala) (Chen et al. 2019).

Cell wall remodeling agents: Arabinogalactan-proteins (AGPs)—a type of cell surface glycoproteins enriched in arabinose and galactose residues—are a group of cell wall proteins that involved in cell growth, division and expansion, embryo pattern formation, modulation of cell wall mechanics or defense. Changes in the composition of these cell wall proteins have been reported during both microspore embryogenesis (Corral-Martínez et al. 2019) and somatic embryogenesis (Pérez-Pérez et al. 2019). It is modifications of AGPs and pectins, as two main components of cell wall, which causes remodeling of cell wall during somatic embryogenesis (Pérez-Pérez et al. 2019). Cell wall remodeling is one of the cellular processes that involved in stress-induced microspore embryogenesis. AGPs are involved in first embryogenic divisions of the microspore (Testillano 2018). The stimulating effect of AGPs, secreted from cells into the culture medium, on embryo development has been reported in microspore culture of maize (Testillano et al. 2010). Makowska et al. (2017) reported the regeneration rate of up to 2.8 times in the anther culture of barley (*Hordeum vulgare* L.) by adding 37.54 μM/L gum arabic, as a carrier of AGPs. In anther culture of tomato it was found that gum arabic has more important role than cold pretreatment and kinetin in callus induction and regeneration (Niazian et al. 2019).

Other applied additives: Silver nitrate, polyvinylpyrrolidone (PVP), colchicine antimitotic agent, and activated charcoal are other additives used for enhancing gametophytic embryogenesis.

Silver nitrate ($AgNO_3$) is an effective anti-browning additive that can promote in vitro regeneration through inhibiting ethylene production and phenolic secretion (Kumar et al. 2016). Silver nitrate was reported to have positive effect in induction of morphogenic callus in anther culture of rice (Sarao and Gosal 2018). Abdollahi and Rashidi (2018) reported a positive and significant effect of silver nitrate (88.75 μM/L) on the number of regenerated embryos and final regenerated plants in the anther culture of recalcitrant chickpeas.

Another anti-browning agent with antioxidant activity is PVP (Phillips and Garda 2019). The positive effect of PVP on somatic embryogenesis has been reported previously (Parthibhan et al. 2018). Callus browning was reduced, and subsequently the androgenic response was significantly enhanced, when PVP (2 mg/L) was added to culture media in anther culture of borage (*Borago officinalis* L.) (Abdoallahi et al. 2017).

The positive effect of the antimitotic agent, colchicine, in the anther culture of borage (*Borago officinalis* L.) has been reported (Hoveida et al. 2017), however, its application was not effective in inducing embryo-like structures and green plants in the anther culture of winter and spring triticale hybrids (Ślusarkiewicz-Jarzina et al. 2017).

One of the signaling molecules involved in stress response and activation of the embryogenic program is calcium (Ca^{2+}). Rivas-Sendra et al. (2017a) reported a dramatic increase in Ca^{2+} level during in vitro-induced microspore embryogenesis of *B. napus* and progressively decrease in its level after the heat shock-based inductive treatment. These results reflect the relationship between changes in Ca^{2+} level and subcellular distribution, and microspore embryogenesis (Rivas-Sendra et al. 2017a). Application of calcium in culture medium can improve the efficiency of microspore embryogenesis (Ahmadi et al. 2018).

In addition to applied chemical enhancers, there are other additives that participate in biotic and abiotic stress tolerance. Materials that involve in preventing accumulation of ROS, ethylene and gibberellin inhibitors, and activators of antioxidant enzyme activity, detoxification capacity, and defense response signal pathway, could potentially be used against applied embryo-inducing stresses in IVB haploid induction (Fig. 7). In addition to aforementioned ROS scavenging materials, another component that can decrease the stress caused by ROS is boron (H_3BO_3), as its positive effect has been reported in

