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Haploid and Double Haploids in Ornamentals – A Review

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A B S T R A C T

Sporophytic plants having gametophytic chromosome number are termed as haploid. These plants can be produced either spontaneously in nature or by *in vitro* or *in vivo* induction technique. By doubling chromosome number of haploid either spontaneously or artificially, double haploid (DH) could be produced. Double haploid are homozygous and this homozygosity is achieved in one generation by using DH production systems. This production system is used for fixation of heterosis. Genetic map construction is relatively easy with DH population. DH can be used as new variety or as parental inbred line in self-pollinated crops and cross-pollinated crops respectively. Haploids can be used for isolation of mutants, particularly where the mutant allele is non-functional in diploid. True breeding diploid transgenic plants can be produced in one step, if haploids are transformed directly following doubling of chromosomes. Using biotechnological tools along with conventional methods, it is possible to achieve main goals of crop improvement in short time. The present paper discusses various developments in haploid breeding with respect to economically important ornamental species. This review article describes the advances achieved, and provides practical tips that would allow researchers new to the field to establish a new field of research to derive haploid plants for breeding programs of ornamental crop improvement.

Introduction

Haploids are sporophytic plants having gametic chromosome number (n). When chromosome duplication of a these plant occurs, the resulting plant becomes doubled haploid (DH). Haploid can originate spontaneously in nature as well as by various induction techniques. Spontaneous occurrence of haploids occurs at a low frequency (Kasha and Maluszynski, 2003). Haploid produced from an autotetraploid (4x) is termed as dihaploid (2n = 2x) and haploids produced from diploid species (2n=2x) is known as monoploids. Haploids can be induced by *in vivo* (pollination with irradiated pollen and wide hybridization) and *in vitro* (culture of immature gametophytes) technique. In gametic embryogenesis, male or female gametophytes switch from their gametophytic

pathway of development towards a sporophytic one. This results in haploid plants because such plants are derived from the regeneration of gametes or products of meiotic segregation. Haploids are sterile and their fertility is restored by chromosome doubling. The obtained DHs are homozygous at all loci. These homozygous or pure lines can represent a new variety or parental inbred line for the production of hybrid varieties in self-pollinated crops and cross-pollinated crops respectively (Murovec and Bohanec, 2012; Forster *et al.,* 2007).

History of haploids

First natural haploid was described by Dorothy Bergner in *Datura stramonium* during 1921 (Blakeslee *et al.,* 1922). This was followed by reports of natural haploids in tobacco, wheat and several other crop species. Haploid embryos development from *in vitro* culture of immature anthers of *Datura anoxia* was reported first time by Guha and Maheswari (1964). This was a advances and breakthrough in the development of haploids. Later, efforts were made for development of DH through *in vitro* methods, resulting in published protocols for over 250 plant species (Maluszynski *et al.,* 2003). Of late, various researchers directed their attention to use this technique for ornamental crop improvement.

Use of haploids

Using DH production systems, homozygosity is attained in one generation. The time saving is significant when compared with conventional method of selfing particularly in biennial and long juvenile period crops. For dioecious species, self incompatible species, and species that suffer from inbreeding depression, haploidy may be the only technique to develop inbred lines. Haploids have value in allowing the isolation of mutants, particularly where the mutant allele is non-functional in diploid. True breeding diploid transgenic plants can be produced in one step, if haploids are transformed directly following doubling of chromosomes. Doubled haploid plants produce viable seed and the desired character is passed on to successive generations. DH production technique is also used for fixation of heterosis. DH lines do not segregate after self-pollination and can be propagated indefinitely by seeds. This enables efficient physical and genetic mapping and genetic dissection of quantitative traits. DH population is used as an eternal mapping population. Moreover, in recent years, haploids are of great interest for structural genomics (Aleza *et al.,* 2009).

Methods of haploid production in ornamentals

Wide hybridization

Inter-specific and inter-generic pollinations exploit haploidy from the female gametic line. In most of the cases, fertilization of ovules is followed by paternal chromosome elimination from hybrid embryos. Embryo rescue and further *in vitro* culture of these hybrid embryos is used as a method for haploid production in many agricultural crops including ornamentals.

