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In Vitro Haploidy Techniques in Ornamental Plants

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Abstract:
The ornamental plant industry is very dynamic and diverse sector having nearly $90 billion production value around the world. Plant breeders develop a great number of new cultivars each year in order to increase production amount and supply market demands of ornamental plants. Various methods have been used in plant breeding, but conventional methods such as selection and crossing are greatly time-consuming processes. In recent years, haploidization techniques based on plant tissue culture are commonly used in ornamental plant breeding in order to overcome disadvantages of conventional methods. The haploid plants described as chromosome numbers in somatic cell of the plant are as many as its in gametic cells. These plants can be formed spontaneously in nature via developing egg cell or synergids into an embryo without fusion of gametic cells or can be obtained using different techniques. At present, *in situ* and *in vitro* haploid/double haploid techniques have been successfully utilized in many plants to accelerate breeding process. *In vitro* haploidy techniques that are gynogenesis (ovule, ovary, flower bud culture) and androgenesis (anther and microspore culture) presented in the present review and some of the successful studies on *in vitro* haploidy induction in ornamental plants were illustrated.
Keywords: Androgenesis, Breeding, Floriculture, Gynogenesis

INTRODUCTION

The ornamental plant industry is very dynamic and diverse sector that comprises cut flowers and cut foliage, flower bulbs, potted flowering as well as foliage plants and bedding plants. Production value in world is approximately $55 billion for cut flowers, cut foliage and flower bulbs which are traded globally. Another $35 billion has been estimated for production value of trees, shrubs and other hardy plants [1].

Plant breeding aims to change and improve genetic structure of plants taking into account the economic benefits. Plant breeders develop a plenty of new varieties of flower altering plant traits such as pigmentation, flower shape or prolonged shelf life in view of market demands in each year. There are various conventional methods such as selection and hybridization methods that are used in plant breeding programs, but conventional methods are greatly time-consuming processes. In recent years, haploidization techniques based on plant tissue culture have been used commonly in ornamental plant breeding in order to overcome disadvantages of conventional methods.

The haploid plants described as chromosome numbers in somatic cell of the plant are as many as its in gametic cells [2]. These plants can be formed spontaneously in nature via developing egg cell or synergids into an embryo without fusion of gametic cells or can be obtained using different techniques [3]. Formation of spontaneous haploids has low frequency and so it is not easy to determine them in nature. Haploids are relatively smaller than normal diploids in terms of morphological traits and they are not able to produce seed due to sexual sterility [2, 3 4, 5, 6, 7]. Therefore, chromosome folding in haploids is needed to obtain fertile plants which are called doubled haploids or homozygous diploids [8]. To fold chromosome set of haploid plants, either spontaneous doubling of the chromosome or the application of ploidy inducing chemicals such as colchicine or oryzalin can be used [9]. The first time occurrence of spontaneous haploid plant was reported in *Datura stramonium* in 1922 [10] and then similar reports were introduced in to tobacco and wheat respectively [11]. Moreover, developing haploid plant in *Datura* from *in vitro* anther culture provided to increase potential of haploid plant production for plant breeding studies and many haploidy studies were introduced since then [3].

Doubled haploid techniques provide many advantages comparing to conventional techniques in plant breeding programs. One of the most important advantages of doubled haploidy in plant breeding is to facilitate obtaining complete homozygosity in short time. The efficiency of selection for both qualitative and quantitative characters is increased since recessive alleles are fixed in one generation and directly expressed due to complete homozygosity [3]. In this way, the technique allows to breeders to avoid time-consuming conventional requirement for extensive selfing or backcrossing before true-breeding lines [14].

In vitro Haploid Techniques and Some Studies in Ornamental Plants

Although haploid plants occur in nature spontaneously, this event is very rare. Therefore, various *in situ* and *in vitro* techniques have been used to obtain haploid plants [12]. Crossing among distance relatives, delaying pollination, pollinating with irradiated pollens, applying different chemicals or X and UV light are used for induction of *in situ* haploidisation [13] while gynogenesis and androgenesis methods are performed for *in vitro* haploidy. Induction

Table 1. *Some researches on ornamental haploid plants successfully obtained via androgenesis*

