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Breeding Triploid Plants: A Review

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Abstract

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Triploid plants have larger organs, greater biomass, and strong stress resistance by preserving relatively larger amounts of photosynthetic energy. The undesirable spread of non-native invasive crop and horticultural plants into natural areas can also be reduced or eliminated by the use of triploids, because they tend to be sterile and seedless. Triploid plants have great economic value and have been useful for developing new agronomic, horticultural, and forestry plant varieties. Because of rapid advances in DNA sequencing technology, triploids may become a focus of genomic research in the future, and will create unprecedented opportunities for discovering and monitoring genomic and transcriptomic changes in unbalanced genomes. One of the new trends in genomics research is to create synthetic triploid plants as materials for the study of first genomic responses that occur immediately after triploid formation. Here, we summarize recent progress in the use of triploid plants, approaches for obtaining triploid plants, including natural selection, artificial hybridization, and endosperm regeneration, the obstacles to obtain triploids, and possible ways to overcome these difficulties. This summary of the scientific progress on triploid plants will promote understanding of how they can be generated and assist plant breeders to design new strategies for triploid breeding.

Keywords: embryo rescue; endosperm; genomics; hybridization

Polyploidy occurs in many taxa, is particularly widespread in flowering plants, and is a prominent feature of the chromosome evolution of higher plants. At least half of the known angiosperm species have experienced polyploidy in their evolutionary history (Hieter & Griffiths 1999; Shaked *et al*. 2001; Xing *et al*. 2010). Compared to their diploid counterparts, polyploid organisms often express specific characteristics such as larger cell and body sizes (Sugiyama 2005). Miller *et al*. (2012) reported that ploidy level affects many morphological and fitness traits, including stomatal size, flower size, and seed weight in *Arabidopsis thaliana* (L.) Heynh. Triploid plants have three sets of chromosomes, and many desirable characteristics, including greater vigor; broad, thick, dark green leaves; and larger flowers or fruit, which result in higher yield or higher harvest index. For example, the Vertigo watermelon variety ($2n = 3x = 33$) has produced the highest watermelon yields (41 000 lb/acre) (Cushman *et al*. 2003). Triploid cassava also has a high yield with outstanding culinary and industrial qualities (Hoshino *et al*. 2011). Triploid plants produce seedless fruits in different species like citrus, banana and watermelon. Only in citrus, international markets demand fruits without seeds and this characteristic is one of the most important for citrus and with special emphasis in mandarins. Sterile triploid crop and horticultural plants can reduce or eliminate the undesirable spread of nonnative invasive crop plants that produce numerous seeds into natural areas (Li *et al*. 2004). Thus, triploid plants will play an even more important role in agriculture, forestry, and ecology in the future.

One of the new trends in genomic research is to create synthetic polyploid plants to provide materials for studying initial genomic responses immediately after polyploid formation. Thus triploid plants have attracted more attention and there has recently been great progress in understanding the details of their formation after decades of investigation. In this review, we will summarize applications of triploid plants,

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ways to generate triploid plants, possible obstacles to generating triploids, and some solutions to these obstacles.

Formation of triploid plants

Errors occur sometimes during meiosis in regular diploid plants and chromosomes fail to segregate properly to the daughter cells. Such an unreduced 2*n* gamete can unite with a normal, haploid gamete, resulting in a triploid zygote that may develop into a triploid plant. Triploid cells have three complete sets of chromosomes, and are designated 3*n*. When meiosis occurs, the probability of obtaining 2*n* and *n* gamete is only $(1/2)^{x-1}$ (Su *et al.* 2012). For chromosome numbers $x > 8$, this probability is reduced by 1% (Su *et al.* 2012). Hybridization between one parent with unreduced gametes (2*n* gametes) and another diploid parent is the typical way to triploid formation. Both 2*n* megagametophyte and 2*n* microgametophyte occur in both wild and cultivated hybrid and non-hybrid species. There are four mechanisms by which triploids form (Figure 1) in addition to somatic fusion. The female parent with unreduced gametes plays a particularly important role in triploid plant formation (Ramsey & Schemske 1998). Two sets of chromosomes in the triploid plant in Figure 1A, B and D are derived from the female parent. Further, the triploid embryo needs nutrition provided by endosperm, which consists of two polar nuclei from female parent and sperm cell from the male parent. Successful hybridizations between different mating types showed that 2*n* female gamete is more efficient than the 2*n* pollen for the formation of triploid plants during hybridization. Embryo-endosperm balance number can determine the viability of seeds and the exit of cross direction (Lu *et al.* 2013). These various triploid formation mechanisms result in different levels of offspring fertility and phenotypes.