Some of the ornamental crops where haploid production through this method has been tried are reviewed here.

Petunia

Deverna and Collins (1984) attempted hybridization of *Petunia axillaris* and *P. parodii* with *Nicotiana tabacum* by *in vitro* pollination and fertilization. In this experiment a low frequency of haploids was produced with crosses involving *P. axillaris* as the maternal parent. In their next experiment of culturing placenta prior and after the time of

anthesis with or without pollination, they reported that; *In vitro* pollination with distant species in petunia had no significant effect on the frequency of haploids produced. They reported production of 6.5 haploid from *in vitro* culture of 100 un-pollinated ovaries. This technique provides an alternative to anthers culture of petunia as a means for haploid production.

Chrysanthemum

Chrysanthemum is highly heterozygous crop. Due to its highly heterozygous state, breeding as well as molecular analysis in this crop is very complicated. So, it is of great interest to develop haploid forms in chrysanthemum in order to develop doubled haploid or true breeding line. Watanabe (1977) developed protocol for pseudo-fertilized ovary culture of chrysanthemum and was successful in the production of androgenic haploids and F_1 hybrids in Japanese *Chrysanthemum* species. Wang *et al.,* (2014) did *in vitro* culture of 2579 unfertilized chrysanthemum ovules pollinated by *Argyranthemum frutescens* to isolate haploid progeny. This experiment resulted in production of one true haploid. The haploidy was confirmed by cytological studies and microsatellite fingerprinting technique.

Use of ionizing radiation for haploid production

Ionizing and non ionizing radiation has been used for pollen irradiation to induce *in situ* haploid plants. Radiation destroys the generative function of pollen without affecting its capacity in egg cell stimulation, thus allowing development of parthenogenic embryo. It has been used successfully in several species. Irradiation dose is the key factor controlling *in situ* haploid production. If irradiation dose is low, the generative nucleus in pollen maintains its ability to fertilize the egg cell. Therefore obtained embryos will be hybrid and mutant phenotype. An increase in the dose of irradiation will causes a decline in the number of embryos developed but these embryos will be frequently of haploid origin. For most plant species, *in vitro* embryo rescue is necessary to recover haploid plants. Haploid has been obtained in apple, cacao, melon, barley, onion (Sestili and Ficcadenti, 1996), petunia, rose, sunflower and carnation by this method.

Petunia

Raquin (1985) pollinated three different F_1 genotype of *petunia hybrida* with irradiated pollen (gamma ray dose 6-100 kR) and ovaries were harvested 9-14 days after pollination and cultured *in vitro*. At dose upto 30 kR hybrid plants were produced, whereas only gynogenic haploids were produced at dose of 60 kR or above. Using *Petunia hybrida* or *Petunia parodii*, Raquin *et al.,* (1989) reported induction of androgenesis by *in vitro* culture of ovaries irradiated with 50-1000 Gy gamma ray doses before pollination. Their result indicated that gamma ray doses ranging from 200-1000 Gy lead to the development of two type of plants: haploids 2n=x=7 and over-diploids 2n>2x=14, the androgenic origin of haploid was confirmed with genetic marker, androgenic haploid contained the chloroplast of irradiated female parent without visible change to the cp DNA pattern after irradiation.

Carnation

In carnation (*Dianthus caryophyllus* L.), most commercially important varieties are vegetatively propagated, and are not F_1 hybrids. From the perspective of commercial production of rooted cuttings and seeds, there exist some disadvantages with clonal propagation compared with seed propagation. First, the production cost per plantlet is much higher in clonal propagation. Second, perfect

