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Production of double haploids in ornamental crops

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Abstract
Double haploids are the plants, generated spontaneously or induced by chromosome duplication of a haploid plant. They are homozygous at all loci which make them a unique line from the parent in respect of its genome constituent. There is a significant utilization of double haploids in breeding program like production of homozygous lines for cross pollinated crops and mutation breeding. Due to haploidization of chromosomes and again doubling them can produce some novel traits, which cannot be expressed under existing diploid condition. This technique has a significant impact on the improvement program of floricultural crops like chrysanthemum, carnation, petunia, rose, iris, lily, phlox etc. Haploids occur spontaneously at a lower frequency but they can be induced by several methods, such as modified pollination methods *in vivo* (Wide hybridization, Chromosome elimination, Pollination with irradiated pollen, *etc*.) and by *In vitro* culture of immature gametophytes. They are made chromosome doubled to achieve stability in trait expression. The newly advanced techniques for production of double haploids not only made the breeding process faster, but opened up a new dimension of crop improvement.

Keywords: Chromosomes, Double haploids, Meiotic segregation, Ornamental plants, Somaclonal variation

Introduction
Haploid plants are sporophytes carrying the gametic chromosome number (n instead of 2n). When spontaneous or induced chromosome duplication of a haploid occurs, the resulting plant is called doubled haploid (DH). In comparison, dihaploid plants (2n = 2x) are haploid plants obtained from an auto-tetraploid (4x). Haploid can originate spontaneously in nature or as a result of various induction techniques. Haploids produced from diploid species (2n=2x), known as monoploids. They are smaller and exhibit a lower plant vigor compared to donor plants and are sterile due to the inability of their chromosomes to pair during meiosis. In order to propagate them through seed and to include them in breeding programs, their fertility has to be restored with spontaneous or induced chromosome doubling. The obtained DHs are homozygous at all loci and can represent a new variety (self-pollinated crops) or parental inbred line for the production of hybrid varieties (cross-pollinated crops). In fact, cross pollinated species often express a high degree of inbreeding depression. For these species, the induction process can serve not only as a fast method for the production of homozygous lines but also as a selection tool for the elimination of genotypes expressing strong inbreeding depression. Haploids from polyploid species have more than one set of chromosomes and are polyhaploids. Dihaploids and trihaploids are not homozygous like doubled haploids, because they contain more than one set of chromosomes. They cannot be used as true-breeding lines but they enable the breeding of polyploid species at the diploid level and crossings with related cultivated or wild diploid species carrying genes of interest. Haploids occur spontaneously at a lower frequency, or they can be induced by several methods, such as modified pollination methods *In vivo* (Wide hybridization, Chromosome elimination, Pollination with irradiated pollen, *etc*.) and by *In vitro* culture of immature gametophytes. Gametic embryogenesis is one of the different routes of embryogenesis present in the plant kingdom, and it consists in the capacity of male (microspore or immature pollen grain) or female (gynogenesis) gametophytes to irreversibly switch from their gametophytic pathway of development towards a sporophytic one. Differently from somatic embryogenesis, which provides the clonal propagation of the genotype (unless the somaclonal variation), gametic embryogenesis results in haploid plants (unless spontaneous or induced chromosome duplication occurs), because such plants are derived from the regeneration of gametes, products of meiotic segregation.

History of haploids

Dorothy Bergner was credited with describing the first natural haploid in *Datura stramonium* (Blakeslee *et al*., 1922) [3]. This was followed by reports of natural haploids in tobacco, as well as wheat, and subsequently, in many other species. (Clausen and Mann, 1924; Gaines and Aase, 1926, Forster *et al*., 2007) [6, 14, 16]. The breakthrough in haploid research came when Guha and Maheswari (1964, 1966) [20, 21] reported the development of haploid embryos from *In vitro* culture of immature anthers of *Datura innoxia*. This was the first report of inducing a change in normal gametophyte development into sporophyte development and embryo with haploid chromosome number can be obtained. Subsequently, a lot of work was carried out for development of haploid and doubled haploids in higher plants through artificial methods, resulting in published protocols for over 250 plant species belonging to almost all families of the plant kingdom (reviewed in Maluszynski *et al*., 2003) [40]. Of late, various researchers directed their attention to ornamental improvement.

Use of haploids

The production of pure lines using doubled haploids has several advantages over conventional methods. Using DH production systems, homozygosity is achieved in one generation, eliminating the need for several generations of self-pollination. The time saving is substantial, particularly in biennial crops and in crops with a long juvenile period. For self-incompatible species, dioecious species and species that suffer from inbreeding depression due to self-pollination, haploidy may be the only way to develop inbred lines. The induction of DH lines in dioecious plants, in which sex is determined by a regulating gene, has an additional advantage. Such a case is well studied in asparagus, in which sex dimorphism is determined by a dominant gene M. Female plants are homozygous for the recessive alleles (mm), while male plants are heterozygous (Mm). Androgenically produced DH lines are therefore female (mm) or 'supermale' (MM). An advantage of supermales is that, when used as the pollinating line, all hybrid progeny are male.