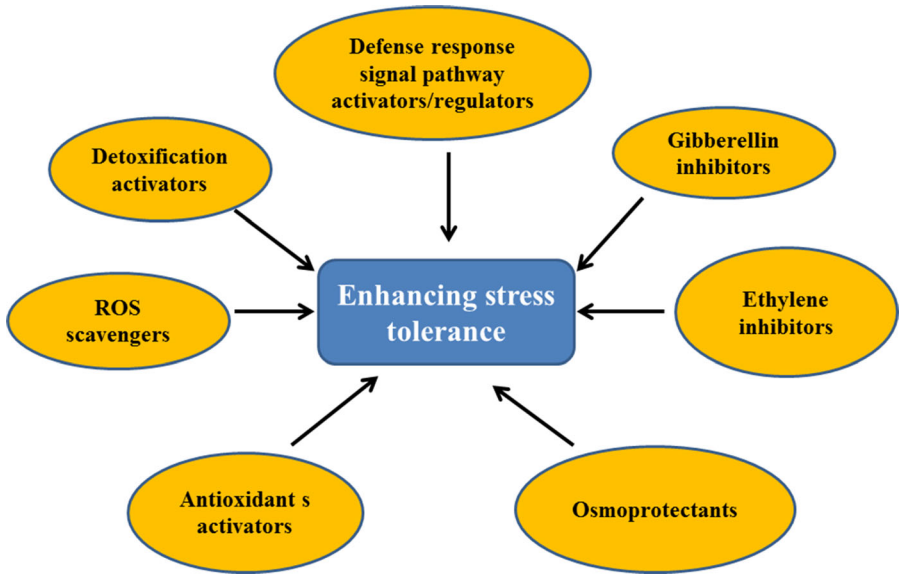

Fig. 7 Chemical enhancers applicable in in vitro haploid induction methods

microspore embryogenesis of *B. napus* (Mahasuk et al. 2017).

Besides aforementioned phytohormones, including auxins, cytokinins, abscisic acid, salicylic acid, polyamines, and jasmonates, other phytohormones such as brassinosteroids and strigolactones are involved in imparting stress tolerance in plants (Khan et al. 2020). The positive effect of hydrogen sulfide and brassinosteroids has been reported during chilling stress (Fang et al. 2019; Zhang et al. 2020).

Other gibberellin inhibitors, such as triiodobenzoic acid, benzothiazole-2-oxyacetate, and N-dimethylamino succinamic acid (Agustí and Primo-Millo 2020), are applicable in IVB haploid induction.

Some recent IVB haploid induction studies where different additives have been used, in response to applied inductive stresses, to enhance the regeneration and final haploid induction efficiency in different plant species are presented in Table 1.

In vivo haploid induction methods

The alternative methods of haploid induction, which avoid in vitro procedures, are in vivo methods of haploid induction (Watts et al. 2018). Irradiated pollen induced parthenogenesis, intra and interspecific hybridization, and haploid inducer line methods, are the main in vivo approaches of haploid induction (Fig. 1). One of the recently highlighted in vivo haploid inducer line methods is known as "targeted centromere manipulation (CENH3) method". The centromeric loss of CENH3 protein is responsible for centromere inactivity and uniparental elimination of *H. bulbosum* (Sanei et al. 2011). The targeted manipulation of *CENH3* can lead to a defective spindle attachment and subsequently haploid inducer line in different crops (Ishii et al. 2016), similar to the inducer line in maize. Haploid induction through CENH3 method has been reported in maize (Kelliher et al. 2016), carrot (*Daucus carota*) (Dunemann et al. 2018), tomato, rice, cucumber, and melon (Kalinowska et al. 2019). State-of-the-art and novel developments of in vivo haploid induction methods has been discussed in detail by Kalinowska et al. (2019) and Watts et al. (2018).

IVB versus *in planta* CENH3 method

IVB methods of haploid induction have their own specific complexities. Huge numbers of factors are involved in induction phase of these methods. In addition, optimization of in vitro regeneration is a complicated and difficult step. These are highly technical, labor-intensive, time and costly consuming procedures, on top of their species and genotype dependency and low rate of haploid induction (Dwivedi et al. 2015).

The efficiency of the in vivo CENH3 method can be divided into two steps. The first step is to develop a haploid inducer line and the second step is the cross of

Table 1 Examples of applied inductive stresses and corresponding additives in in vitro-based haploid induction in different plants