control of diseases in the nursery is essential. Third, the shelf life of cuttings is far shorter than that of seeds. To breed F_1 varieties, producing inbred lines as the parental lines is necessary. Inbreeding depression is a problem in carnation and it is almost impossible to produce S4 seeds. Production of doubled haploids is another way to produce pure lines. Sato *et al.,* (2000) tried the pseudofertilized ovule culture of carnation and succeeded in DH production. In this research emasculated flower buds were pollinated with pollen inactivated by X ray irradiation (100 kR or 200 kR). After 2±3 weeks, the ovaries were cultured on solid MS medium having 2 mM NAA, 2 mM BAP and 6% sucrose. Regenerated plants (R0) were morphologically different from the mother plants. Root tip cells contained both 2n = 30 cells and 2n = 15 cells. The R1 plants of each R0 plant were identical to their respective R0 plants. From these observations they concluded that the R0 plants were doubled haploids. Dolcet-Sanjuan *et al.,* (2001) summarize the protocol for the production of DHLs in carnation, by *in situ* induced parthenogenesis and *in vitro* embryo rescue. They proved the homozygosity of some DHLs by determining the resistance of the progeny to *F. oxysporum* obtained with a susceptible variety.

Standard carnation varieties and other accessions resistant to *F. oxysporum*, were used as mother plants. Collected pollen was irradiated at 1000 Gy with a Co^{60} gamma ray source. Pollination was performed 2-4 days after emasculation. Three to four weeks after pollination, embryos were extracted from seeds and rescued *in vitro*. Flow cytometry was used to determine the ploidy level of growing plantlets during *in vitro* micropropagation and after acclimation to greenhouse. Haploid plants were treated *in vitro* with colchicine previous to their acclimation to soil. DHLs with a good growth habit were acclimated and transferred to a greenhouse. Cuttings for the assays of *Fusarium* resistance were harvested from mother plants. Each DHL was rooted and inoculated with *F. oxysporum f. sp. dianthi* race 2 of Spanish origin. All experiments included a set of cultivars of known resistance or susceptibility. Susceptible DHLs, or those resistant but with poor growth, were eliminated, while resistant and vigorous lines were crossed with the susceptible standard carnation variety 'Persa'. The progeny of each cross was evaluated for resistance to *F. oxysporum*. Fertile DHLs ('D220', 'D504' and 'D524'), resistant to *F. oxysporum*, proved to be homozygous for the three genes involved in this resistance.

Sunflower

DH was obtained from the sunflower hybrids 'Albena' and 'Viki', following the pollination with irradiated pollen and *in vitro* culture of immature embryos (Todorova *et al.,* 1994). Todorova *et al.,* (1997) found that the efficiency of method was affected by the interaction between pollen source genotype, γ-radiation strength and the genotype of the initial forms for parthenogenic induction. Todorova and Ivanov, (1999) reported that genotype specificity of the pollen donor was partially overcome by using mixed pollen from the best pollen sources. A total of 762 new lines were already produced by this method and had been used in the crop improvement of sunflower in the Dobroudja Agricultural Institute.

Rose

Modern roses (2n = 4x = 28) are generally considered an autotetraploid species. It is important to obtain and analyse the dihaploid (2n = 2x= 14) of cultivated roses to study the genetic structure and the heredity of its traits. Meyneta *et al.,* (1994) produced Dihaploid plants of roses (*Rosa* x *hybrida*, cv 'Sonia') by

parthenogenesis induced using irradiated pollen and *in vitro* culture of immature seeds. They found that a 500-Gy minimum dose was sufficient to inactivate pollen and induce *in situ* parthenogenesis. The dihaploid plants produced a small amount of pollen of reduced size.

Bush monkey-flower

Mimulus aurantiacus (bush monkey-flower), is a perennial sub-shrub and is rapidly emerging as a model system for evolutionary and ecological functional genomics studies (Wu *et al.*, 2008).

Murovec and Bohanec, (2013) developed a haploid regeneration technique through gynogenesis in *M. aurantiacus* and they also developed reliable marker for homozygosity testing of putative DH. Among several methods of haploid induction, pollination with gamma-irradiated pollen proved to be the only efficient method. This method produced 4 haploids, 5 triploids, 3 aneuploid and 319 diploid plants.

Lily

Vassileva-dryanovska (1966) pollinated flowers of *Lilium speciosum* with pollen irradiated with X rays (1-500 kR). They collected pistils from different stages of development (1-30 days after pollination) for cytological studies.