Dihaploids and trihaploids are not homozygous like doubled haploids, because they contain more than one set of chromosomes. They cannot be used as true-breeding lines but they enable the breeding of polyploid species at the diploid level and crossings with related cultivated or wild diploid species carrying genes of interest.

Haploids have value in allowing the isolation of mutants, which may be masked in a diploid, particularly where the mutant allele is nonfunctional. Haploids also have value in transformation programmes. If haploids are transformed directly, then true breeding diploid transgenic plants can be produced in one step, following doubling of chromosomes. As such, haploid plants (and doubled haploids) reveal all their genetic information, or in other words, their genotype is completely displayed by their phenotype. Doubled haploid plants (transgenic plants) produce viable seed and the desired trait is passed on to successive generations. Some of the genetically determined traits can be introduced into plants by a single gene or possible a small cluster of genes, including insecticidal activity, protection against viroid infection, resistance to herbicides, delay of senescence, tolerance to environmental stresses and improved nutritional quality of plant products. Resistance to pest and diseases or unfavorable external factors (drought, salinity, heavy metal toxicity etc.) can thus be directly recognized and selected. Haploid plants allow the detection of mutants that are unable to pass through the embryonic phases. For similar reasons, haploid plant tissue make ideal vehicles for genetic transformation, by whatever gene manipulation techniques are relevant, to give genetically modified material that on doubling give homozygous versions of the introduced gene or genes.

Doubled haploid technologies are widely used in genetic research because, in addition to complete homozygosity, they offer a simplified segregation pattern, enabling efficient physical and genetic mapping and genetic dissection of quantitative traits. DH lines do not segregate after self-pollination and can be propagated indefinitely by seeds. Moreover, in recent years, haploids are of great interest for structural genomics, because homozygous lines provide a significant facilitation for assembly of whole genome sequences (Aleza *et al*., 2009) [1].

Genetics of double haploids

In the doubled haploid (DH) method, only two types of genotypes occur for a pair of alleles, "A" and "a", with frequency of AA and aa, while in the diploid method, three genotypes occur with frequency of AA, Aa and aa. Thus, if AA is a desirable genotype, the probability of obtaining this genotype is higher in the haploid method than in the diploid method. If n loci are segregating, the probability of getting the desirable genotype is (1/2) n and (1/4) n by the haploid and diploid method, respectively. Thus, the efficiency of the haploid method is obviously high when the number of genes concerned is large.

Compared to some agronomic species [i.e. *Brassica napus* L. (canola), *Hordeum vulgare* L. (barley)], there has been very little work on doubled haploidy in the ornamental species despite the tremendous potential benefits. Although anther culture has been the main approach for haploid production, we will review various methods of haploid production in ornamentals.

1. Methods of haploid production in ornamentals

1.1 Wide hybridization

Wide crossing between species has been shown to be a very effective method for haploid induction and has been used successfully in several cultivated species. It exploits haploidy from the female gametic line and involves both inter-specific and inter-generic pollinations. The fertilization of polar nuclei and production of functional endosperm can trigger the parthenogenetic development of haploid embryos, which mature normally and are propagated through seeds (e.g., potato). In other cases, fertilization of ovules is followed by paternal chromosome elimination in hybrid embryos. The endosperms are absent or poorly developed, so embryo rescue and further *in vitro* culture of embryos are needed (e.g., barley).

Petunia

Hybridization of *Petunia axillaris* and *P. parodii* with *Nicotiana tabacum* was attempted using the method of *In vitro* pollination and fertilization. Seedlings were produced when the *Petunia species* and *N. tabacum* were used as the maternal parents; however, most of these had the identical somatic chromosome complement of the maternal parent. With crosses involving *P. axillaris* as the maternal parent, a low frequency of haploids was also produced. The culture of placenta attached ovules provides an alternative to anther culture as a means for haploid production (Deverna and Collins, 1984) [9].

Chrysanthemum

Chrysanthemum is one of important ornamental species in the world. Its highly heterozygous state complicates molecular analysis, so it is of interest to derive haploid forms. A total of 2579 non-fertilized chrysanthemum ovules pollinated by *Argyranthemum frutescens* were cultured *in vitro* to isolate haploid progeny. One single regenerant emerged from each of three of the 105 calli produced. Chromosome counts and microsatellite fingerprinting showed that only one of the regenerants was a true haploid. Nine doubled haploid derivatives were subsequently generated by colchicine treatment of 80 *in vitro* cultured haploid nodal segments. Morphological screening showed that the haploid plant was shorter than the doubled haploids, and developed smaller leaves, flowers, and stomata. An *in vitro* pollen germination test showed that few of the haploid's pollen were able to germinate and those which did so were abnormal. Both the haploid and the doubled haploids produced yellow flowers, whereas those of the maternal parental cultivar were mauve.