Species	Explant Source	Year	References
African violet (*Saintpaulia ionantha* Wendl.)	Anther	1981	[34]
Annual Phlox (*Phlox drummondii*)	Anther	2007	[35]
Asiatic Hybrid Lily	Anther	1999	[36]
Calla Lily (*Zantedeschia aethiopica* (L.) Spreng.)	Anther	2011	[37, 38]
Chrysanthemum (*Dendranthema grandiflorum*)	Anther	2014	[39]
Easter Lily (*Lilium longiflorum* L.)	Anther	1997	[40]
Gentian (*Gentiana triflora*)	Anther	2010	[41]
Geranium (*Pelargonium hortorum* Bailey.)	Anther	1973	[42]
Ornamental Pepper (*Capsicum annuum* L.)	Microspore	2016	[43, 44]
Petunia (*Petunia hybrida* L.)	Microspore	1975	[45]
Poppy Anemone (*Anemone Coronaria*)	Anther	2006	[46]
Tulip (*Tulipa gesneriana*)	Microspore	1994	[47]

of gynogenic haploidy comprises culture of unfertilized ovaries, ovules, or flower buds, while anther and microspore cultures are used in androgenic ways [14].

Androgenic Pathways

Anther culture was first discovered *in vitro* haploid induction method which was efficient for the plant breeding purposes [15]. Excised anthers from unopened flower buds at the appropriate developmental stage are used for induction of haploid plants in anther cultures [16]. Immature anthers at uni-nucleate microspore stage are mostly used as a starting material [17] and this stage is the most suitable for androgenic response [18, 19, 20, 21, 22, 23]. The developmental stage corresponds to first pollen mitosis and the stage can be determined observing pollen grains under microscope after staining via different methods such as acetocarmine and DAPI [24]. To increase response of *in vitro* anther culture in haploidy induction, various physical or chemical pre-treatments can be applied to flower buds and then isolated anthers from flower buds under aseptic conditions are incubated in different *in vitro* culture mediums.

Another technique is microspore culture also named pollen embryogenesis that has been commonly performed in plant breeding programs in recent years. The first successful microspore culture isolated from anther tissues was performed in *Datura innoxia* by Nitsch and Norreel in 1970s [25]. In the following years, this technique has been developed and tested in other species. At present, microspore culture has been routinely used in annual field crops such as oat [26], rice [27], wheat [28], triticale [29], barley [30] and some vegetables particularly in Brassica [31]. In ornamental plants, the use of microspore culture is relatively low compared to field crops and vegetables. However, it is clear that efforts of obtaining haploid plants have been increased in ornamental plants. Haploid plants obtained successfully in ornamentals are presented in Table 1.

While regeneration can be occurs from anther wall in anther culture, haploid plantlets are obtained through regenerating only a single microspore cell. Therefore, determination of developmental stage of the microspores and isolation from anther are crucial steps for successful microspore culture. Appropriate developmental stage of microspores is determined with similar manner in anther culture and anthers having microspores at the uni-nucleate to early bi-nucleate stage are preferred for further steps. Microspores are isolated from surface sterilized anther mechanically using a mortar and pestle or a blender or anthers are extracted from the buds, placed in a liquid medium, and microspores are allowed to dehisce [26, 32]. Similar treatments previously cited in anther culture that various physical and chemical pre-treatments enhance efficiency of microspore culture before inoculation in order to obtain haploid embryos. Microspore culture has several advantages comparing to anther culture, but it is more difficult to perform from the technical point of view [33]. Basic steps of *in vitro* haploidy induction through androgenesis and gynogenesis are illustrated in Figure 1.

Gynogenic Pathways

Androgenesis is the most frequently used method of haploid production and preferred over gynogenesis. However, obtaining haploid plants by androgenesis is not always a very effective method and sometimes albino plants can be occurred in culture or donor plants do not respond to this method [48]. Moreover, androgenic methods may not be appropriate for a particular species which have some problems such as low regeneration capability, male sterility of donor plants. [49]. In such cases, obtaining haploid plants by the gynogenic pathway is generally able to produce more healthy results. In addition, ovule and ovary culture are frequently used to learn about the physiology of flower and fruit, morphogenesis physiology and biochemical change [48].