From this study he concluded that in *Lilium* there might be two different ways of formation of haploid embryos or tetraploid endosperm nuclei after pollination with pollen irradiated with higher exposures; (a) after a stimulation of the female nuclei to divide by the pycnotic male chromatin. (b) After a stimulation of the egg nucleus to divide pseudogamically under influence of the developing endosperm.

Iris

The first report of successful regeneration of haploid lines in *Iris pseudacorus* developed by *in situ* parthenogenesis followed by embryo rescue was reported by Grouh *et al.*, (2015). Anthers collected from the pollen donor were irradiated by X-ray (100 - 400 Gy). The ovaries from each pollinated flower were cultured and the produced plantlets were then acclimatized. In this experiment the best dose for haploid production in *I. pseudacorus* is found to be 300 or 400 Gy of X-ray.

Androgenesis

Process of regeneration of haploids from male gametic cells is termed as androgenesis. It has outstanding potential in plant breeding and commercial exploitation of DH. This method depend on the ability of microspores and immature pollen grains to convert their gametophytic developmental pathway to sporophytic pathway. This results in cell division at a haploid level. This is achieved with *in vitro* culture of immature anthers and isolated microspore. Isolated microspore is an improved technique. During isolation of microspores, the anther wall tissues are removed, thus preventing regeneration from somatic tissue. Androgenesis is influenced by several factors; genotype, developmental stage of male gametes, stress treatments (temperature pre-treatment to flower bud, osmotic stress, nitrogen and sucrose starvation in growing media) and growing condition of donor plant. Majority of studies have focused on effect of culture media constituents on androgenesis.

The most commonly used carbohydrate is sucrose (13%), particularly in microspore media. Under optimal *in vitro* culturing conditions, androgenetic plants are regenerated by embryogenesis or organogenesis. Direct embryogenesis is

favored, since regeneration through the callus might induce undesired gametoclonal variation.

Anther culture

Anemone

Laura *et al.,* (2006) cultured anthers of *Anemone coronaria* on double layer medium; the lower solid layer consisted of NN medium with activated charcoal, whereas the top layer was liquid NN medium without charcoal. Embryos were observed after 12-14 weeks of culture. The regenerated plants had various ploidy levels, including some plants which were haploid. Their androgenetic origin were confirmed by RAPD- based DNA fingerprinting.

Carnation

Attempts have been made to develop an anther culture protocol in carnation. Callus was produced from anthers cultured on MS medium with 2,4-D and BA, 2,4-D and NAA, TDZ and NAA (Mosquera *et al.,* 1999; Nontaswatsri *et al.,* 2008). All resulting plants were diploid or tetraploid and it was determined that the plants originated from the anther wall (Fu *et al.,* 2008).

Purple coneflower

Zhao *et al.,* (2006) developed 19 haploid plant of *Echinacea purpurea* by anther culture. They found that, in callus induction cultures, N6 basal medium was more effective than MS media, and a combination of BA at 2.22 µM with NAA at 0.054 µM was more effective than 2,4-D alone.

Hepatica

Anther culture techniques for *Hepatica nobilis* Schreber var. *japonica* Nakai were developed by Nomizu *et al.,* (2004). The best embryogenic response was found when anthers containing uninucleate microspores were cultured on solid NN media with 1% activated charcoal. Germination of the embryos was found higher at temperatures of 8°C or 15°C rather than 25°C. All plants regenerated were haploid.

Phlox

Phlox drummondii usede as an annual garden plant and for cut flowers. It suffers from inbreeding depression. Razdan *et al.,* (2008) devised an efficient anther culture protocol for production of haploid plants of *Phlox drummondii*. Anthers with microspores at early- to late-uninucleate stages were inoculated on MS basal medium containing 9% sucrose, 10 µM 2,4-D + 5 µM BA in the dark for callus induction. The callus (~2 mm) was transferred to MS medium containing 3% sucrose + 10 µM BA + 5 µM NAA under 16 h photoperiod for multiplication. 60 plants were produced of which 50% were haploid, 30% were diploid, and 20% were aneuploid