Use of ionizing radiation for haploid production

Because of the interest in haploids for both basic and applied genetics and their low occurrence in natural population, several different approaches have been used to produce haploid in large number. Physical agent such as 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 parthenogenic embryo to develop. It has been used successfully in several species. The production of maternal haploids stimulated by irradiated pollen requires efficient emasculation, which has in some cases been shown to limit its use because the method is too laborious. To overcome such an obstacle in onion, for instance, only male sterile donor plants were used as donor plants, but such lines, possessing cytoplasmically inherited male sterility, are of very limited practical use. Apart from the factors affecting haploid production, the dose of irradiation is the main factor controlling *in situ* haploid production. At lower doses, the generative nucleus is partly damaged and therefore maintains its capacity to fertilize the egg cell. It results in large numbers of obtained embryos but all of hybrid origin and abnormal (mutant) phenotype. An increase in the irradiation dose causes a decrease in the total number of developed embryos but the obtained regenerants are mostly of haploid origin. For most plant species, *in vitro* embryo rescue is necessary to recover haploid plants. The successful use of pollen irradiation has been rare. However low number of haploid has been obtained in apple, cacao, melon, petunia, barley, onion (Sestili and Ficcadenti, 1996) [61], rose (Meynet *et al*., 1994) [42], sunflower (Todorova *et al*., 1997) [67] and carnation (Sato *et al*., 2000) [60].

Petunia

Raquin (1985) [57] pollinated three different F1 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 (mostly diploids) were produced, whereas only gynogenic haploids were produced at dose of 60 kR or above. Using *Petunia hybrid* or *Petunia parodii*, Raquin *et al*. (1989) [58] reported induction of androgenesis by in vitro culture of ovaries irradiated with gamma ray doses ranging from 50-1000 Gy before pollination.

Carnation

In carnation (*Dianthus caryophyllus* L.), most commercially important varieties are vegetatively propagated, and are not F1 hybrids. Anther culture of carnation has been tried (Murty and Kumar, 1976), but to our knowledge, no reports exist of the successful production of haploids or doubled haploids. Likewise, no reports exist of microspore cultures of carnation. Ovules are a possible alternative source material for haploid (or doubled haploid) production. Sato *et al*., (2000) [60] tried the pseudo-fertilized ovule culture of carnation and succeeded in the reproducible production of doubled haploids. This is the first report to succeed in producing doubled haploids of carnation. In this research emasculated flower buds of carnation were pollinated with pollen inactivated by X- ray irradiation (100 kR or 200 kR).

Dolcet-Sanjuan *et al*., (2001) [11] summarize the protocol for the production of DHLs from standard carnation clones, by in situ induced parthenogenesis and *in vitro* embryo rescue, in sufficient number to be incorporated in a breeding program for resistance to *F. oxysporum*. They also prove the homozygosity of some DHLs by determining the resistance of the progeny obtained with a susceptible variety. Pollen irradiation at 1000 Gy with a Co^{60} gamma ray source use to induce haploid in this experiment. Haploid plants generated and were treated *in vitro* with colchicine to produce double haploids. Fertile DHLs ('D220', 'D504' and 'D524'), resistant to *F. oxysporum*, proved to be homozygous for the three genes involved in this resistance after transferring this character to all the progeny of a susceptible clone.

Sunflower

In a preliminary experiment (Todorova *et al*., 1994) [66] following the pollination with heavily irradiated pollen and *in vitro* culture of immature embryos, doubled haploids were obtained from the sunflower hybrids 'Albena' and 'Viki' by pollen irradiation with doses of 300 Gy, 600 Gy and 900 Gy. In total, 2279 embryos were cultivated *in vitro* and 296 of the plantlets obtained were haploids. It was found that the efficiency of the method was affected by the interaction between the genotype of the pollen source, the strength of γ-radiation and the genotype of the initial forms for parthenogenic induction.

By using mixed pollen from the best pollen sources studied, the genotype specificity of the pollen donor was partially overcome (Todorova and Ivanov, 1999) [68]. In a series of consequtive studies, radiation doses which suppress the fertilization ability of the pollen, but preserve its stimulating effect on the embryo sac of sunflower, were determined (Todorova and Ivanov, 2000) [69]. A total of 762 new lines were already produced by this method; these were included in the breeding programs of the Dobroudja Agricultural Institute.