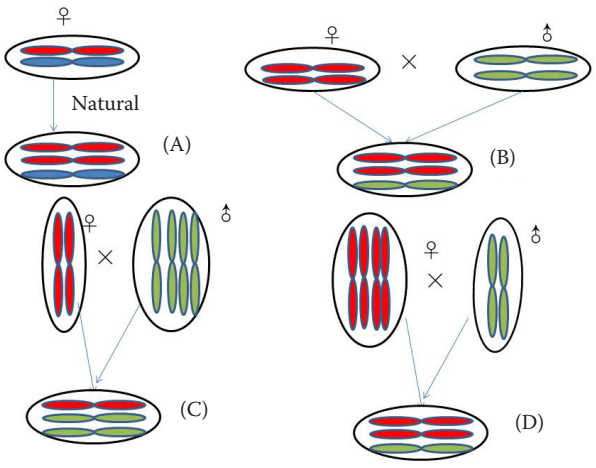

Figure 1. Triploid formation by hybridization: triploid endosperm recovered from a normal 2*x* × 2*x* hybridization (A), sexual hybridization between diploid parents with one parent producing 2*n* gametes (female or male) (B), sexual hybridization between a diploid female parent and tetraploid male parent (C) and sexual hybridization between a tetraploid female parent and diploid male parent (D)

Characteristics and application of triploid plants

Seedlessness of triploid plants and neutralizing the invasive plants

Prolonging flowering period. Although one of the chief characteristics of true triploids is partial or total sterility, this sterility can be horticulturally useful. Flowers of triploid plants are generally larger and more colorful than those of their diploid counterparts partly because the energy that is normally devoted to seed formation is used for flowers or other organs (Miyashita *et al.* 2009; Tiku *et al.* 2014). Triploid flowers often have longer shelf life and the triploid plants require little or no 'dead-heading' (the removal of faded or dead flowers from plants to maintain both a plant's appearance and to improve its overall flowering performance).

Neutralizing invasive plants. Weedy invasive plants have been a problem in the United States for years. The first comprehensive assessment of weedy invasive plants in the continental United States has found that nonnative plants are more widely distributed than are native plants (http://news.sciencemag.org/biology/2015/01/invasive-plants-taking-over-us). Gene flow mediated by pollen has also been demonstrated between commercial cultivars and weedy relatives (Deng *et al.* 2014). Thus, sterile triploid cultivars can be a vital strategy for reducing the invasiveness of crop plants. Many invasive plant species are considered noxious because they produce massive amounts of seeds, which can be dispersed by birds or other means and colonize surrounding areas of native flora (Evans *et al.* 2005), resulting in major transformation of ecosystems such as forests, roadsides, parks, preserves, wildlife refuges, and urban areas. However, if this seed production can be blocked, these plants may behave well as crops or high-quality ornamentals without this invasive

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tendency. One potential solution good for both the horticultural industry and for the environment is to create seedless versions of plants that have been shown to be, or that have potential to be invasive (Li *et al*. 2004). Thus, seedless triploid varieties can play an important role in neutralizing the invasiveness of introduced plants.

Triploid plants with larger organs and greater biomass

Triploid production increases the size of somatic cells and guard cells (Jones & Reed 2007), and increases chloroplast number, which results in strengthening photosynthesis (Padoan *et al.* 2013; Tapan 2014). Therefore, many triploid plants are relatively more vigorous; have short internodes; broad, thick, dark green leaves, resulting in greater biomass or crop yield per plant. Hoshino *et al*. (2011) found that triploids, including cassava (*Manihot esculenta* C.), watermelon, little gourd (*Coccinia grandis* (L.) J. Voigt), had higher yields and higher starch content. Today, over 80% of the watermelons produced in the US are seedless triploid (www.watermelon.org). The triploid seedless watermelon commands premium prices because of its high-quality flesh that is virtually free of seeds (Acton 2013). The protein content of triploid mulberry leaves is 4.14% higher than that of diploid mulberry (Yu *et al*. 2004). Therefore, the edibility and digestibility of triploid mulberry leaves is higher for silkworms. When fed triploid mulberry leaves, silkworms grow more rapidly, which reduces the length of their life cycle by about 2–3 days and increases whole cocoon weight, cocoon layer weight, and pupal weight over those fed with diploid leaves (Yang *et al*. 2000). Cocoon production is also increased by 14–16%, and fecundity improved by about 11%. As in these examples, the use of triploid plants can result in economic benefits in several kinds of farming systems (Hoshino *et al*. 2011).