Gentians

Gentians also suffer from inbreeding depression. Doi *et al.,* (2011) develop an anther culture system to produce doubled haploid (DH) lines of gentian (*Gentiana triflora*). Embryos were detected 2-4 months after anthers were cultured. Embryogenesis was induced from anther cultures incubated on half-strength modified Lichter (NLN) medium containing a high concentration of sucrose (130 g/l) and subjected to heat shock treatment. Anthers collected from buds 9–12 mm in length induced the highest frequency of androgenesis. A total of 138 plants were regenerated: 5% of these were haploid, 25% were diploid, and the majority were triploid (70%). Confirmation by ISSR (Inter Simple Sequence Repeat) analysis indicated that the

one diploid analyzed was a doubled haploid. In another study, protocols were developed for the generation of haploid or doubled haploid plants from anther and ovules of *Gentiana triflora* (Pathirana *et al.,* 2011). Plant regeneration was achieved using mid to late uninucleate microspore treated at 4°C for 48 h prior to culture. Anthers and ovaries were cultured on modified NN medium supplemented with NAA and BA. Haploid, diploid, triploid and tetraploid plants were regenerated and all were confirmed by RAPD bands to be of gametophytic origin.

Anthurium

The application of anther culture for producing DH lines of *Anthurium andreanum* can be of great importance because of its high frequency of cross-pollination, high heterozygosity in seed-derived progenies and long growth period of plants after pollination. Anther culture of anthurium was first attempted by Custers (2004) but without any positive results. A novel half-anther culture method has been developed by Winarto *et al.,* (2010) wherein anthers were isolated from the plant, the middle to top part of the anther was excised and cultured with the adaxial side on solid media. Explants were cultured on Winarto–Teixeira basal medium (WT-1) containing 0.01 mg/l NAA, 0.5 mg/l TDZ and 1.0 mg/l 6- BAP, or on New Winarto–Teixeira basal medium (NWT-3) supplemented with 0.02 mg/l NAA, 1.5 mg/l TDZ, and 0.75 mg/l BAP for callus initiation. Regenerated calli produced multiple shoots on WT-1, which were then rooted in NWT-3 supplemented with 1% activated charcoal. Callus regenerated from half anthers displayed a variety of colours with differing regeneration frequencies. The ploidy also ranged from haploid, diploid and triploid to aneuploid. These differences could be exploited in breeding this ornamental species. This led to the following successful development of anther culture for a local Indonesian accession of anthurium (Winarto and Mattjik 2009b), *A. andraeanum* 'Carnaval' (Winarto and Mattjik 2009a), as well as *A. andraeanum* 'Casino', 'Safari' and 'Laguna' (Winarto, 2014). Further research work should focus on improving protocols to ensure that regeneration is from haploid tissue.

Marigold

Li *et al.,* (2007) reported the highest callus inducement and organ differentiation of marigold anther in B5 basic medium with Kt/NAA and 6-BA/NAA, Kn/NAA, Kn/NAA respectively. Yingchun *et al.,* (2011) reported that the highest callus induction rate and regeneration frequency of line 21605 was obtained when inflorescence buds were stored at 4°C for 4 days, and anthers with microspores at the mid to late uninucleate stage were cultured on MS basal medium containing BA (2.2 μM) and NAA (1.82 or 2.7 μM).

Chrysanthemum

Anthers and microspores of six cultivars of chrysanthemums were cultured. Good callus initiation were obtained with the modified MS medium supplemented with NAA 0.1-0.5 mg/l and BAP 0.5-1.0 mg/l, or 2,4-D 0.5 mg/l and BAP 2.0 mg/l. Histological observation of anthers under culture conditions showed that, connective tissue between the thecae developed callus, and microspores degenerated after 10 days of culture (Yang, 2005). Gao *et al.,* (2011) developed haploid chrysanthemum. They reported that the most effective induction medium was basal MS + 2.0 mg/l of BA + 1.0 mg/l of 2, 4-D + 9% sucrose, while the best differentiation medium was MS basal medium + 2.0 mg/l of BA + 0.1 mg/l of NAA + 2% sucrose. Khandakar *et al.,* (2014) cultured anthers of three Korean cultivars 'Yes Morning', 'Hi-Maya' and pot

cultivar 'Peace Pink'. Good callus induction were obtained with the basal MS medium supplemented with 1 mg/l 2, 4-D, 2 mg/l BA, 250 mg/l casein hydrolysate and 45 g/l sucrose. They reported that a pretreatment of anthers in media at 4°C for 48h enhanced the callus induction. Calli were allowed to differentiate on basal MS medium supplemented with 2 mg/l BA and 0.1 mg/l NAA. In this experiment haploid plantlet was detected for the garden cultivar 'Yes morning'.