Rose

Modern roses with constant flowering belong to Rosa x hybrida (2n = 4x = 28) generally considered an autotetraploid species. Although this is commercially one of the major species that undergo selection processes, the genetic structure and the heredity of its traits remain unclear. On the other hand, new objectives of selection aim at obtaining an insect and disease-resistant or tolerant material. Hence, botanic diploid germplasm can be used as the genetic source. Finally, the present tetraploid level, the result of 200 years of uncontrolled interspecific crossing is not necessarily the best ploidy level for breeding efficiency. For all these reasons, it is important to obtain and analyse the dihaploid (2n = 2x= 14) of

cultivated roses. Meynet *et al*, 1994 [42] 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 originated from small embryos which occupied only a part of the carpel cavity; they were clearly distinguished from tetraploid plants by miniaturization of all organs. The dihaploid plants were observed under glass-house conditions until flowering presented a normal gynoecium. They produced a small amount of pollen of reduced but regular size; growth and development were faster than the tetraploid controls in summer.

Bush monkey-flower

Mimulus aurantiacus Curtis, commonly known as bush monkey-flower, is a perennial sub-shrub native to southwestern North America. Due to the high ecological and phenotypic diversity of *M. aurantiacus* natural populations, this species has been the subject of numerous studies of adaptive differentiation and speciation (Streisfeld and Kohn, 2005, 2007; Streisfeld and Rausher, 2009) [63, 64, 65]. It is a member of the genus *Mimulus*, which is rapidly emerging as a model system for studies of evolutionary and ecological functional genomics (Wu *et al*., 2008) [85]. Murovec and Bohanec, 2013 [85] developed a haploid induction and regeneration technique from female gametes of *M. aurantiacus*, which would be useful in plant breeding and further genetic research and they also developed reliable marker (s) for homozygosity testing of putative doubled haploids. Among several methods of haploid induction tested, pollination with gamma-irradiated pollen proved to be the only effective method and produced 4 haploid, 319 diploid, 5 triploid and 3 aneuploid plants.

Lily

Vassileva-dryanovska (1966) [71] pollinated Flowers of *Lilium speciosum* with mature pollen irradiated with X- or gamma rays of the exposures 1-500 kR. They collected pistils from different stages of development (1-30 days after pollination) for cytological studies. Pollen tube growth inhibition was found to increase after the exposures of 50 to 500 kR. With the exposures of 1-10 kR most of the pollen tubes passed into a metaphase stage. After 50 to 500 kR the generative nuclei turned elongated and did not divide. After completion of the tube mitoses and during the later stages of the tube growth to the embryo sac the following pictures could be seen: (a) only two sperm nuclei of equal or unequal size (after 1-5 kR), or (b) two sperm nuclei of equal or unequal size with or without extra fragments, free or bridge-connected, or an undivided generative nucleus (after 5-50 kR). More than one pollen tube may penetrate into the embryo sac until 10-12 days after pollination as in control material. After exposures to more than 50 kR the penetration of two pollen tube into the embryo sac is not often found. Depending on the abnormalities in the male chromatin the process of fertilization is realized as a double or a single fertilization. The double fertilization occurs when in the embryo sac has arrived a pollen tube carrying two sperm nuclei or two tubes with undivided generative nuclei (1-50 kR). The single fertilization occurs here as a fertilization of only the polar nuclei and is found when in the embryo sac only one pollen tube with an undivided generative nucleus has arrived (50-500 kR). In many cases, more often than in control material, a fertilization of one synergid was found (1-50 kR). From this study Vassileva-Dryanovska 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

Grouh *et al*., (2015) [19] evaluated the function of pollen grains, irradiated by X-ray, with the aim of producing haploid plants in the iris (*Iris pseudacorus*). The viability of irradiated pollens and the development of parthenogenetic embryos were also investigated. This is the first report of successful regeneration of haploid lines in *Iris pseudacorus* developed by *in situ* parthenogenesis followed by embryo rescue. Native genotypes of *I. pseudacorus* (2n = 38) were prepared as female parents and *I. spuria* was set as the males. Anthers collected from the pollen donor were irradiated by X-ray with doses of 0, 100, 200, 300, and 400 Gy in 2012. Irradiation at 100 and 200 Gy was found to be insufficient to cause pollen sterility. The best dose for haploid production in *I. pseudacorus* is hereby found to be 300 or 400 Gy of X-ray.