Use of triploid plants for studying genome evolution and genome plausibility

Yang *et al*. (2011) has reviewed the genomic aspects of research involving polyploid plants. Triploid plant species provide some unique characteristics for genome studies. After genome doubling, genomic characteristics at the individual or population level can be affected, including imbalances in gene dosage, genetic or epigenetic changes, genome size, genomic diversity and genomic rearrangements (Shaked *et al*. 2001; Edger & Pires 2009). For example, some gene loss often occurs following polyploidization. Town *et al*. (2006) found that 35% of the genes inferred to be present when genome triplication occurred in the *Brassica* lineage have been lost in an interspersed pattern, most likely *via* a deletion mechanism. And they found genes encoding proteins involved in signal transduction or transcription were not preferentially retained in a triploidized *Brassica oleracea* genome. This result was not consistent with other studies which have indicated that dosage-sensitive genes involved in signal transduction and transcription may be preferentially retained after duplication. The DNA methylation status of newly formed species appears to be consistently affected following polyploidization. The genetically identical offspring of asexual triploid F_1 dandelion plants (a cross between a diploid sexual seed parent (TJX320) and a triploid apomict pollen donor (A681)) showed a modest level of methylation variation, indicating that *de novo* methylation was triggered by the formation of triploids (Verhoeven *et al*. 2010). Triploids, which carry three complete sets of chromosomes, in particular produce offspring with different chromosome numbers, including diploid and tetraploid progeny, as well as a swarm of aneuploid progeny. Henry *et al*. (2009) reported that most of aneuploid gametes were viable and the chromosomal composition of swarms in *Arabidopsis thaliana* are strongly influenced by the dosage effect of the other gamete. There is thus strong selection against imbalance in gamete ploidy in crosses between triploids and diploids, even in the absence of aneuploidy (Henry *et al*. 2009).

Ways to produce triploid plants

Triploid plants can also be produced by natural selection, sexual hybridization, endosperm culture *in vitro* and fusion of somatic diploid protoplasts with haploid microspore cells. There are few reports on protoplast fusion to produce triploid plants, so here we mainly talk about the other three ways to produce triploid plants.

Natural selection

Natural triploid poplar, especially *Populus tremula* L., is widely present in nature. In 1936, Nilsson discovered a natural triploid of *Populus tremula* L. in Sweden. It is named gigas form of *P. tremula* due to its huge leaves, rapid growth, and tall stature.

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Compared with other trees of the same age, it has obvious advantages, therefore, it aroused the interest of scholars in various countries who carried out research on this variant. Subsequently, many triploid *P. tremula* L. variants were found in other countries (Johnsson 1940; Yablokov 1941; Sylven 1943; Sarvas 1958; Li 2001). Triploid *P. alba* L. and *P. balsamifera* L. were also found by Dillewijn (1939) and Gurreiro (1944), respectively. These triploid poplars also had more desireable characteristics of volume growth, resistance, stem straightness, and fiber than did diploids of the same species. Triploid oak has also been found in a mixed stand of *Q. petraea* (Matt.) Liebl and *Q. robur* (Matt.) Liebl at a frequency of 0.48% (Dzialuk *et al*. 2007).

Unreduced gametes seem to occur more frequently when plants experience environmental stresses, such as frost, wounding, herbivory attack, and water or nutrient shortage (Mason *et al*. 2011). Noticeably, the frequency of unreduced gamete production occurs up to 50-fold more often in hybrids between divergent genomes than in non-hybrid systems (Zhang *et al*. 2010). Nishiwaki *et al*. (2011) found that new, naturally derived *Miscanthus* (*Poaceae*) triploid genotypes were identified more efficiently by flow-cytometry screening of seeds harvested from areas where tetraploid *M. sacchariflorus* F. plants grow sympatrically with diploid *M. sinensis* F. plants, than by random identification of triploids in the field. Triploid *Arachis pintoi* K. *(Leguminosae)* arose by autopolyploidy as evidenced by FISH and meiotic behaviour (Lavia *et al*. 2011).

Artificial hybridization

Triploid plants can be recovered by $2x \times 2x$, $2x \times 4x$, $4x \times 2x$ or $2x \times 3x$ sexual hybridization. Most conventional methodology is from $2x \times 4x$ and $4x \times 2x$ hybridization (Kesara 2003; Aleza *et al*. 2010, 2012; Moreno *et al*. 2010; Zhou *et al*. 2012; Ji *et al*. 2013). Since 1996, the Plant Protection and Biotechnology Center of the Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada, Spain) has developed an extensive citrus triploid breeding program based on interploid sexual hybridizations. A lot of citrus triploid hybrids have been successfully recovered from different $4x \times 2x$ and $2x \times 4x$ sexual hybridizations (Esen & Soost 1971; Williams & Roose 2004; Viloria & Grosser 2005; Aleza *et al*. 2012). Most natural species and hybrids are diploid and spontaneous tetraploidy is extremely rare. So aritifical induction of tetraploid lines is necessary. Doubling of the diploid chromosome number may be achieved by the use of spindle inhibitors, mutation breeding (Smith *et al*. 1993), protoplast fusion mediated by electricity or PEG (Liu *et al*. 2002, 2010; Guo *et al*. 2006; Eeckhaut *et al*. 2013). Mechanical damage such as top pinching over and over again can also achieve tetraploidy (Tao *et al*. 2007). Colchicine was one of the most commonly used spindle inhibitors and has been used to good effect in numerous plants either *ex vitro* or *in vitro* (Vainola 2000; Blakesley *et al*. 2002; Kadota & Niimi 2002; Shao *et al*. 2003). Oryzalin, and trifluralin were also used to disrupt spindle formation and preventing nuclear and cell division (Ascough & van Staden 2008; Aleza *et al*. 2009). The effectiveness of these compounds depends strongly on the concentration applied, the duration of treatment, the type of explant, and the penetration of the compound (Allum *et al*. 2007). Colchicine has been used effectively in concentrations ranging from 0.25 mM (Chen *et al*. 2006) to 38 mM (Stanys *et al*. 2006). Dimethyl sulfoxide (DMSO) can improve the permeability of drugs (Tao *et al*. 2007). But colchicine-induced tetraploids *ex vitro* were confronted with low mutation rate, high chimeric rate and reverse mutation, were difficult to select and vulnerable to environmental disturbance. Fusion of protoplasts together with colchicine-induction created homogenous tetraploid of *Citrus reticulata* Blanco (Dutt *et al*. 2010). But fusion of protoplasts is technically demanding (Eeckhaut *et al*. 2013).