Calla lily

The heterozygosity and long juvenile period of calla lily hinder its crop improvement. Haploid plant production would be of great advantage to breeding and studying genetics in calla lily. Ko *et al.,* (1996) produce haploid of *Z. aethiopica* by anther culture, but it was a long and quite inefficient procedure. Only one haploid plantlet was obtained from 296 cultured anthers (0.34%). Zhang *et al.,* (2011) describe a faster and more successful anther culture procedure for haploid production of this ornamental. They observed that important factors for improvement as compared to the earlier procedure are: (1) using flowers bud from plant developed at relatively low temperature, (2) high temperature stress treatment (32°C for 2 days) in the beginning of the culture, (3) use of Gamborg B5 medium, and (4) 8% sucrose in the culture medium. In this research frequency of anthers producing calli was around 4–5% and 87% of the calli gave regenerants, out of which 52% were haploid, 36% were diploid.

Lupinus

Kozak *et al.,* (2012) investigated different parameters for anther culture of three genotypes of *Lupinus angustifolius*. They found that yellowish green anthers of buds from the central segment of inflorescence contained maximum number of uninucleate microspores. Cytological investigation shows these anthers were the most responsive to induction. Among three genotypes, Emir showed the highest number of multicellular and embryo-like structures on MS medium supplemented with 2.0 mg/l 2,4 D and 0.5 mg/l Kinetin. These calli continued their growth on regeneration medium and produced roots. This study provided a good foundation for further research for the development of haploid plants for *L. angustifolius*.

Baby primrose

Primula (Baby primrose) is used as a pot flower and garden plant. Improvement of this valuable species is hampered by its self incompatible and high heterozygosity nature. DH production from anther culture of primula accelerate cultivar development in this crop. A protocol for successful callus induction and plant regeneration from *Primula forbesii* Franch anthers was described (Jia *et al.,* 2014). They reported highest callus induction ratio (2.4%) on MS supplemented with 1.0 mg/l BAP + 0.5 mg/l 2,4-D and highest induction and proliferation of indefinite buds (55.2%) on MS supplemented with 0.2 mg/l BAP + 0.01 mg/l NAA. In this research 2% haploid, 65% diploid, 9% triploid, 5% tetraploid, 2% hexaploid and 17% mixoploid has been identified based on flow cytometry and cytological analysis.

Microspore culture

Camalia

Three method of microspore culture were tested for the induction of microspore embryogenesis in *Camalia japonica* L. cv. Elegans (Pedroso and Pais,1994). Microspore suspension plated over solid MS medium containing 4.5 µM 2,4- D and 0.5 µM kinetin with sucrose and glucose were seen as the best

culture condition for induction of embryogenesis.

Sunflower

Gurel *et al.,* (1991) carried out experiment for the embryogenesis of successful microspore culture of sunflower. Both division of uninucleate microspores and embryogenesis were achieved, although in low rates. Hoekstra *et al.,* (1993) conducted an experiment to find out the influence of density and osmolality on microspore culture of *Hordeum vulgare* cv. Igri. They reported that optimum plating density is achieved by adjusting the density to 2 x 10^4 embryogenic microspores per ml. When microspore of cv. Igri were cultured under optimised condition the green/ albino ratio increased from 1:1 to 34:1 and 50 green plant per anther were developed.