1.2 Androgenesis

Androgenesis is the process of induction and regeneration of haploids and double haploids originating from male gametic cells. Due to its high effectiveness and applicability in numerous plant species, it has outstanding potential for plant breeding and commercial exploitation of DH. Its major drawbacks are high genotype dependency within species and the recalcitrance of some important agricultural species, such as woody plants, leguminous plants and the model plant *Arabidopsis thaliana*. The method relies on the ability of microspores and immature pollen grains to convert their developmental pathway from gametophytic (leading to mature pollen grain) to sporophytic, resulting in cell division at a haploid level followed by formation of calluses or embryos. Androgenesis can be induced with *in vitro* culture of immature anthers, a technically simple method consisting of surface sterilization of pre-treated flower buds and subsequent excision of anthers under aseptic conditions. The anthers are inoculated and cultured *in vitro* on solid, semi-solid or liquid mediums or two-phase systems (liquid medium overlaying an agar-solidified medium). Anther culture was the first discovered haploid inducing technique of which efficiency was sufficient for plant breeding purposes (Maluszynski *et al*., 2003) [40]. It is still widely used, although isolated microspore culture is an improved alternative. During isolation of microspores, the anther wall tissues are removed, thus preventing interference of maternal sporophytic tissue during pollen embryogenesis and regeneration from somatic tissue. Androgenesis, is influenced by several biotic and abiotic factors. The developmental stage of male gametes at the time of anther or microspore isolation, in combination with suitable stress treatments, are the main factors determining the androgenetic response. It can be triggered within a relatively wide developmental window around the first pollen mitosis, when uninucleate microspores divide asymmetrically resulting in a generative cell embedded in a vegetive cytoplasm. The application of suitable physiochemical factors promotes a stress response, which arrests the microspores or young pollen grains in their gametophytic pathway. Their development is triggered through embryogenesis by

promoting cell divisions and the formation of multicellular structures contained by the exine wall. Finally, the embryo-like structures are released from the exine wall (Maraschin *et al*., 2005) [41]. The most widely used triggering factors are temperature pre-treatment, sucrose and nitrogen starvation and osmotic stress. Depending on the plant species and genotype, temperature stress can be applied by subjecting excised flower buds, whole inflorescences or excised anthers to low (barley, wheat, maize, rice, triticale, rye) or high (rapeseed, *Brassica* species, tobacco, wheat) temperatures for several hours or days. As demonstrated in rapeseed and tobacco, different triggering factors can promote embryogenesis from microspores or immature pollen cells at different developmental stages. Several other triggering factors such as irradiation, colchicine, auxin and water stress are also used for reprogramming microspores, but to a limited extent. The androgenetic response can be enhanced by *in vivo* pre-treatments of donor plants with nitrogen starvation, short days and low temperature culture conditions.

In addition to stress treatments, the majority of studies have focused on culture media constituents. In general, the concentration of salts in the culture media is lower compared to micropropagation media, but there is no general rule. Choices and concentrations of carbohydrates are often essential. The most commonly used carbohydrate is sucrose, particularly in microspore media, and is added in high concentrations (i.e., 13%), while substitution of sucrose by maltose (Hunter, 1987) has been an important innovation, first discovered for barley anther culture. Although not always required, plant growth regulators might be essential. The influence of all groups of growth regulators has been tested, with positive effects of polyamines being among the latest studied. Under optimal *in vitro* culturing conditions, androgenetic plants are regenerated from embryo-like structures (direct microspore embryogenesis) or from microsporial callus cells (organogenesis). Direct embryogenesis is preferred, since regeneration through the callus stage might induce undesired gametoclonal variation and might also cause albinism.

1.2.1 Anther culture

Anemone

Early work by Johansson and Eriksson (1977) [30] established anther culture protocols for wild *Anemone* species. Further experimentation focused on the economically important *Anemone coronaria* (Laura *et al*. 2006) [35]. A double layer medium was used in the experiments; the lower solid layer consisted of NN medium (Nitsch and Nitsch 1969) [50] 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. RAPD- based DNA fingerprinting showed that all the regenerants tested differed genetically from their anther donor, confirming their androgenetic origin.

Purple coneflower

Zhao *et al*. (2006) [89] developed haploid of purple coneflower, *Echinacea purpurea* by anther culture. They found that, in callus induction cultures, N6 basal medium was more effective than Murashige and Skoog (MS), and a combination of benzyladenine (BA) at 2.22 µM with naphthaleneacetic acid (NAA) at 0.054 µM was more effective than 2,4-dichlorophenoxyacetic acid (2,4-D) alone at 4.52, 9.05 and 13.57 µM. A total of 30 plants were regenerated and 19 were determined to be haploid.