Triploid production efficiency is determined by pollen viability, parents' compatibility and the frequency of unreduced gametes. The pollen germination rates were more dependent on genotypes than polyploidy (Aleza *et al*. 2012). Luro *et al*. (2004), Viloria and Grosser (2005) and Aleza *et al*. (2010) reported that the male parent and environmental conditions influence the production of citrus triploid plants. But Zhang *et al*. (2001) thought that the female parent has a great influence on the success rate of hybridization. They found that $4x \times 2x$ crosses could produce 40.13% triploid seeds but $2x \times 4x$ crosses produce sterile seeds in False Pakchoi (*Brassica parachinensis* L.H.Bailey). Yang *et al*. (2000) found that both $4x \times 2x$ and $2x \times 4x$ crosses could generate a small number of triploid mulberry but the germination percentage of seeds from $4x \times 2x$ was small. Aleza *et al*. (2012) reported 12/114 citrus seeds from $2x \times 4x$ were developed and 123/142 seeds from $4x \times 2x$ were developed. The efficiency

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of triploid regenerated plants produced from 4*x* × 2*x* was higher (114/116) than from 2*x* × 4*x* (1/12) by *in vitro* embryo rescue. Environmental conditions, especially temperatures can affect pollen production, pollen size, pollen germination and pollen tube growth rate and the response to temperature during the reproductive phase is genotype-dependent (Young & Stanton 1990; Hedhly *et al*. 2005). Aleza *et al*. (2010) demonstrated that the frequency of unreduced gametes is an intrinsic characteristic of each genotype. It suggested that the recovery efficiency in triploid plants was more dependent on the genotype and combination.

In 4*x* × 2*x* or 4*x* × 2*x* hybridisations, three seed types are obtained: undeveloped seeds, developed seeds (normal seeds) and developed small seeds. Only the developed normal seed can germinate. Seeds tend to abort due to endosperm degeneration during early embryogenesis (Esen & Soost 1973; Sanford 1983). Esen and Soost (1973) proposed that the 3/4 ploidy ratio of embryo and endosperm caused the induction of seed abortion and endosperm degeneration. Aleza *et al*. (2012) confirmed those triploid plants could be originated as a consequence of unreduced megagametophyte with haploid pollen grain with a most suitable endosperm/embryo ploidy ratio (3/5) or maternal/paternal contribution. So embryo rescue is an indispensable technique for triploid breeding programs that are based on interploid hybridizations (Hiramatsu *et al*. 2003; Guo 2011; Aleza *et al*. 2012). Embryo rescue was utilized by plant breeders to rescue inherently weak, immature and/or abortive embryos, breeding seedless crosses and triploid plants, and distant hybridization between different species (Sharma 1995). Thus far, embryo rescue was extentively applied in rescuing many fruit crops, including apple (Dantas *et al*. 2006), banana (Uma *et al*. 2011), citrus (Viloria & Grosser 2005; Aleza *et al*. 2012), grape (Sun *et al*. 2011), etc. Li *et al*. (2015) provided an overview of the factors that affect its efficiency, including genotype, the time point of removing ovules, medium, culture method and condition, plant growth regulators, etc. According to the results of citrus embryo rescue, the undeveloped seeds were 49–75% smaller than normal seeds and the undeveloped seeds had either one (monoembryonic) or multiple embryos (polyembryonic) which is difficult to individualize or isolate. All normal seeds contained only one well-formed embryo. But the efficiency of triploid plants recovered from small seeds was higher than from normal seeds (Aleza *et al*. 2012). These results provided great help for breeding of triploid plants. In the future, maker-assisted selection technique together with embryo rescue technique will continuously play an important role in the efficient evaluation and selection of the triploid hybrids.

Endosperm culture in vitro

As endosperm is a triploid tissue, it would be reasonable to assume that natural triploids could be successfully regenerated plants from endosperm tissues. The first attempts at endosperm culture *in vitro* took place in the 1930s (Lampe & Mills 1936). Endosperm culture has now been attempted for triploid plant regeneration in nearly 64 species, but successful initiation of buds or shoots from endosperm explants has been reported in only 32 species, 30 of which are listed in Table 1. Triploid plantlets have been regenerated only from 15 of these species. Thus, regeneration from endosperm tissues is often technically challenging.