Plant species	Pathway	Inductive stress(s)/applied additive(s)	Regeneration (%)[a]	Haploid induction (%)[b]	Reference
Barley (*Hordeum vulgare* L.)	Anther culture	Cold pretreatment/ arabinogalactan-proteins	98.00	–	Makowska et al. (2017)
Borage (*Borago officinalis* L.)	Anther culture	Cold pretreatment/Colchicine, n-butanol	35.00–40.00	65.12	Hoveida et al. (2017)
	Anther culture	Cold pretreatment/Activated charcoal, ascorbic acid, silver nitrate, PVP	–	–	Abdollahi et al. (2017)
Broccoli (*Brassica oleracea* L.)	Microspore culture	Cold pretreatment, heat shock/ Ascorbate, glutathione	79.00	–	Zeng et al. (2017)
Cabbage (*Brassica oleracea*)	Microspore culture	Heat shock/Activated charcoal	–	–	Li et al. (2020)
Chickpea (*Cicer arietinum* L.)	Anther culture	Cold pretreatment, heat shock/ Silver nitrate	–	72.00	Abdollahi and Rashidi (2018)
Chinese cabbage (*Brassica campestris* L.)	Microspore culture	Cold pretreatment, heat shock/ Ascorbic acid	44.30	21.81	Niu et al. (2019)
Cucumber (*Cucumis sativus* L.)	Anther culture	Cold pretreatment/PGRs (2,4-D, BAP, kinetin), amino acids (arginine, asparagine, cysteine, glutamine)	23.30	–	Amirian et al. (2020)
Eggplant (*Solanum melongena* L.)	Anther culture	Heat shock/PGRs (NAA, BAP)		–	Rivas-Sendra et al. (2017b)
Kale (*Brassica oleracea*)	Microspore culture	Heat shock/Methylene blue	95.79	37.32	Chen et al. (2019)
Kiwifruit (*Actinidia arguta* Planch.)	Anther culture	Cold pretreatment/PGRs (2,4-D, kinetin, NAA)	13.70–64.55	3.36	Wang et al. (2018)
Lemon balm (*Melissa officinalis* L.)	Anther/Microspore culture	Cold pre-treatment, heat shock/ PGRs (NAA, 2,4-D)	–	–	Kästner et al. (2016)
Pakchoi (*Brassica rapa* L.)	Microspore culture	Cold pretreatment/trichostatin A, suberoylanilide hydroxamic acid, sodium butyrate	75.01–87.30	–	Zhang et al. (2016)
Pepper (*Capsicum annuum* L.)	Anther culture	Cold pretreatment, heat shock/ PGRs (2,4-D, Kin, BA), $AgNO_3$	19.05	–	Popova et al. (2016)
Pepper *Capsicum* spp	Anther culture	Heat shock/2,4-D pre-treatment	27.00	–	Nowaczyk et al. (2016)
Perennial ryegrass (*Lolium perenne* L.)	Anther culture	Cold pre-treatment/PGRs (2,4-D)	86.00	42.48	Begheyn et al. (2017)
Pumpkin (*Cucurbita moschata* Duch.)	Gynogenesis	Heat shock/PGRs (2,4-D, BAP, NAA, TDZ)	–	57.10	Kurtar et al. (2018)
Rapeseed (*B. napus* L.)	Microspore culture	Cold pretreatment/Boron	–	–	Mahasuk et al. (2017)

Table 1 continued

Plant species	Pathway	Inductive stress(s)/applied additive(s)	Regeneration (%)[a]	Haploid induction (%)[b]	Reference
Rice (*Oryza sativa* L.)	Anther culture	Cold pretreatment/PGRs (2,4-D, BAP)	68.20	–	Naik et al. (2017)
Stevia (*Stevia rebaudiana*)	Anther culture	Cold pretreatment, heat shock/ PGRs (2,4-D, BAP, NAA)	37.50	–	Uskutoğlu et al. (2019)
Sugar beet (*Beta vulgaris*)	Gynogenesis	Cold pretreatment/PGRs (BAP)	37.80	11.30	Pazuki et al. (2018a)
	Gynogenesis	Cold pretreatment/colchicine, PGRs (kinetin)	–	–	Pazuki et al. (2018b)
Sweet pepper (*Capsicum annuum* L.)	Microspore culture	Mannitol starvation, heat shock/ Putrescine, ascorbic acid	49.70	–	Heidari-Zefreh et al. (2018)
Tea plant (*Camellia assamica*)	Anther culture	Cold pretreatment, heat shock/L-glutamine, L-serine, PGRs (2,4-D, kinetin)	65.00	90.00	Mishra et al. (2017)
Triticale (X *Triticosecale* wittmack)	Anther culture	Cold pretreatment, heat shock/ Ascorbic acid, gibberellic acid	19.50	26.70	Yerzhebayeva et al. (2017)
	Microspore culture	Cold shock/Glutathione	–	–	Żur et al. (2019)
Wheat (*Triticum aestivum* L.)	Microspore culture	Cold pretreatment/Trichostatin A, scriptaid, BIX-01294, sodium butyrate	–	70.00	Wang et al. (2019)
	Microspore culture	Cold pretreatment/Trichostatin A	–	–	Jiang et al. (2017)
Winter Squash (*Cucurbita maxima* Duch.)	Gynogenesis	Heat shock/PGRs (2,4-D, BAP, NAA, TDZ)	–	74.46	Kurtar et al. (2018)