Cow cockle

A microspore culture protocol has been developed for *Saponaria vaccaria* L. (Kernan and Ferrie 2006), a member of the Caryophyllaceae family. Genotypic differences were observed among five lines evaluated. The most embryogenic line (cv. White Beauty) produced more than 350 embryos/100 buds and buds that were 4-7.9 mm produced the most embryos/100 buds. Of several media compositions investigated, full-strength NLN with 15% sucrose resulted in the most embryos. They also reported that microspores required pretreatment of 32 °C for 3 days for production of microspore-derived embryos. In this study over 800 DH plants were regenerated.

Zantedeschia

Wang *et al.,* (2011) revealed an essential and novel role of monosaccharides in sporophytic development of *Zantedeschia aethiopica* microspores, whereas, it is known that monosaccharides are less effective for many other crops.

Ornamental kale

Wang *et al.,* (2011) studied the effect of solid medium, developmental stage, embryonic age, cold treatment and additives to the medium on plant regeneration from microspore-derived embryos of ornamental kale (*Brassica oleracea* L. var. acephala). Best result was found when the embryos were cultured in solidified B5 medium having 1% agar. Cold treatment (4°C for 2 days or 5 days) significantly improved plant regeneration with a frequency of up to 79.0%. They also observed that addition of 3.0 or 5.0 mg/l silver nitrate increased the frequency of plant regeneration.

Roselle

Maarup *et al.,* (2012) evaluated the effects of different media and hormonal combinations on microspore culture of roselle. They reported that pretreatment of microspores at 4°C and 35°C for 3 days in the dark had significant effect on callus induction. In this study 1 haploid, 4 mixploids and 25 diploids were detected using flow cytometry combined with propidium iodide. Polymerase chain reaction study using M13 universal primer showed the regenerated haploid plant having four unique bands which were absent in the microspore donor plant UKMR-1. This is the first report of the development of haploid plants in roselle *via* microspore culture.

Anemone

Paladine *et al.,* (2012) establish a procedure to obtain haploid plant from microspore culture of *Anemone coroneria* L., an important ornamental known worldwide due to its commercial value in the cut flower industry. Microspores were isolated from two genotype

of *A. coronaria* 'Blue' and Lilac. Achieved microspore- derived embryo formation was 0.53% for 'Blue' and 0.06% for 'Lilac'. They identified 18 haploid plants and 9 doubled haploid plants of 'Blue', and 4 haploid plants and 3 doubled haploid plants of 'Lilac'.

Gynogenesis

Gynogenesis is the production of haploid embryos from a female gametophyte. It can be achieved with the *in vitro* culture of various un-pollinated flower parts, such as ovules, placenta attached ovules, ovaries or whole flower buds. Gynogenesis has been shown to be a possible alternative source for haploid production in plants, particularly in plants which are recalcitrance to androgenesis, male sterilite and dioecious. The efficiency of the method is greatly influenced by a number of biotic and abiotic factors. Donor plants genotype, growth conditions, developmental stage of gametes, pre-treatment of flower buds, *in vitro* culture media composition and culture conditions are some of the factors affecting the embryogenic response of gametes in culture. These all factors are genotype dependent and no universal protocol exists for *in vitro* gynogenesis. The female gametophyte is usually immature at inoculation and, its development continues during *in vitro* culture, leading to a mature embryo sac (Musial *et al.,* 2005). These contain several haploid cells theoretically (egg cell, synergids, antipodal cells and non-fused polar nuclei) competent of forming haploid embryos. They can develop into haploid plants directly or through an intermediate callus phase.

Gerbera

Gerbera is responsive to gynogenic methods (Kanwar and Kumar, 2008). Cappadocia *et al.,* (1988) reported the formation of callus when they cultured unfertilized ovules of *Gerbera jamesonii in vitro*. When these calli were transferred to the regeneration medium, shoot differentiation occurred in about 2 weeks. Chromosome and chloroplast counts revealed that 76% of the regenerants were haploid and 24% were diploid. Gynogenesis in gerbera is genotype dependant (Tosca *et al.,* 1990; Miyoshi and Asakura, 1996). Haploid plants of gerbera by unfertilized ovule culture has been produced using three different cultural protocols by Honkanen *et al.,* (1991). They reported that regeneration depends on the medium, cultivar and time of the year when ovules were excised. In another study, two of four genotypes evaluated produced more callus in the spring than in the summer and responded poorly in autumn. The third genotype had the worst response in the spring and summer and produced the most callus in the autumn, whereas the fourth genotype gave a poor response regardless of the season (Tosca *et al.,* 1999).