Genotype, sampling times, and culture media are important aspects of endosperm culture systems. First, the efficiency of endosperm response has been found to be genotype-dependent in many species (reviewed by Popielarska-Konieczna *et al*. 2013).

Second, because either immature and mature endosperms have been used for successful endosperm culture (18 out of 30 species from mature endosperm and 14 out of 30 species have successfully used immature endosperm), it is not clear how critical endosperm developmental stage is for regeneration (Figure 2). But Bajaj *et al*. (1980) found striking differences in the growth responses of immature or mature rice endosperm of various cultivars cultured on different media. The immature endosperm underwent two modes of differentiation, *i.e.*, direct regeneration of plants without callus phase, and indirect regeneration after the differentiation of callus. The mature endosperm, however, first proliferated to form callus, and the plants differentiated 4–6 weeks later. Thammina *et al*. (2011) compared the efficiency of regeneration from immature or mature endosperm in *Euonymus alatus*. They found that the mature endosperm formed callus at a lower rate than did immature endosperm tissues, but that after being transferred to bud induction medium, mature endosperm-derived calli initiated buds more easily. The stage of immature endosperm, usually calculated as days after pollination (DAP), varies from plant

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Table 1. Response of endosperm culture in vitro on various basic culture media supplemented with different concentrations of plant growth regulators

Species	Status of endosperm	Basic media	The best combination of plant growth regulator	Regeneration	Reference
Zea mays	mature	White	YE	no calli	Straus and La Rue (1954)
Exocarpus cupressiformis	mature	White	9.04 µM 2,4-D + 23.2 µM KT + 2.5g/l CH	calli	Johri and Bhojwani (1965)
Dendrophthoe falcate	mature	White	24.6 µM IBA 23.2 µM KT or 148 µM Ad	callus shoot 80%	Johri and Nag (1968)
Taxillus vestillus	mature	White	50 µM KT	shoot	Johri and Nag (1970)
Ricinus communis	mature	WB	9.04 µM 2,4-D + 23.2KT + 2.5 g/l YE	calli	Srivastava (1971)
Croton bonplandianum	mature	White	9.04 µM 2,4-D + 23.2 µM KT + 2.5 g/l YE 0.1 µM IBA	callus root	Bhojwani and Razdan (1971)
Lolium multiflorum	mature	White	5.7 µM IAA + 117 mM Sucrose + 5 g/l YE	calli	Smith and Stone (1973)
Codiaeum varigatum	mature	White	4.5 µM 2,4-D + 4.65 µM KT + 0.5 g·/l CH + 10% CM 4.5 µM 2,4-D + 0.5 g/l CH + 10% CM	calli root; shoot	Chikkannaiah and Gayatri(1974)
Oryza sativa	immature	White	2,4-D 10^{-5}M + YE IAA + YE or YE + KT YE + 2,4-D	calli shoot root	Nakano *et al.* (1975)
Oryza sativa	mature and immature	MS	9.04 µM 2,4-D 9.29 µM KT + 21.4 µM IAA No growth regulator	calli shoot root; plantlet	Bajaj *et al.* (1980)
Santalum album	mature	MS	4.5–9.0 µM 2,4-D or 2.22–8.88 µM BA + 5.37 µM NAA	calli	Lakshmi Sita *et al.* (1979)
		White	1.33 µM BA + 5.34 µM IAA or 1.39 µM KT + 2.89 µM GA 2.67 µM IAA	shoot root; plantlet	
Hordeum vulgare	immature	MS	4.5 µM 2,4-D +0.5 g/l CH 2.32 µM KT + 1.07 µM NAA	calli shoot	Sun and Zhu (1981)
Emblica officinale	mature	MS	1 mg/l BAP + 1 mg/l IAA or 1mg/l 2,4-D + 1 mg/l KT 0.2 mg/l BAP + 0.1 mg/l IAA 0.002 mg/l NAA	calli shoot root	Sehgal and Khurana (1985)
Annona squamosa Linn	mature	White	0.46 µM KT + 0.89 µM BA + 5.37 µM NAA + 2.89 µM GA_3	callus	Nair *et al.* (1986)
		NT	2.69 µM NAA + 8.88 µM BA	shoot	
Actinidia	mature	LS	13.7 µM zeatin + 0.54 µM NAA + 0.4 g/l CH 4.6 µM zeatin + 0.4 g/l CH + 3% sucrose 490 µM IBA	calli shoot root; plantlet	Kin *et al.*(1990)
Citrus	immature	MT	22.2 µM BA + 9.04 µM KT + 23.2 µM 2,4-D + 1 g/l CH +0.5 g/l ME	calli	Gmitter *et al.* (1990)
		2 MT	5.5 µM BA + 14.8 µM Ad + 5.77 µM GA_3 + 0.5 g/l CH	shoot; plantlet	
Lycopersicon esculentum Mill	mature		0.44 µM BA + 4.5 µM 2,4-D + 28.9 µM GA_3	callus	Kagan-Zur *et al.* (1990)
Acacia nilotica	immature	MS	25 µM BAP + 10 µM 2,4-D + 1 g/l CH	calli	Garg *et al.* (1996)