In gynogenic pathway, ovules, ovaries and flower buds can be used as starting material and gynogenic haploids could be produced as a result of division of unfertilized egg cell or

Table 2. *Haploids successfully obtained via gynogenesis in ornamental plants*

Species	Explant Source	Year	References
Gentian (*Gentiana spp.*)	Ovule	2013	[51, 58]
Gerbera (*Gerbera jamesonii*)	Ovule	1981	[54]
Gerbera (*Gerbera jamesonii*)	Ovule	1986	[59]
Gerbera (Gerbera *jamesonii*)	Ovule	1996	[60]
Petunia (*Petunia axillaris* (Lam.))	Ovule	1984	[61]
Peace lily (*Spathiphyllum wallisii*)	Ovary	2001	[62]

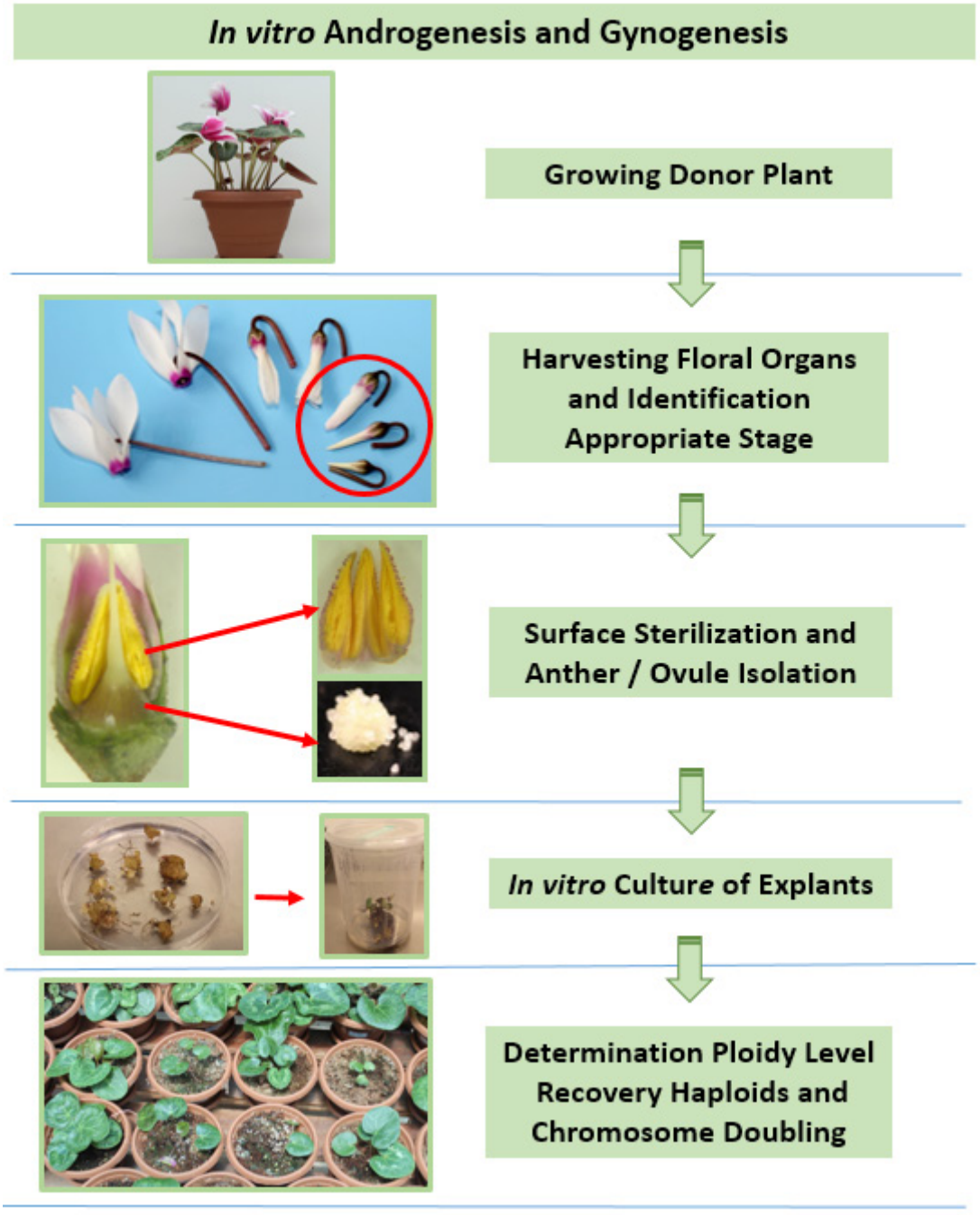

Figure 1. *Basic steps of androgenesis and gynogenesis in Cyclamen*

other haploid cells in the embryo sac, such as synergids or antipodes [50]. Developmental stage of the explants in order to induce haploid plants via gynogenesis is essential as much as in androgenesis. Various developmental stages of ovules are responsive to gynogenesis, and later stages are more responsive. It has been reported that a gynogenic response from ovules occurs at a broad range of developmental stages, in contrast to androgenesis [51, 52, 53]. In ovule culture of *Gerbera jamesonii*, developmental stages of ovules can be classified as young ovules occupying the half of the ovary cavity and ovules near maturity and it is reported that only ovule in second stage is responsive [54]. In addition, pre-treatments such as cold pre-treatment of flower buds or inflorescences before culture can increase the frequency of embryogenesis through gynogenesis as it is in androgenesis [55, 56]. In *Gentiana triflora* cold pre-treatment on anther, ovule and ovary culture were recommended in previous studies [57, 58].

In gynogenic pathways, explants can be cultured in liquid, semi-solid or solid medium after determination of developmental stage of explants and surface sterilization process depending on species. Inoculations of flower buds and ovary into growing medium relatively are easier than ovules, due to larger explant size. It is more difficult to perform unfertilized ovule culture because of ovule isolation from flower buds required. Basic steps of the gynogenesis are presented previously in Figure 1. Studies successful on induction haploidy through gynogenesis in ornamental plants are presented in Table 2.

Determination of Ploidy Level(s)

Determination of ploidy level(s) in plants coming from plant tissue culture is important process. It is required to evaluate ploidy level due to haploid inducing techniques differ in haploid induction rates and undesired regenerants can be obtained. Therefore, isolated microspore culture is more convenient than other *in vitro* haploid techniques because of filtering diploid plant parts during process [3]. There are several methods for determination of ploidy levels that provide to assessment of successfulness of haploidy induction. Chromosome counting is conventional method based on counting mitotic cells of root tip and it has been used in many plants including ornamentals [63]. When a few plants need to be analyzed, chromosome counting is commonly used and successfully