Hepatica

Hepatica or liverwort is a genus of herbaceous perennials in the buttercup family and is used as an ornamental. Anther culture techniques for *Hepatica nobilis* Schreber var. *japonica* Nakai were developed by Nomizu *et al*. (2004) [51]. Anthers containing uninucleate microspores were precultured at 35°C for 4 days then cultured on NN basal medium supplemented with activated charcoal. Microspore divisions were observed after six days and embryos developed after 20 weeks. Four media types were evaluated: liquid, solid, solid with 1% activated charcoal, and double layer (comprised of solid media containing activated charcoal overlaid by liquid media and activated charcoal). The best embryogenic response was found with the solid media with activated charcoal and the double layer. Germination of the embryos was higher at temperatures of 8°C or 15°C rather than 25°C. All plants regenerated were haploid.

Phlox

Phlox drummondii Hook. belongs to the Polemoniaceae family and is grown in North America,

Europe as well as in India as an annual garden plant and for cut flowers. The flowers are usually red, pink and white. Razdan *et al*., (2008) [59] 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. From these experiments, 60 plants were produced of which 50% were haploid, 30% were diploid, and 20% were aneuploidy.

Gentians

Inbreeding depression in perennial gentians makes it difficult to produce homozygous lines for hybrid breeding. The development of haploids and DH's would be beneficial. Doi *et al*., (2011) [10] develop an anther culture system to produce doubled haploid (DH) lines of gentian (*Gentiana triflora*). Half strength NLN medium supplemented with 10, 13, or 16% sucrose was evaluated along with different growth regulators. Embryos were detected 2-4 months after the 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. 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.

Anthurium

Anthurium andreanum Linden ex André (Flamingo lily) is a common house-plant and commercially important cut flower. This plant is propagated by seed but the seeds do not store well and are not uniform. It also takes up to three years of growth before plants can be used in a breeding program. The application of anther culture for producing homozygous double-haploid (DH) lines of Anthurium can be of great importance (Maluszynski *et al*. 2003) [40] because of its high frequency of cross-pollination, high heterozygosity in seed-

derived progenies (Geier 1990; Dufour and Gue´rin 2006) [18, 12], and long growth period of plants after pollination (Higaki *et al*., 1995; Jahan *et al*., 2009) [24, 28]. Anther culture of anthurium was first attempted by Custers 2004 [88], without any positive results. A novel half-anther culture method has been developed by Winarto *et al*., (2010) [80] 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 α-naphthalene acetic acid (NAA), 0.5 mg/l thidiazuron (TDZ) and 1.0 mg/l 6-benzylaminopurine (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 ploidy level ranged from haploid, diploid and triploid to aneuploid.

Successful development of anther culture for *A. andraeanum* 'Carnaval' (Winarto and Mattjik 2009a) [78], a local Indonesian accession of anthurium (Winarto and Mattjik 2009b) [79], as well as *A. andraeanum* 'Casino', 'Laguna' and 'Safari' (Winarto, 2014) [84].

Recently, 20–29 % of the regenerants from Anthurium anther culture were haploids (Winarto *et al*., 2010b) [83]. Regeneration from anthers is also genotype-dependent. Further research work should focus on improving protocols to ensure that regeneration is from haploid tissue without the risk of regeneration from diploid meristematic tissues found in the anther cell walls.

Chrysanthemum

Production of haploid plants of chrysanthemums by anthers and microspores culture was tried by Yang, 2005 [86] without concrete results obtained. Gao *et al*., (2011) [17] developed haploid garden chrysanthemum. In another experiment, producing haploid plants of Chrysanthemum, anthers of three Korean cultivars 'Yes Morning', 'Hi-Maya' and pot cultivar 'Peace Pink' were cultured (Khandakar *et al*., 2014) [33]. Callus induction among cultivars were obtained with the basal MS medium supplemented with 1 mg/l of 2,4-D, 2 mg/l of BA, 250 mg/l of casein hydrolysate, 45 g/l of sucrose; solidified by 2.75 g/l gelrite. Calli were allowed to differentiate on basal MS medium supplemented with 2 mg/l of BA, 0.1 mg/l of NAA, 30 g/l of sucrose; solidified by 2.75 g/l gelrite. Shoot formation from calli in that media slightly differed among cultivars. Multiple shoots elongated from calli were shifted to basal MS medium supplemented with 0.1 mg/l of NAA, 30 g/l of sucrose; solidified by 3 g/l gelrite for rooting. The plantlets with sufficient roots thus obtained were acclimatized and transferred to the soil. Fifty regenerated plantlets from each cultivar were randomly selected for ploidy observation by chromosome counting and haploid plantlet was detected for the garden cultivar 'Yes morning'.