Marigold

Thaneshwari and Aswath (2018) studied the effect of plant growth regulators and sucrose concentration on callus induction and shoot differentiation from ovary culture of marigold (*Tagetes* spp). Maximum callus induction rate has been reported in MS media supplemented with 4.44 µM BAP and 4.52 µM 2,4-D and maximum shoot differentiation rate has been reported when calluses were sub cultured on MS media supplemented with 4.44 µM BAP and 1.07 µM NAA. MS media without plant growth regulator was found to be the best rooting medium. 4% sucrose concentration on MS media was found to be optimum for callus induction. Thaneshwari *et al.,* (2018a) also studied the effect of stress on callus initiation and shoot regeneration in ovary culture of marigold and found that pretreatment of flower buds at 45 °C for 2hr resulted in best callus induction and shoot differentiation when cultured on MS media

supplemented with 4 % sucrose, 4.44 µM BAP and 4.52 µM 2, 4-D and incubated in dark for 4 weeks at 25 °C. Minimum days to callus initiation and highest shoot differentiation rate were also reported when the flower buds pretreated at 4 °C for 24 hr shoot (Thaneshwari *et al.*, 2018b).

Spathiphyllum

Ovule culture method has been reported in *Spathiphyllum wallisii* (Eeckhaut *et al.*, 2001). They observed the genotypic differences and cultivar 'Alfa' gave the best embryogenic response, but the embryos from this genotype were all determined to be somatic in origin. The genotype 'Stefanie', which gave a lower embryogenic response, did yield two homozygous plants as confirmed by AFLP analysis. The basal medium consisted of MS macroelements, NN microelements, as well as myo-inositol, thiamine- HCl, sucrose and agar. They reported that use of TDZ (0.25–1 µM) was important for ovary cultures but was not essential for culture of ovules. Use of a too high TDZ concentration induced diploid parthenogenesis in ovules of cultivar 'Alfa'. The addition of fungicide allowed the ovules to swell making isolation of the ovules easier.

Gentian

Doi *et al.*, (2011) investigated gynogenesis on gentian (*Gentiana triflora*, *G. scabra* and their hybrids). They found that embryo-like structures (ELS) were induced, when unfertilized ovules were cultured in 1/2 NLN medium containing 10% sucrose. The ovules collected from flower buds of later stages (just before anthesis or flower anthesis) tended to exhibit higher response. The dark culture condition produced significantly more ELSs than in 16-h light condition. Flow cytometry of 179 regenerated plants revealing that the majority of them were diploid and haploid (55.9% and 31.3% respectively). Out of 54 diploid plants examined by molecular markers, 52 (96.3%) were reported as doubled haploids (DHs). This is the first report showing successful gynogenesis in gentian.

It is concluded that the ornamentals are produced mainly for their aesthetic value and to enhance both our indoor and outdoor environments. Consumers create a continual demand for new ornamental plant varieties displaying new colours, fragrances, plant architecture and resistance to biotic and abiotic stresses. Doubled haploids have been successfully used in breeding programs to produce new breeding lines and cultivars. Microspore-derived embryos and DH lines can also be used in mutagenesis breeding as well as for genetic modification. Haploids and doubled haploids have been produced in some ornamentals. Different methodologies have been used to generate haploid and doubled haploid plants with each species, and there are very few commonalities between protocols, with media composition and culture conditions varying significantly among species.

Within a species, there are genotypic differences in response to a protocol as has been observed with doubled haploidy protocols in crop species. Progress has been made in developing doubled haploid protocols for a number of ornamentals, although progress has been slow when compared to major crop species. There are many ornamentals that still need to be evaluated for microspore culture response. Further research is required to develop and optimize protocols for production of doubled haploids from ornamentals. Efficient protocols are essential since doubled haploids need to be produced in large numbers to be beneficial to breeding programs. Basic research to further elucidate the factors that control embryogenesis would be of benefit.

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