applied method. However, it is costly, time consuming and laborious method to evaluate big amount of plant material. Therefore, other conventional indirect methods which are rapid and reliable methods to identify the ploidy level have been used in many plants as well as in ornamentals [64]. Chloroplast counting in a pair of stomata guard cells [65, 66, 67], the ratio of length and width of stomata, stomatal size and frequency [68, 69], size of leaves [70] and pollen grain size [71] have been utilized, but these alternative methods are also time-consuming process. At present, flow cytometry method, based on the detection of the fluorescence emitted by compounds that specifically bind to DNA [72, 73] have been used in many ornamental plants such as carnation (*Dianthus caryophyllus* L.) [74] cyclamen (*Cyclamen rohlfsianum*) [75], Chinese redbud (*Cercis chinensis*) [76] phlox (*Phlox paniculata*) [77], pelargonium (*Pelargonium crispum*) [78]. Additionally, ploidy level can be determined utilizing multiallelic molecular markers (microsatellites). Both of the methods (flow cytometry and molecular markers) can help to determine ploidy levels rapidly in closely related taxa in the absence of meristematic tissue [79].

Factors Effecting In Vitro Haploidy Induction

One of the most important factors affecting the process is the donor plants which floral organs will be obtained. The genetic structure of the plant is one of the internal factors that affect donor plant and process. Different cultivars within the same species and even different genotypes belonging to the same cultivars could give different reactions to the process [80, 81]. Environmental factor such as temperature at which the donor plant is grown, the duration of light exposure, the age of plant and nutrition program influence the success in haploidy induction [26, 81, 82]. In further steps, developmental stage of floral organs, pre-treatment (i. e. cold treatment of inflorescences prior to culture, hot treatment of cultured microspores), composition of the culture medium and physical factors during tissue culture are significant factors affecting successfulness of *in vitro* haploidy induction [3].

Although plant breeding is long process, a plenty of new varieties of flower altering morphological (flower shape, color, etc.) and physiological/genetic traits (tolerance of disease and disorders, shelf life etc.) are developed by hybridization and selection in each year. Obtaining completely homozygous lines in plant breeding programs is major issue that allows plant breeders to improve desired cultivars, but classical breeding methods take more time to have homozygous plant lines. At present, haploid/double haploid techniques have been successfully utilized in many plants to accelerate breeding process. Haploidy induction has also been used in ornamental plants to overcome limitations of conventional breeding techniques such as inbreeding depression. However, species or genotypes specific protocols are required in *in vitro* studies and so further researches are needed in this case.

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