Calla lily

Calla lily (Zantedeschia spp.), a genus of the Araceae family, is an important pot and cut flower worldwide. Calla lily is also characterized by a long juvenile phase of 1–2 years. The heterozygosity and long juvenile period hamper genetic analysis of important traits and efficient breeding of this ornamental. Breeding efficiency could be improved by inbreeding programs, but it is time consuming to obtain pure lines by numerous cycles of self-pollination. Therefore, haploid plant production would be of great benefit 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) [88] describe a faster and more successful anther culture procedure for haploid production of this ornamental. They concluded that important factors for improvement as compared to the earlier procedure are: (1) using flowers from inflorescences developed at relatively low temperature during winter, (2) high temperature stress treatment at 32 ◦C for 2 days in the beginning of the culture, (3) use of Gamborg B5 as anther culture medium, and (4) addition of sucrose at high concentration of 8% in the culture medium. In this study plants were obtained via a callus phase. Frequency of anthers producing calli was around 4–5%. About 87% of the calli gave regenerants, of which 52% were haploid, 36% were diploid and the rest had other ploidy levels.

Baby primrose

Primula (Baby primrose) is an ornamental plant popular for its early spring blooms and diversity of colors. It is used as a pot flower and garden plant. Improvement of this valuable species is hampered by its high heterozygosity. Producing homozygous lines by conventional methods is time consuming and difficult, because the species is self-incompatible. The production of haploid plants from anther culture provides Primula breeders with a means of accelerating cultivar development. A protocol for successful callus induction and plant regeneration from *Primula forbesii* Franch anthers was described (Jia *et al*., 2014). Among a total of 516 anther-derived plantlets; flow cytometry and cytological analysis identified 2% haploid, 65% diploid, 9% triploid, 5% tetraploid, 2% hexaploid and 17% mixoploid.

2.3.2. 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) [55]. Culture was performed on 17 different media consisting of Murashige and skoog and N6 basal media with different combination of carbon, growth regulators, serine and glutamine. Microspore suspension plated over solid MS medium containing 4.5 µM 2, 4- dichlorophenoxyacetic acid and 0.5 µM kinetin with sucrose and glucose were seen as the best culture condition for induction of embryogenesis.

Sunflower

Initial steps for the development of successful microspore culture of sunflower have been carried out (Gurel et al., 1991) [2]. Both division of uninucleate microspores and embryogenesis were achieved, although in low rates with two of the four genotypes tested. Hoekstra *et al*., (1993) [25] 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 2×10^4 embryogenic microspores per ml, with a lower threshold at 5×10^3 per ml. By increasing the osmolality of the pretreatment solution to 400 mos per kg and that of the culture medium to 350 mos per kg, up to 15% of the population developed in to embryo like structures. 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) [32], a member of the Caryophyllaceae family. This ornamental also has valuable properties as a nutraceutical and a doubled haploid protocol would assist in breeding lines with specific biochemical properties. 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 (Lichter, 1982) [37] with 15% sucrose resulted in the most embryos. They also reported that Cow cockle microspores required an initial period of 32 °C for 3 days for production of microspore-derived embryos (MDEs) and over 800 DH plants were regenerated and produced seed.

Zantedeschia

Wang *et al*., (2011) [73-74] 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) [73-74] studied the effect of solid medium, developmental stage, embryonic age, cold treatment and additives to the medium on plant regeneration from microspore-derived embryos in four F1 hybrids of ornamental kale (*Brassica oleracea* L. var. *acephala*). They found that all of the cultivars responded best when the embryos were cultured in solidified B5 medium with 1% agar. Optimal regeneration was gained when cotyledonary embryos were cultured for 25 days. Cold treatment significantly improved plant regeneration with a frequency of up to 79.0% under 4°C for 2 days or 5 days. The addition of 3.0 or 5.0 mg/l silver nitrate ($AgNO_3$) increased the frequency of plant regeneration.

Roselle

Attempts to obtain new variants of roselle through conventional breeding were unsuccessful for 358 trials of (UKMR-3 × UKMR-2) and 63 trials of (UKMR-3 × accession 3). Roselle is an autotetraploid plant (2n = 4x = 72). Therefore Ma'arup *et al*., 2012 conducted an experiment and the objectives of this study were to obtain new variants in roselle through the establishment of a plant regeneration system *via* microspore culture followed by detection of the ploidy level of regenerants based on their distinctive PCR patterns. Thirty regenerants were evaluated for their ploidy level using flow cytometry combined with propidium iodide and only 1 was detected as haploid, 4 mixploids and 25 diploids. Polymerase chain reaction (PCR) analysis using M13 universal primer (5'-TTATGAAACGACGGCCAGT-3') showed the regenerated haploid plant having four unique bands at loci M13-01, M13-03, M13-04, and M13-06, 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 [55] 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. Microspore were isolated from two genotype of *A. coronaria* 'Blue' and Lilac. The effect of different treatment to interrupt the gametophytic development of microspore and promote sporophytic development was evaluated. High temperature, culture media composition and developmental stage of microspores at the moment of isolation were the assessed factors. 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'.