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Table 1 to be continued

Species	Status of endosperm	Basic media	The best combination of plant growth regulator	Regeneration	Reference
Passiflora foetida	mature	MS	25 µM BA 5 µM NAA	shoot root	Mohamed *et al.* (1996)
Diospyros kaki	immature	MS	10 µm zeatin + 10 µm IAA + 500 mg/l CH 10 µm zeatin + 0.1 µm IAA	calli shoot	Tao *et al.* (1997)
		1/2MS	1.5 mM IBA	root	
Morus alba	immature	MS	5 µM 2,4-D 1 µM TDZ or 5 µM BA + 15 µM NAA 1/2 MS + IBA	calli shoot plantlet	Thomas *et al.* (2000)
Azadirachta indica A. Juss	immature	MS	2 µM BAP + 5 µM NAA + 0.5 g/l CH 5 µM BAP	callus shoot	Chaturvedi *et al.* (2003)
		1/2 MS	2.46 µM IBA	root; plantlet	
Actinidia deliciosa cv. Hayward	mature	MS	23.2 µM KT + 9.04 µM 2,4-D 2.27 µM TDZ 2.7 µM NAA and 2.22 µM BA	calli shoot root	Goralski *et al.* (2005)
Carthamus tinctorius	immature	MS	4.44 µM BAP or 2.32 µM KT or 4.5 µM 2,4-D; 2.27 µM TDZ	calli; shoot	Walia *et al.* (2007)
Eucalyptus	immature	MS	6.66 µM BA + 5.34 µM IAA 4.44 µM BA + 5.37 µM NAA	calli shoot	Li *et al.* (2008)
		1/2 MS	2.46 µM IBA	root; plantlet	
Lonicera caerulea var. *emphyllocalyx*	immature	MS	4.44 µM BA + 4.92 µM IBA 0.44 µM BA and 2.89 µM GA_3	calli shoot	Miyashita *et al.* (2009)
		1/2 MS	no regulator	root; plantlet	
Euonymus alatus	mature or immature	MS	2.22 µM BA + 2.7 µM NAA 4.4 µM BA + 0.5 µM IBA	calli shoot	Thammina *et al.* (2011)
		WPM	4.9 µM IBA	root; plantlet	
Jatropha curcas	mature	MS	1.82 µM TDZ + 9.04 µM 2,4-D + 1.44 µM GA_3 +1 g/l YE 6.8 µM ZT + 1.33 µM IAA	calli shoot	Zhu (2011)
		1/2 MS	0.49 µM IBA	root; plantlet	
Carica papaya	immature	MS	6.0 µM 2,4-D + 2.5 µM NAA + 4.0 µM KT 3.0 µM TDZ + 1.5 µM NAA or 1.5 µM BA + 3.0 µM IAA	calli shoot 93.8%	Sun *et al.* (2011)
		1/2MS	2.0 µM IBA	root 90%; plantlet	
Sapium sebiferum	mature	MS	4.44 µM BA + 5.37 µM NAA 8.88 µM BA + 1.07 µM NAA 9.84 µM IBA + activated charcoal	calli 80% shoot 40–66.7% root 80%; plantlet	Tian *et al.* (2012)
Phlox drummondii	immature	MS	5 µM BAP + 10 µM NAA 10 µM BAP + 2.5 µM IAA	calli shoot; plantlet	Tiku *et al.* (2014)

MS – Murashige and Skoog (1962); White – White (1954); WB – modified White's semi-solid (agar 0.8%) medium containing 2% sucrose; NT – Nitsch and Nitsch (1969); MT – Murashige and Tucker (1969); WPM – woody plant medium; LS – Linsmaier and Skoog (1965); CH – casein hydrolysate; 2,4-D – 2,4-dichlorophenoxyacetic acid; GA3 – gibberellic acid; IAA – indole acetic acid; IBA – indole butyric acid; 2iP – 2 isopentenyl adenine; NAA – naphthalene acetic acid; ZT – zeatin; BAP (BA) – benzylaminopurine; TDZ – thidiazuron; PAA – phenyl acetic acid; PEG – polyethylene glycol

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to plant, as reviewed by Thomas and Chaturvedi (2008). Miyashita *et al.* (2009) investigated the histology of endosperm development and explained different stages of development in haskap (*Lonicera caerulea*). They confirmed the endosperm was at best stage for callus formation (88.9%) when developing embryos had reached the globular to early torpedo-stage.