[a]Regeneration percentage according to ratio of regenerants after hardening to the total regenerants

[b]Haploid induction percentage according to ratio of haploids obtained to the total obtained plant after hardening

Table 2 The comparison of commonly used in vitro-based methods versus in vivo-CENH3 method of haploid induction

	In vivo CENH3 method	In vitro-based methods
Advantages	Single step for haploid induction	Cost effectiveness
	Universality	The genetic stability of DHs
	In planta haploid embryos	
Disadvantages	Complexity of inducer line creation	Complexity of in vitro experiments
	Optimization of gene transformation protocol	Genotype dependency
	Searching for null mutants	Optimization of in vitro regeneration protocol
	Very Low efficiency	Accurate identification of buds with embryogenic microspores and female flowers contain responsive ovules
		Albinism (especially in androgenesis of cereals)
		Synchronizing the flowering time of two parents from different species
		The absence of spontaneous chromosome doubling
		Low efficiency
Output(s)	Optimized in vitro regeneration protocol	Optimized in vitro regeneration protocol
	Optimized gene transformation protocol	Haploid plants
	Haploid plants	

the haploid inducer line with a wild-type parent. Haploid inducer line either must be found in TILLING population or created through genetic engineering methods. Both pathways have their own difficulties. In vitro regeneration and gene transformation need to be optimized for second pathway of haploid inducer line production (genetic engineering). For gene transformation, *Agrobacterium*-mediated is the most effective method, which can be done in both in vitro and *in planta* forms (Niazian et al. 2017b).The second step of haploid induction through haploid inducer line methods is also critical. The cross of produced haploid inducer and wild-type plants is important. In the CENH3 method, it is suggested that the maternal haploid inducer and paternal wild-type can lead to better results (Ravi and Chan 2010).In both in vitro and in vivo methods of haploid induction we faced a complex and multifactorial situation (Żur et al. 2019). However, there are some automated and predictive methods that can help us to a better management of multivariable researches and increase the efficiency of in vitro studies, including image processing, algorithm genetics, and artificial neural networks (Abdipour et al. 2018; Hesami et al. 2019; Niazian et al. 2018a, b, c). A coupled image processing-artificial neural network system has been used to find the best developmental stage of donor plants and most effective factor on callus induction and regeneration in anther culture of tomato (Niazian et al. 2019).

Some advantages and disadvantages of the IVB methods versus in vivo-*in planta* haploid inducer line methods are presented in Table 2.

Conclusions

The completely homozygous genome of DHs is a valuable platform for many research areas, including hybrid varieties production, genetic transformation, RB, linkage maps in QTL, GWAS, and marker assisted selection studies. In addition, the homozygous DH lines are suitable substrates for new methods of plant gene function programs including TILLING, TALENs, ZFNs, and CRISPR/Cas. There are two main categories of plant haploid induction including in vitro and in vivo methods. IVB methods of haploid induction consist of induction and regeneration phases. Application of inductive stresses is crucial for haploid induction. However, cell death, oxidative stress and production of ROS are the consequences of inductive stress for gametophytic embryogenesis. Autophagy, cell death, epigenetic modifications, and cell wall remodeling are different cellular processes involved in stress-induced embryogenesis. Treatment with some additives, whose effects are modulators of

the involved cellular processes, can regulate and increase the efficiency of gametophytic embryogenesis. Application of some components that are able to reduce stress-induced cell death during microspore embryogenesis, such as autophagy and/or protease modulators, DNA demethylating agents and histone deacetylase inhibitors, and cell wall remodeling agents can enhance the efficiency of IVB haploid induction. In addition, using some chemical additives can enhance tolerance to inductive stress and improve the efficiency of IVB methods of haploid induction. In recent years, the positive effect of some additives (chemical enhancers), including PGRs, stress hormones, polyamines, compatible solutes, cellular antioxidants, silver nitrate, colchicine, ethylene inhibitors, and active charcoal, has been reported in IVB haploid induction of different plant species. Knowledge about mechanisms through which these additives increase tolerance to inductive stresses can help researchers choose other potentially applicable additives and enhance efficiency of IVB-recalcitrant genotypes of important crops. Selection of proper embryo-inducing stress and its corresponding additive(s) is crucial for improving the efficiency of IVB methods. It is difficult to find the proper combination of inductive stress-additive(s), because of multifactorial nature of IVB studies. However, some modeling methods, such as artificial neural network, can predict and facilitate this process.

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Authors contribution MES conceived the idea and corrected the whole body of manuscript, M N wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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