1.3 Gynogenesis

In vitro induction of maternal haploids, so-called gynogenesis, is another pathway to the production of haploid embryos exclusively 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 species where androgenesis is recalcitrance or where the level of albino regenerated plants is high (reaching in most cases 100%), or due to male sterility and dioecious nature of plants. Gynogenic induction using un-pollinated flower parts has been successful in several species, such as onion, sugar beet, cucumber, squash, gerbera, sunflower, wheat, barley *etc*., but its application in breeding is mainly restricted to onion and sugar beet. The success of the method and its efficiency is greatly influenced by several biotic and abiotic factors. The genotype of donor plants, combined with growth conditions, is the crucial factor. Developmental stage of gametes, the pre-treatment of flower buds prior to inoculation, *in vitro* culture media and culture conditions are other factors affecting the embryogenic response of gametes in culture. The female gametophyte is usually immature at inoculation and, in contrast to androgenesis, its development continues during *in vitro* culture, leading to a mature embryo sac (Musial *et al*., 2005). Mature embryo sacs contain several haploid cells theoretically capable of forming haploid embryos, such as the egg cell, synergids, antipodal cells and non-fused polar nuclei. However, under optimal conditions, the egg cells in most gynogenetic responsive species undergo sporophytic development (haploid parthenogenesis) (Bohanec, 2009) [4]. They can develop into haploid plants directly, avoiding the risk of gametoclonal variation, or through an intermediate callus phase. Media components, mainly the type and concentration of carbohydrates and plant growth regulators, play an important role in reprogramming haploid cells from gametophytic to the sporophytic pathway. The requirements are species and genotype dependent and no universal protocol for *in vitro* gynogenesis exists.

Gerbera

Gerbera (Barberton daisy) is used as a cut flower or potted plant and has been increasing in popularity over the last few years. It is grown throughout the world and is native to South Africa and Asia. *Gerbera jamesonii* is the only Gerbera species grown commercially. Gerbera can be propagated sexually and asexually, but vegetative methods are predominant to maintain purity. These methods are very time consuming, hence tissue culture techniques have been developed to accelerate propagation. Genetic transformation methods have also been developed for Gerbera (Nagaraju *et al*., 1998) [49]. In addition, Gerbera is very responsive to gynogenic methods (Kanwar and Kumar, 2008) [31]. The unfertilized ovules of one clone of *Gerbera jamesonii*

cultured in vitro w/v of activated charcoal, were able to sustain the formation of callus. When these calli were transferred to the regeneration medium, shoot differentiation occurred in about 2 weeks. Chromosome and chloroplast counts performed on randomly selected plants revealed that 76% of the regenerants were haploid and 24% were diploid (Cappadocia *et al*., 1988) [5]. One of the major factors influencing embryogenesis is genotype: 12 out of 21 *G. jamesonii* genotypes evaluated produced callus and six of those generated shoots. In another study, 13 out of 17 genotypes produced callus (Miyoshi and Asakura, 1996) [43]. Haploid plants of gerbera by unfertilized ovule culture has been produced using three different cultural protocols by Honkanen *et al*., (1991) [26] They reported that regeneration depends on the medium, the cultivar and the time of the year when ovules were excised. Double haploids were produced with an efficiency of 60% using two colchicines treatment methods (0.025% for 72 hrs and 0.1 % for 2 to 2.5 hrs).

Spathiphyllum

Spathiphyllum wallisii a member of the Araceae family can be easily propagated by *in vitro* methods. Although success with haploidy methodology has been limited, an ovule culture method has been reported (Eeckhaut *et al*., 2001) [13]. Genotypic differences were observed among three genotypes evaluated. '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 (Amplified Fragment Length Polymorphism) analysis. They reported that use of TDZ (0.25–1 µM) was important for ovary cultures but was not essential for culture of ovules. On the contrary, cytokinins were not crucial during ovule culture; in fact, the 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

Anther culture methods were attempted with *G. scabra* (Japanese Gentian) but these were not successful, therefore unfertilized ovule culture was evaluated with this species. Gynogenesis was investigated on gentian (*Gentiana triflora*, *G. scabra* and their hybrids). When unfertilized ovules were cultured in 1/2 NLN medium containing a high concentration of sucrose (100 g/l), embryo-like structures (ELS) were induced. The ploidy levels of 179 regenerated plants were determined by flow cytometry, revealing that the majority of them were diploid (55.9%) and haploid (31.3%). When a total of 54 diploid plants were examined by molecular genetic markers, 52 (96.3%) were considered as doubled haploids (DHs). This is the first report showing successful gynogenesis in gentian (Doi *et al*., 2011) [10].

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