Third, culture media composition can be the decisive factor that determines the success of triploid plant development. Most experiments on endosperm culture attempt to determine the most efficient media for the given species. The following three points can be concluded from Figure 1B: MS (Murashige & Skoog 1962), White (White 1954), LS (Linsmaier & Skoog 1965) and MT (Murashige & Tucker 1969) were used as basic medium and (1) MS is the most commonly used basal medium. (2) Plant growth regulators are essential for regeneration from immature endosperm in most species. About 70% of the reports we have identified have found that 2,4-D is the most effective plant growth regulator to induce callus from endosperm. And BAP appears to be the most popular cytokinin in endosperm culture (Table 1). Successful callus induction without cytokinin has been reported, but there have been no reports of successful callus induction without auxin. The concentrations and ratios of cytokinins (BA, KT) and auxins (2,4-D, NAA) are key factors for different genotypes or endosperm stage. Thammina *et al.* (2011) reported that callus was not induced with *Euonymus alatus* endosperm on hormone-free MS medium. Sun *et al.* (2011) also reported that all papaya (*Carica papaya*) explants turned brown and died without any sign of growth on basal medium. Tian *et al.* (2012) reported that high callus formation rates (approx. 80%) were obtained on media containing both BA and NAA. BA is the most popular cytokinin and IAA and NAA are the most popular auxins used for shoot induction. Auxin is not indispensible, but cytokinin is almost always present in shoot induction media (Figure 1B). Tian *et al.* (2012) also found that increasing BAP concentration from 4.44 to 22.2 μM did not significantly affect callus induction rates, but that higher concentrations were more effective for shoot induction. Stimulation of endosperm metabolism (increasing alpha-amylase activity and hydrolysis) by the addition of exogenous gibberellic acid (GA) has been reported by many workers. Mohamed *et al.* (1996) found that a medium supplemented only with GA_3 and casein hydrolysate could increase the development of shoot primordia in *Passiflora foetida* L. from 2 shoots per explant on all other treatments to more than 20 shoots per explant. Kin *et al.* (1990) found that the presence of GA_3 in the medium was apparently not necessary and did not enhance callus production for *Actinidia*.

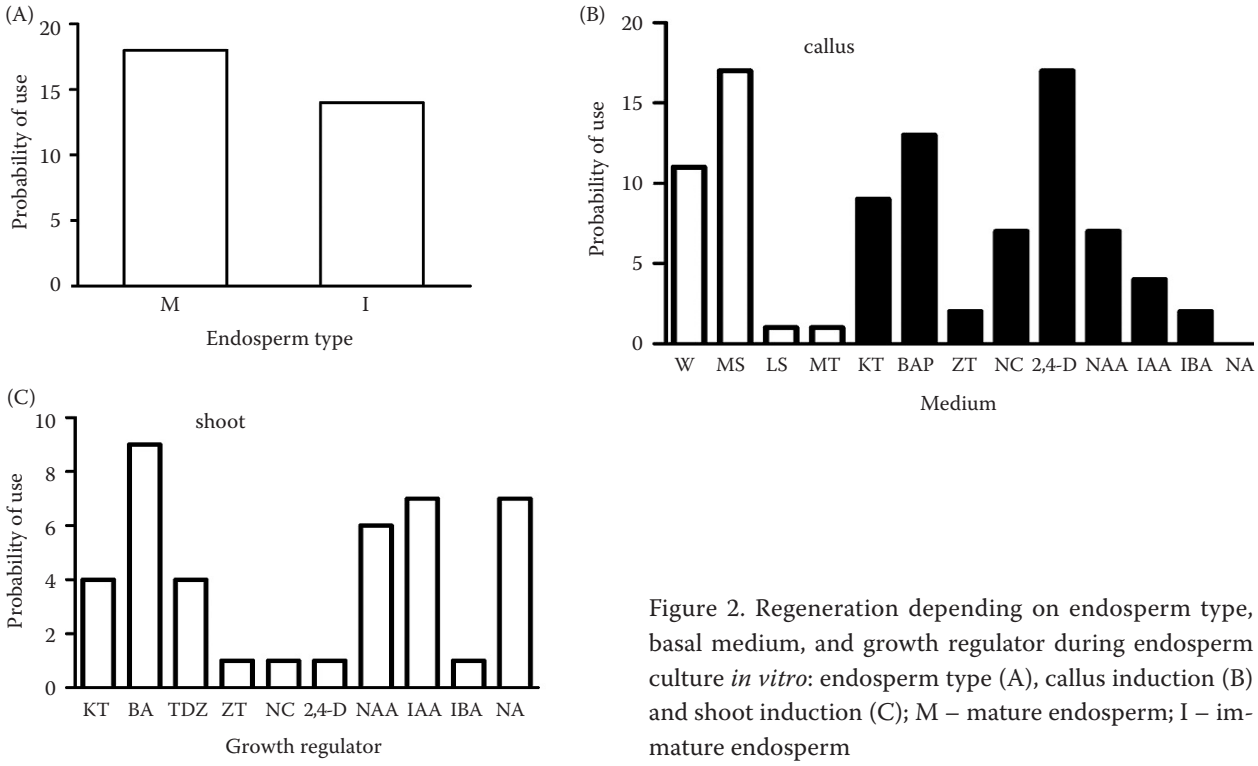

Figure 2. Regeneration depending on endosperm type, basal medium, and growth regulator during endosperm culture *in vitro*: endosperm type (A), callus induction (B) and shoot induction (C); M – mature endosperm; I – immature endosperm

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Many obstacles can decrease the probability of obtaining viable triploid plants in endosperm culture. But despite its challenges, endosperm culture is still a common method for producing triploid plants, but a limited number of species are amenable to endosperm culture.

Albinism. Albinism often occurs in progeny of interspecific crosses and in endosperm culture regenerants. Albino seedlings typically do not survive because they lack chlorophyll pigments and chloroplast membranes (Kita *et al*. 2005; Eeckhaut *et al*. 2007; Kumari *et al*. 2009). Kumari *et al*. (2009) suggested that the important post-zygotic barrier to successful *in vitro* embryo or ovule cultures could be due to any one or a combination of factors including genotype, environment, meiotic abnormalities, hormonal imbalance, nuclear-plastid genome incompatibility, deletions in plastid DNA, mutations in genes responsible for chlorophyll biogenesis, or a metabolic block in pathways leading to chlorophyll biosynthesis. Genetic factors are one of the most important causes of albinism in plants. Also, the degree of genetic relatedness between parents of a cross can also affect the chances of recovering albino seedlings (Sharma 1995).

Mixoploid chimeras. Mixoploid chimeras are one of the common outcomes of interploidy crosses or endosperm culture. The majority of cells in endosperm culture have several ploidy levels, and aneuploidy is frequently seen (Tiku *et al*. 2014). For example, plants generated from apple ($2n = 2x = 34$) endosperm had chromosome numbers ranging from 29 to 56, most of which ranged from 37 to 56, while only 2% to 3% of cells were true triploids (Wu 1978). Ploidy levels, however, have been found to be relatively stable in some endosperm-generated plantlets and these kinds of endosperm cells often can maintain a long-term ability to differentiate organs. Sun *et al*. (2011) reported that over 75% of endosperm-derived papaya plants were triploid with chromosome number $2n = 3x = 27$. Tiku *et al*. (2014) analyzed the chromosome number of 80 *Phlox drummondii* plants derived from endosperm cultures. Of these plants, 70% were triploids ($2n = 3x = 21$) and 5% were diploids ($2n = 2x = 14$), whereas the remaining 25% were aneuploids. Huang *et al*. (1982) reported that kiwi fruit could generate triploid and diploid plants from endosperm from the same plant. When 0.5 mg/l NAA was added to the medium, the plantlets were diploid ($2n = 2x = 58$); whereas when 1 mg/l 2,4-D was substituted in the medium, the plantlets were triploid ($2n = 3x = 87$). The authors suggested that the internal structure of the endosperm together with appropriate hormone components in the culture medium could affect the proliferation and differentiation of certain cell types, and affect the likelihood of recovering plantlets with particular ploidy levels.

Conclusions and perspectives

Triploid plants are rare in nature because of their inviable seeds and resulting lack of progeny, so it is challenging to detect naturally occurring triploid plants. However, due to their faster growth and seedlessness, they will be useful for improving biomass, fruit and flower traits, and other qualities of economically important food, medicinal, bioenergy, and ornamental plants, reducing or eliminating the invasiveness of many crop and horticultural plants. So scientists have intentionally bred triploids through traditional and modern technologies. Natural selection, interploid sexual hybridization, endosperm culture, protoplast fusion were used for production of triploids. A lot of plant species produced triploid plants and popular application. There are more talks about interploid sexual hybridization and endosperm culture. As endosperm is a triploid tissue, it is thought that endosperm culture is the most direct and efficient method for production of triploid plants Although endosperm culture is not yet entirely routine, many successful protocols have been developed over the last 15 years. In suitable media, 82% shoot and 80% root regeneration can be achieved from endosperm cultures of *Phlox drummondii* (Tiku *et al*. 2014). This paper reviewed effect of many factors on the endosperm culture, which may help further study. Protoplast fusion technology has been utilized in many crops to generate allotetraploid somatic hybrids and sometimes triploids can be produced (Fu *et al*. 2003; Cai *et al*. 2009; Grosser & Gmitter 2011). It is important to combine the traditional methods with modern methods to promote development of breeding triploid. In the future, marker-assistant selection technique, which has already been used in grape breeding (Akkurt *et al*. 2012), together with embryo rescue technique will continuously play an important role in the breeding triploid plants.

Other new strategies might be developed to induce triploid plants. With the rapid development of genomics research and advanced biology technologies, perhaps new methods to induce formation of triploids and new avenues of research into and using triploid plants will become possible.

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